

**Human Telomerase Holoenzyme Assemblage as an  
Anticancer Drug Target**

by  
Brian Raymond Keppler

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill  
in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the  
School of Pharmacy (Pharmaceutical Sciences).

Chapel Hill  
2005

Approved By:

Advisor: Professor Michael B. Jarstfer

Reader: Professor Kenneth F. Bastow

Reader: Professor Jian Liu

Reader: Professor Scott Singleton

Reader: Professor Kevin Weeks

© 2005

Brian Raymond Keppler  
ALL RIGHTS RESERVED

## ABSTRACT

Brian Raymond Keppler: Human Telomerase Holoenzyme Assemblage as an  
Anticancer Drug Target

(Under the direction of **Michael B. Jarstfer, Ph.D.**)

Telomerase is an RNA-dependent DNA polymerase that extends the 3' ends of linear chromosomes. Almost 90% of all cancers require telomerase activity in order to maintain their immortal phenotype. This thesis describes a novel platform for human telomerase inhibition as an anticancer approach. Preliminary studies included testing the feasibility of perturbing proper human telomerase assemblage as a means of inhibiting enzymatic activity. This methodology was validated using oligonucleotides targeted at specific domains of the telomerase RNA subunit (hTR), which were found to inhibit telomerase activity in a direct assay by preventing the association of hTR with the telomerase protein subunit (hTERT) when added prior to assembly. Following these proof-of-principle studies, this approach was further authenticated with the use of known nucleic acid-binding ligands. Various compounds, including DNA minor groove binders and aminoglycoside antibiotics, were found to decrease telomerase activity to a greater extent when added prior to hTR/hTERT assembly as compared to their addition after hTR and hTERT were allowed to associate.

A small library of compounds including various tanshinone natural products and some synthetic derivatives was tested for telomerase inhibition using the pre- and post-assembly parallel screen. Results indicate that the most potent inhibitor was tanshinone

II-A. This compound completely inhibited activity prior to assembly, however, could only partially inhibit activity after assembly indicating that its mode of action could be preventing telomerase assemblage.

Studies here also further characterize and elucidate the roles of the telomerase-associated protein Hsp90 and reveal that the presence of Hsp90 is unremittingly required in order to maintain telomerase in an active conformation. Results show that the N-terminus of Hsp90 may be involved in preparing telomerase for telomeric primer loading while the role of the C-terminus may be to stabilize the telomerase holoenzyme complex.

This new technique of identifying novel telomerase inhibitors was converted into a high-throughput format. Scintillation proximity assay technology was utilized in order to design and develop a screen capable of identifying compounds that perturb a specific interaction between hTR and hTERT. The assay was optimized and validated using an oligonucleotide previously shown to preclude this interaction.

These studies indicate that human telomerase assemblage is a viable anticancer drug target. The further development and optimization of telomerase assemblage exploitation methods could lead to a new class of clinically relevant telomerase inhibitors.

This dissertation is dedicated to  
my wife, Caterina, whose unconditional love and unyielding support  
is a true source of inspiration  
and  
to my mother, father and three sisters, for their support and encouragement not only  
during my graduate career but throughout my life.

## **ACKNOWLEDGMENTS**

The author wishes to express his sincere gratitude to:

Dr. Michael B. Jarstfer for his tutelage, guidance, patience, enthusiasm and encouragement throughout my graduate career

Drs. Kenneth Bastow, Jian Liu, Scott Singleton and Kevin Weeks for their participation and invaluable contribution to this endeavor

Dr. Eric J. Jarvi for his mentorship and friendship

The American Foundation for Pharmaceutical Education (AFPE) for their fellowship during my graduate career

Lab members from the Jarstfer laboratory for their comments, collaboration and friendship

## TABLE OF CONTENTS

Chapter I. Introduction.....	1
A. Telomerase.....	1
1. Significance and background.....	1
2. The catalytic subunit of human telomerase, hTERT ( <u>h</u> uman <u>T</u> elomerase <u>R</u> everse <u>T</u> ranscriptase) .....	3
3. The RNA subunit of human telomerase, hTR ( <u>h</u> uman <u>T</u> elomerase <u>R</u> NA) .....	5
4. Telomere structure and function .....	6
5. Telomerase-associated proteins .....	7
a. Hsp90 .....	8
i. Structure and function.....	9
ii. Hsp90-associated proteins .....	10
iii. Hsp90 and cancer.....	12
iv. Hsp90 inhibition .....	13
6. The secondary role of telomerase in tumorigenesis.....	15
7. Regulation of telomerase .....	16
a. Transcriptional regulation .....	16
i. Positive regulators of hTERT transcription .....	16
ii. Negative regulators of hTERT transcription .....	19
b. Posttranslational regulation.....	21

c. Telomeric accessibility.....	23
B. Previously documented telomerase inhibitors .....	25
1. Reverse transcriptase inhibitors .....	26
2. Antisense oligonucleotides directed against hTR.....	27
3. G-quadruplex stabilizing compounds .....	29
4. Natural products.....	30
5. Small molecules.....	31
6. Pitfalls of previous approaches to inhibit telomerase .....	33
C. Targeting human telomerase assemblage .....	33
1. Significance.....	33
D. Specific aims of this research .....	34
1. (Chapter II) Determine the feasibility of inhibiting human telomerase activity by affecting assemblage with hTR-targeting oligonucleotides in a direct assay .....	34
2. (Chapter III) Test the hypothesis that known RNA-binding ligands will affect human telomerase assemblage and activity.....	34
3. (Chapter IV) Evaluate the human telomerase assemblage inhibiting effects of the natural product tanshinone II-A and a group of novel derivatives.....	34
4. (Chapter V) Characterize the roles of Hsp90 in human telomerase assemblage and activity .....	34
5. (Chapter VI) Design and develop a high-throughput screen capable of identifying novel inhibitors of telomerase assemblage using scintillation proximity assay (SPA) technology .....	34
Chapter II. Inhibiting telomerase assemblage with specific hTR-targeted oligonucleotides.....	35
A. Introduction.....	35



B. Results.....	36
1. Identification of susceptible regions of hTR.....	36
2. Pre- and post-assemblage assays .....	39
a. Concentration dependence of hTRas009 and hTRas010 inhibition.....	39
b. Direct telomerase assay in the presence of hTRas009 and hTRas010 .....	41
3. hTRas009 and hTRas010 affect the association of hTR with hTERT .....	42
4. hTRas009 affects hTR homo-dimerization.....	45
C. Discussion .....	46
D. Materials and methods .....	53
1. Oligonucleotides .....	53
2. pET-28c-hTERT and phTR+HH expression plasmids .....	53
3. In vitro transcription and purification of hTR.....	54
4. Synthesis of hTERT .....	55
5. Reconstitution of telomerase.....	55
6. Direct telomerase assay.....	55
7. Assemblage assay .....	57
8. Synthesis of <sup>32</sup> P-labeled CR4-CR5 and pseudoknot RNA fragments and full-length hTR.....	57
9. Immunoprecipitation/RNA pull-down.....	58
10. Inhibition studies.....	59
11. Nondenaturing polyacrylamide gel electrophoresis of <sup>32</sup> P-hTR .....	60

Chapter III. RNA-binding ligands affect telomerase assemblage .....	61
A. Introduction.....	61
B. Results.....	63
1. Pre- and post-assemblage assays .....	63
2. Binding studies of Hoechst 33258 to hTR.....	65
C. Discussion .....	66
D. Materials and methods .....	69
1. Chemical reagents.....	70
2. <i>T. thermophila</i> telomerase assays .....	70
3. <i>E. aediculatus</i> telomerase assays .....	70
4. Fluorescence spectroscopy.....	70
5. Calculation of dissociation constant .....	71
Chapter IV. The natural product tanshinone II-A and novel derivatives inhibit telomerase assemblage .....	72
A. Introduction.....	72
B. Results.....	74
1. Tanshinone II-A is a potent and specific inhibitor of telomerase assemblage.....	74
2. Tanshinone II-A inhibition of various nucleic acid polymerases.....	76
3. The tanshinone quinone moiety is essential for inhibition .....	76
4. Tanshinone II-A inhibits telomerase in cultured cells and is selectively cytotoxic towards telomerase positive cells.....	77
C. Discussion .....	78
D. Materials and methods .....	82

1. Chemical reagents.....	82
2. Cells and cell culture.....	82
3. Telomerase activity of cell extracts .....	83
4. Cytotoxicity assay.....	84
5. Testing the effects of tanshinone II-A on T7 RNA polymerase, M-MLV reverse transcriptase and the Klenow fragment of DNA polymerase I .....	84
6. Natural product isolation and derivative synthesis .....	85
 Chapter V. Hsp90 is required to maintain telomerase in an active conformation.....	86
A. Introduction.....	86
B. Results.....	88
1. Hsp90 inhibitors affect in vitro reconstituted human telomerase activity both before and after assembly.....	88
2. Telomerase maintains hTR-hTERT interactions in the presence of geldanamycin and novobiocin after hTERT translation.....	90
3. Primer binding overcomes GA, but not NB, inhibition of human telomerase .....	93
4. Hsp90 is associated with hTERT after telomeric primer binding .....	95
5. Hsp90 inhibition alters telomerase holoenzyme structure .....	96
6. NB destabilizes hTERT .....	98
7. Hsp90 is associated with hTERT following geldanamycin or novobiocin treatment .....	99
C. Discussion .....	100
D. Materials and methods .....	105

1. Antibodies and chemical reagents .....	106
2. In vitro transcription and purification of hTR.....	106
3. Synthesis of [ <sup>32</sup> P]-labeled pseudoknot and CR4-CR5 RNA fragments and full-length hTR.....	106
4. Synthesis of biotinylated pseudoknot or CR4-CR5 RNA fragment .....	107
5. Inhibition studies.....	107
6. Association of hTR and hTERT in the presence of GA and NB .....	108
7. Association of hTR and hTERT when GA or NB was present during translation of hTERT .....	108
8. Immunoprecipitation of telomerase using an Hsp90 antibody .....	110
9. Lys-C proteolysis of hTERT.....	111
10. Pulse-chase.....	111
 Chapter VI. Development of a high-throughput screen capable of identifying novel inhibitors of a specific hTERT/hTR interaction .....	 113
A. Introduction.....	113
B. Results.....	116
1. Biotin-labeled CR4-CR5 domain binds hTERT .....	116
2. A Scintillation Proximity Assay for hTERT-hTR interactions.....	118
3. Validation of SPA.....	119
4. Optimization of SPA.....	120
C. Discussion .....	122
D. Materials and methods .....	127
1. Oligonucleotides .....	127

2. Biochemical assay for the interaction between hTERT and the CR4-CR5 domain of hTR .....	128
3. Scintillation Proximity Assay .....	128
4. Inhibition studies.....	130
5. Z-factor determination .....	130
Chapter VII. Conclusions and future directions of this research .....	131
Representative works .....	136
Literature cited.....	137

## LIST OF TABLES

Table 2.1	Summary of inhibition data with hTR-targeted oligonucleotides .....	37
Table 3.1	Percent inhibition of telomerase by selected nucleic acid-binding ligands .....	65
Table 4.1	Natural tanshinones affect telomerase activity .....	77

## LIST OF FIGURES

Figure 1.1 Mechanism of telomerase-mediated telomere extension .....	2
Figure 1.2 Telomere length versus time for different cell types.....	3
Figure 1.3 Human telomerase RNA.....	5
Figure 2.1 Assemblage assay using hTR-targeted oligonucleotides .....	38
Figure 2.2 Concentration dependence of telomerase inhibition by hTRas009 and hTRas010 when added before assemblage .....	39
Figure 2.3 High concentrations of hTRas009 in an assemblage assay produce telomerase-independent artifacts .....	40
Figure 2.4 Concentration dependence of telomerase inhibition by hTRas009 and hTRas010 when added after assemblage .....	41
Figure 2.5 Inhibition of hTR/hTERT interactions by hTRas009 and hTRas010 .....	43
Figure 2.6 Oligonucleotide and RNA specificity .....	44
Figure 2.7 hTRas009 inhibits hTR homo-dimerization .....	45
Figure 2.8 hTRas009 and hTR010 prevent proper telomerase assemblage by blocking essential interactions between hTR and hTERT .....	52
Figure 3.1 Compounds tested as telomerase inhibitors .....	62
Figure 3.2 RNA binding ligand-induced inhibition of telomerase pre- and post-assemblage .....	64
Figure 3.3 Hoechst 33258 binds to hTR but not to other nucleic acid components present in the telomerase reaction .....	66
Figure 4.1 Effectors of telomerase assemblage .....	72
Figure 4.2 Tanshinones tested in this study .....	74

Figure 4.3	Inhibition of telomerase by tanshinone II-A .....	75
Figure 4.4	Effects of tanshinone II-A on different nucleic acid processing enzymes .....	76
Figure 4.5	Consequences of treating immortalized cells with tanshinone II-A .....	78
Figure 5.1	GA and NB inhibit telomerase when added before or after assembly .....	89
Figure 5.2	p23 has a slight rescue effect on GA-induced telomerase inhibition .....	90
Figure 5.3	hTR/hTERT interactions are unaffected by the presence of GA and NB after translation of hTERT, but hTR/hTERT interactions are effected by the presence of GA and NB during translation .....	92
Figure 5.4	Pre-Incubation with primer rescues GA-induced but not NB-induced telomerase inhibition .....	94
Figure 5.5	Hsp90 remains associated with hTERT after primer loading .....	95
Figure 5.6	NB, but not GA, increases the proteolysis rate of hTERT .....	97
Figure 5.7	hTERT stability, but not RNA/protein complex half-life, is affected by NB .....	98
Figure 5.8	Hsp90/hTERT interactions are unaffected by the presence of GA and NB after hTERT translation .....	99
Figure 5.9	Conceptual model for Hsp90-mediated human telomerase maturation and the effects of GA and NB .....	103
Figure 6.1	Structure and hTERT-binding inhibition of the CR4-CR5 domain of hTR .....	117



Figure 6.2 Assay schematics for scintillation proximity assay .....	118
Figure 6.3 Concentration dependence of hTRas010 in an affinity purification and a scintillation proximity assay .....	119
Figure 6.4 Effects of hTRas010 and hTRas010MM on the interaction between hTERT and the CR4-CR5 fragment of hTR.....	121
Figure 6.5 Precision of scintillation proximity assay for assembly of hTERT with CR4-CR5 RNA under optimal conditions .....	122

## LIST OF ABBREVIATIONS

17-AAG	17-allylamino-17-demethoxygeldanamycin
2'-MOE	2'- <i>O</i> -(2-methoxyethyl)
5-FAM, SE	5-carboxyfluorescein, succinimidyl ester
5-TAMRA, SE	5-carboxytetramethylrhodamine, succinimidyl ester
ALT	Alternative Lengthening of Telomeres
AZT	3'-azido-3'-deoxythymidine
BIBR1532	2-[(E)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid
BRACO-19	9-[4-( <i>N,N</i> -dimethylamino)phenylamino]-3,6-bis(3-pyrrolidinopropionamido) acridine
DAPI	4',6-diamidino-2-phenylindole
DHBV	Duck Hepatitis B Virus
GA	Geldanamycin
HDAC	Histone deacetylase
Hsp70	Heat shock protein-70
Hsp90	Heat shock protein-90
hTERT	human Telomerase Reverse Transcriptase
hTR	human Telomerase RNA
HTS	High-throughput screen
NB	Novobiocin
NPS	N3'→ P5' <i>thio</i> -phosphoramidate
PKC	Protein Kinase C
PNA	Peptide Nucleic Acid

RNP	Ribonucleoprotein
RRL	Rabbit Reticulocyte Lysate
RT	Reverse Transcriptase
SPA	Scintillation Proximity Assay
Tan II-A	Tanshinone II-A
TRAP	Telomeric Repeat Amplification Protocol
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labeling
WT1	Wilms' Tumor 1

## **Chapter I. Introduction**

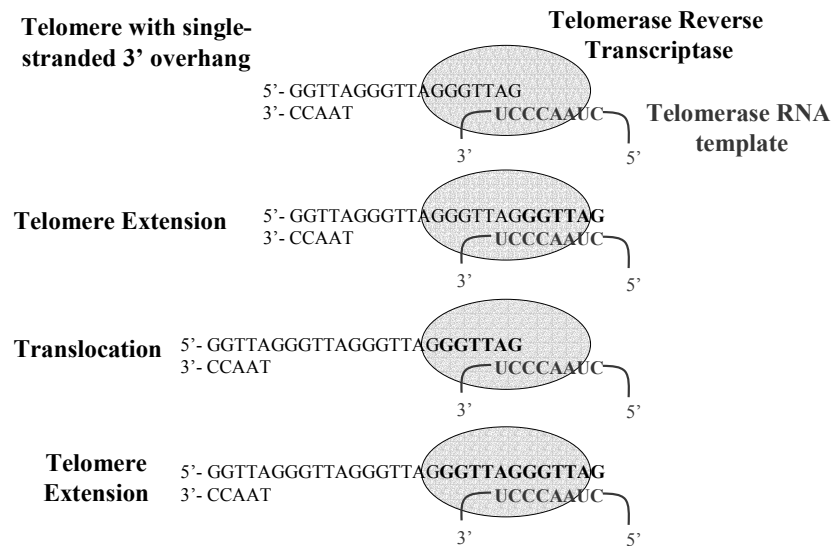
### **A. Telomerase**

#### **1. Significance and background**

Cancer is a growing health problem for people all over the world. Most cancer research focuses on curing or subduing a specific type of cancer (e.g. breast, prostate, lung, colon, etc.). Recently, though, a large proportion of cancer cell types were found to share a common trait as approximately 90% of all human tumors express telomerase activity (Cong et al., 2002). Telomerase, or telomere terminal transferase, is a ribonucleoprotein complex responsible for the maintenance of telomeric DNA. Telomeres are DNA-protein complexes, which possess a 3' single-stranded overhang of short, repeated, guanosine-rich sequences (5'-TTAGGG for humans). Human telomerase is composed minimally of a protein (hTERT) and an RNA (hTR) subunit (Feng et al., 1995; Meyerson et al., 1997; Nakamura et al., 1997). The RNA subunit is used as a template for the G-rich sequence addition while the protein subunit employs its reverse transcriptase activity to extend telomeric DNA (Figure 1.1).

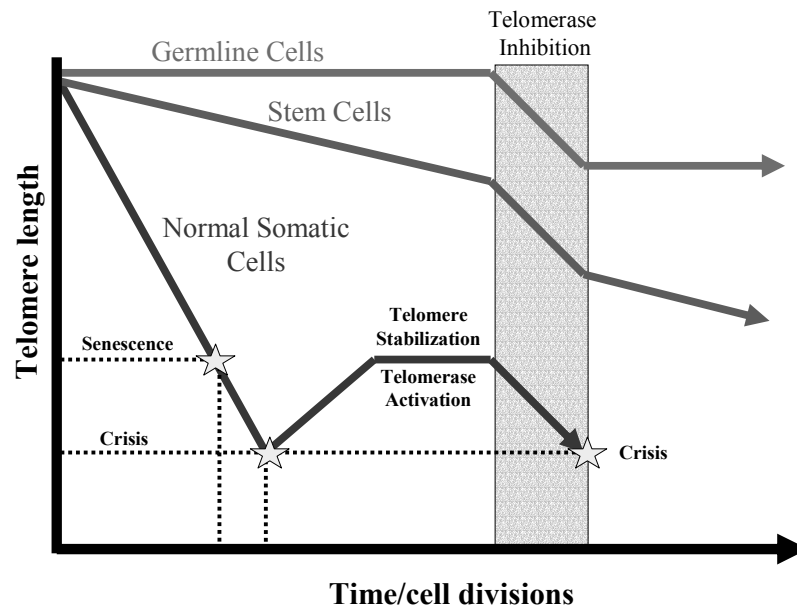
Telomerase activity is extinguished in most human tissues during embryonic development (Wright et al., 1996b), however, certain cells in the human body express telomerase activity through all stages of life such as germline cells and some stem cells (Cong et al., 2002). Most normal healthy human somatic cells, on the other hand, are deficient in telomerase activity. The telomeres of these somatic cells therefore undergo telomere shortening after each cell cycle as the result of the end-replication problem

(Olovnikov, 1971; Watson, 1972). When a cell's telomeres get too short, the cell will either senesce or enter crisis and die. The majority of cancerous cells, however, can escape crisis



**Figure 1.1 Mechanism of telomerase-mediated telomere extension.** The telomerase reverse transcriptase (hTERT) and the telomerase RNA (hTR) work together to extend the 3' end of the telomere. Nucleotides added to the telomere by telomerase are bold. This figure was adapted from White et al., 2001.

by up-regulating telomerase expression (White et al., 2001) (Figure 1.2). As a result, telomerase promotes tumorigenicity in part by helping maintain telomeric DNA length. Importantly, inhibiting telomerase activity in cancer cells causes telomere shortening and cessation of cell growth (Hahn et al., 1999). The successful exploitation of this common trait could therefore lead to the development of a universal anticancer agent based on inhibiting telomerase activity.



**Figure 1.2 Telomere length versus time for different cell types.** Germline cells constitutively express telomerase activity and thus their telomeres do not decrease in length over time. Most normal somatic cells do not express telomerase and thus their telomeres decrease in length over time. The telomeres of certain stem cells also decrease in length over time, but at a slower rate than that of most normal somatic cells because they express low levels of telomerase activity. Though the administration of a telomerase inhibitor to the human body would affect all telomerase-positive cells, the cancerous cells would die off first before the healthy cell's telomeres get critically short. This figure was adapted from White et al., 2001.

## 2. The catalytic subunit of human telomerase, hTERT (human Telomerase Reverse Transcriptase)

The hTERT gene, discovered in 1997 (Harrington et al., 1997; Kilian et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997), is found on the short arm of chromosome 5 (5p15.33). The hTERT protein is 1132 amino acids in length and contains a number of distinguishable features including reverse transcriptase motifs within the C-terminal half of the gene, a conserved telomerase-specific region (T-motif) located just 5' of the reverse transcriptase motifs, and a large N-terminal region (Cong et al., 2002; Harrington, 2003). The telomerase catalytic subunits from different organisms represent a distinct subgroup within the reverse transcriptase family (Cong et al., 2002). Their reverse transcriptase motifs

are phylogenetically conserved with other reverse transcriptases, but are more closely related within their own subgroup than with other members of the reverse transcriptase family (Eickbush, 1997; Nakamura et al., 1997; Nakamura and Cech, 1998). The T-motif has been found to be required for hTR binding in vitro (Bryan et al., 2000) and is also thought to be involved in the recruitment of hTERT to the nucleolus, which has been proposed to be an important step in telomerase biogenesis (Etheridge et al., 2002; Bosoy et al., 2003). The large N-terminal region contains conserved residues that are functionally important for hTR binding, telomerase complex assemblage, and catalysis, as well as a number of other functions (Cong et al., 2002; Harrington, 2003).

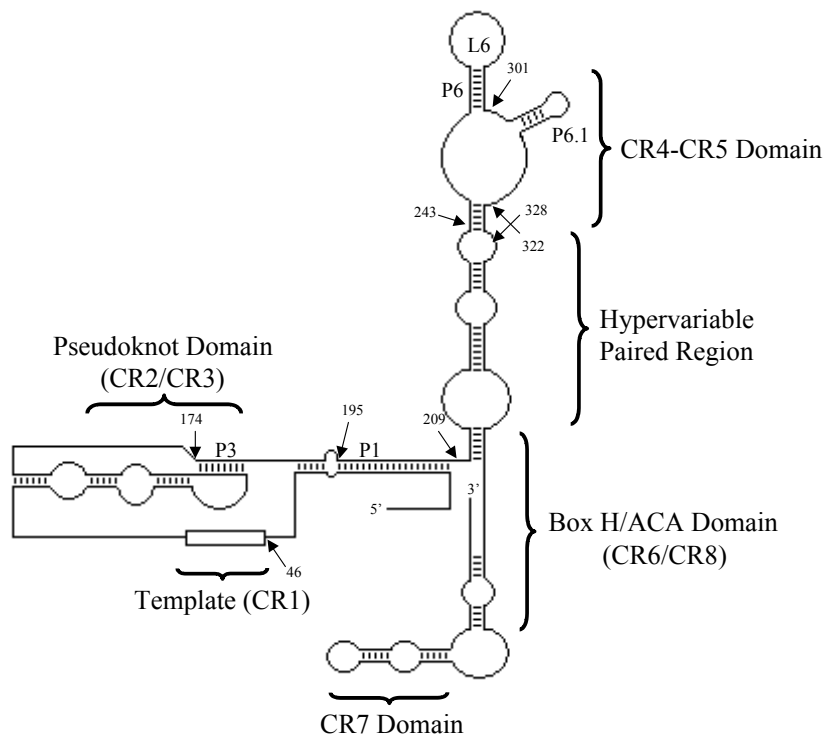
Importantly, hTERT expression usually correlates closely with telomerase activity, cancer initiation, progression, and metastasis (Cong et al., 2002). In fact, while hTR and other required subunits of the telomerase holoenzyme are ubiquitously expressed in most cells, many independent studies have shown that hTERT is the limiting determinant of telomerase activity (Meyerson et al., 1997; Weinrich et al., 1997; Ito et al., 1998; Kanaya et al., 1998; Nakayama, J. et al., 1998; Takakura et al., 1998). Furthermore, elevated telomerase levels oftentimes directly correlate with poor tumor prognosis and vice versa, though this is not the case for all cancer cell types (Granger et al., 2002).

The hTERT gene contains 16 exons and 15 introns and can be differentially spliced (Kilian et al., 1997) to generate both the full-length protein as well as multiple splice variants, however, only the full-length transcript has been found to correlate with telomerase activity (Ulaner et al., 1998; Ulaner et al., 2000). Although the expression of the variants is deliberate and may serve a dominant negative function, the role of the alternately spliced hTERT mRNAs remains unknown (Ulaner et al., 2001).

### 3. The RNA subunit of human telomerase, hTR (human Telomerase RNA)

hTR was the first core component of human telomerase to be cloned (Blasco et al., 1995; Feng et al., 1995). Unlike hTERT, hTR is typically expressed ubiquitously in most tissues, even those lacking telomerase activity (Blasco et al., 1995; Avilion et al., 1996; Harrington, 2003). The mature human transcript is 451 nucleotides long and is transcribed by RNA Polymerase II (Feng et al., 1995).

Extensive comparative phylogenetic analysis of the vertebrate telomerase RNA allowed a proposed secondary structure of hTR (Chen, J. L. et al., 2000). Vertebrate telomerase RNAs contain several conserved regions including the template-containing pseudoknot domain and the CR4-CR5 domain (Figure 1.3). Previous studies have shown that the



**Figure 1.3 Human telomerase RNA.** Conserved regions (CR) of hTR are indicated. Pairing regions (P) indicated include the P3 pairing region, which is part of the pseudoknot domain, and the P6.1 stem-loop, which is part of the CR4-CR5 domain. Arrows indicate nucleotides that are referred to in the text. This structure was adapted from Chen, J.L. et al., 2000 and 2002.



pseudoknot and CR4-CR5 domains are essential for telomerase activity and interact separately with hTERT (Tesmer et al., 1999; Beattie et al., 2000; Mitchell and Collins, 2000; Bachand and Autexier, 2001; Martin-Rivera and Blasco, 2001; Chen, J. L. et al., 2002b; Chen, J. L. and Greider, 2003). In addition, Chen et al. revealed the presence of an additional secondary structure within the CR4-CR5 domain of hTR, the P6.1 stem-loop (nucleotides 302-314), which appears to be essential for telomerase activity as well as for binding to hTERT (Chen, J. L. et al., 2002b). The roles of hTR were further extended by the work of Ly et al. (Ly et al., 2003) and Ren et al. (Ren et al., 2003). Ly et al. showed that hTR can homo-dimerize via the P3 pairing region to form a *trans*-pseudoknot and that mutations preventing the P3-*trans* interaction led to loss of enzymatic activity but did not appear to prevent binding of hTR to hTERT (Ly et al., 2003). Similarly, Ren et al. propose that hTR can homo-dimerize via the internal loop J7b/8a within the CR7 domain to form a "kissing complex," which may be functionally important for hTR accumulation and telomerase holoenzyme assemblage in vivo (Ren et al., 2003). Theimer et al., however, reported evidence contradictory to the previously proposed hTR dimerization models (Theimer et al., 2005). Using NMR spectroscopy to solve the solution structure of a fragment of the human telomerase RNA pseudoknot they showed that telomerase activity actually correlates with the presence of a conserved pseudoknot tertiary structure and not with pseudoknot-mediated hTR dimerization (Theimer et al., 2005).

#### 4. Telomere structure and function

The human telomere ranges in length from 5-15 kilobases (White et al., 2001) of the repeated sequence 5'-TTAGGG/AATCCC and 75-600 nucleotides of a 3' single-stranded

overhang (McElligott and Wellinger, 1997; Wright et al., 1997). Telomeric DNA, which is not replicated efficiently by the normal DNA replication machinery, is lost at a rate of 50-100 base pairs per cell cycle in cells with limited telomerase activity. With the help of various specialized telomere-binding proteins, such as TRF1, TRF2 and Pot1, the single-stranded portion of the telomere can be inserted into the double-stranded portion to form what is called a t-loop (Griffith, J. D. et al., 1999). The telomeric repeat binding factors, TRF1 and TRF2 (van Steensel and de Lange, 1997; van Steensel et al., 1998; Griffith, J. D. et al., 1999; Smogorzewska et al., 2000; Stansel et al., 2001), bind to the double-stranded region of the telomere while Pot1 (protection of telomeres) (Baumann and Cech, 2001; Colgin et al., 2003; Loayza and De Lange, 2003) binds to the single-stranded region and plays a critical role in both t-loop integrity and regulating telomere length. The t-loop therefore appears to function as a protective "cap" to guard the telomere from degradation, chromosome end-to-end fusions and mistaken DNA repair (Griffith, J. D. et al., 1999; Cong et al., 2002). Though telomere shortening has been found to induce cellular senescence, it is actually the disruption in the telomeric capping structure that alters the cell's replicative potential (Karlseder et al., 2002). In other words, when a cell's telomeres get too short the t-loop cannot form and a signal triggers the cell to enter senescence. Therefore, telomere capping is extremely important for cell viability as loss of capping can lead to genetic instability and cell death (Rhodes et al., 2002).

## 5. Telomerase-associated proteins

Though human telomerase is minimally composed of hTR and hTERT, there are other proteins that associate with the holoenzyme and are required for in vivo telomerase regulation and activity (Cong et al., 2002). The active telomerase complex is actually a

multi-subunit holoenzyme containing hTERT, hTR, Hsp90, p23, Hsp70, p60 and Hsp40/ydj, Ku, TEPI and dyskerin, as well as other telomerase-associated proteins (Forsythe et al., 2001; Cong et al., 2002; Harrington, 2003). The addition of these and other factors to in vitro experiments, however, is not necessary when expressing hTERT in reticulocyte lysate as the reticulocyte lysate already contains these factors (Holt et al., 1999; Cong et al., 2002).

#### a. Hsp90

The molecular chaperone Hsp90, which directly associates with hTERT, has a demonstrable role in establishing telomerase activity both in vitro and in vivo, and previous reports indicate that Hsp90 is required for the reconstitution of telomerase activity from recombinant hTERT and hTR (Holt et al., 1999). Hsp90, perhaps the most abundant housekeeping protein, is believed to account for almost 2% of all cytosolic proteins. The human chaperone exists as two isoforms, Hsp90 $\alpha$  and Hsp90 $\beta$ , which share 85% identity, however, no functional differences have been identified (Hickey et al., 1986). The main function of Hsp90 is to stabilize its clients in denaturing environments and times of stress, however, the unwavering involvement of this heat shock protein in membrane translocation, turnover, folding and activation of numerous targets reveals a much larger responsibility. Hsp90 is highly conserved in both prokaryotes and eukaryotes, and its client list includes proteins involved in signal transduction (Richter and Buchner, 2001; Pratt and Toft, 2003), steroid receptors (Cheung and Smith, 2000; Kimmins and MacRae, 2000; Smith, 2000; Bledsoe et al., 2002), kinases (Rose et al., 1987; Matts and Hurst, 1989; Stancato et al., 1993; Palmquist et al., 1994; Wartmann and Davis, 1994; Jaiswal et al., 1996; Sato et al., 2000; Pratt and Toft, 2003) and reverse transcriptases (Hu and Seeger, 1996; Holt et al., 1999), as well as many others (Pratt and Toft, 2003).

### i. Structure and function

The Hsp90 protein consists of three main domains; the N-terminus, the C-terminus and a central charged region. The N-terminal of Hsp90 is the most conserved and therefore the most studied domain in the chaperone (Prodromou et al., 1997). Though the C-terminal portion of Hsp90 is less well understood, it has been shown to contain a dimerization domain that is required to achieve an active functional homodimer (Iannotti et al., 1988; Minami et al., 1991). Both the N-terminal (Grenert et al., 1997; Prodromou et al., 1997; Stebbins et al., 1997) and C-terminal (Marcu et al., 2000a; Garnier et al., 2002; Soti et al., 2002) contain ATP-binding pockets. The central charged region acts as a flexible linker and is believed to mediate cross-talk between the two termini (Scheibel et al., 1999; Marcu et al., 2000a). There is also some evidence that this middle segment regulates access of nucleotides to the N- and C-termini by interacting with the  $\gamma$ -phosphate of ATP (Marcu et al., 2000a; Soti et al., 2002; Meyer et al., 2003).

The N-terminal pocket shares sequence homology with MutL proteins, histidine kinase and bacterial gyrase B, an ATP-dependent DNA topoisomerase (Dutta and Inouye, 2000). Hydrolyzable ATP must be able to bind to this domain in order to convert Hsp90 into its active conformation capable of binding client proteins and co-chaperones (Grenert et al., 1997; Prodromou et al., 1997; Stebbins et al., 1997). In fact, binding of a non-hydrolyzable ATP analog to Hsp90 or chaperone mutations resulting in the loss of ATP binding capabilities eradicates Hsp90 function both in vitro and in vivo (Obermann et al., 1998; Panaretou et al., 1998; Grenert et al., 1999). When the N-terminal ATP binding site is unoccupied, the chaperone is “open” and able to bind clients. As ATP binds, the chaperone changes conformation to a “closed” state and “clamps” down on its client (Prodromou et al.,

2000; Richter et al., 2002). In fact, it is believed that the two N-termini of the homodimer interact transiently upon ATP binding, and that this interaction is required for ATP hydrolysis (Maruya et al., 1999; Prodromou et al., 2000). Furthermore, additional evidence suggests that the capturing of clients is also dependent on the participation of Hsp90-associated co-chaperones (Wegele et al., 2004; Young et al., 2004).

The C-terminal of Hsp90 is responsible for holding the homodimer in an antiparallel arrangement (Maruya et al., 1999). The dimer formation of this chaperone is required for functionality as C-terminal truncations have been shown to prevent ATP hydrolysis (Prodromou et al., 2000). This terminus, like the N-terminal, is capable of binding ATP. Some data suggests that the C-terminal binding site only becomes available for nucleotide binding once the N-terminal ATP binding site is occupied (Chiosis et al., 2004). Because Hsp90 exists as a dimer and both termini are capable of binding substrate, it is possible that the functional homodimer may be capable of binding four substrates simultaneously (Pratt and Toft, 2003).

## ii. Hsp90-associated proteins

Although Hsp90 is typically the major player in chaperoning cellular activities it does not act alone, but rather functions in larger multi-protein complexes with accessory proteins and other chaperones. One of the most important partners of Hsp90 is the acidic phosphoprotein p23 (Johnson and Toft, 1994). This 23-kDa protein is known to bind to the N-terminal domain of ATP-bound Hsp90 (Sullivan et al., 1997; Fang et al., 1998; Chadli et al., 2000). Studies have also provided evidence that additional p23 binding sites on Hsp90 exist outside of the N-terminus though the precise locations have yet to be identified (Grenert et al., 1997; Chadli et al., 2000). Like Hsp90, p23 is also required for the reconstitution of

active telomerase from recombinant sources (Holt et al., 1999). The principal function of p23 is to suppress Hsp90's ATPase activity and thus aid Hsp90 in binding and holding onto its clients by maintaining the chaperone in a closed state (McLaughlin et al., 2002; Panaretou et al., 2002). The presence of p23, however, is not essential for functional assembly of Hsp90 complexes (Johnson and Toft, 1994; Bohen, 1998).

Hsp70, another cellular chaperone involved in protein folding and preventing protein aggregation, is another Hsp90-associated protein. This heat shock protein binds to the C-terminus of Hsp90 (Young et al., 1998; Carrello et al., 1999; Marcu et al., 2000a; Murphy, P. J. et al., 2001) and is necessary to run the ATP hydrolysis cycles of Hsp90 (Wegele et al., 2004; Young et al., 2004). In fact, both Hsp90 and Hsp70 are known to work in conjunction in order to prepare certain clients, such as steroid receptors, for ligand binding. It is believed that, in the case of steroid receptors, Hsp70 first binds to the client in an ATP-dependent step to "prime" the receptor, followed by transfer of the client to Hsp90 in a second ATP-dependent step, which fully activates the receptor (Morishima et al., 2001; Hernandez et al., 2002; Kanelakis et al., 2002). Furthermore, Hsp70 is released from the Hsp90 multi-protein chaperone complex during the assembly of steroid receptors (Smith, 1993). Similarly, in the case of human telomerase, Hsp70 is only transiently associated while Hsp90 and p23 are stably associated with the holoenzyme (Forsythe et al., 2001). As for p23, the precise binding site(s) for Hsp70 on Hsp90 have not been mapped out, however, recent studies are beginning to shed some light on this question (Young et al., 1997; Scheibel et al., 1998).

Evidence reveals that both Hsp70 and Hsp90 are essential components of the active chaperone complex while p23 is non-essential, though the presence of p23 has the potential to increase the kinetics of substrate binding. There are a number of other Hsp90-associated

proteins that are involved in client maturation, however, they will not be discussed in detail here.

### iii. Hsp90 and cancer

Over-expression of Hsp90 and other heat shock proteins have been found to lead to poor prognosis in a variety of cancers (Jameel et al., 1992; Ciocca et al., 1993; Yano et al., 1996; Conroy et al., 1998). Targeting Hsp90 as an anticancer approach has therefore received much attention (Beliakoff and Whitesell, 2004; Chiosis et al., 2004; Workman, 2004). Because Hsp90 is ubiquitously expressed in all cell types, normal and cancerous, it is also the functions of Hsp90 and not only its presence that defines its involvement in cancer (Bagatell and Whitesell, 2004). Hsp90 has been shown to stabilize mutated proteins found in cancer cells which contribute to apoptotic pathways. For example, this heat shock protein chaperones Akt (Sato et al., 2000; Basso et al., 2002), NF- $\kappa$ B (Chen, G. et al., 2002a), survivin (Fortugno et al., 2003), Raf-MAPK (Schulte et al., 1995), Apaf-1 (Pandey et al., 2000), IKK $\alpha/\beta$  (Lewis et al., 2000), TNF- $\alpha$  (Zhao and Wang, 2004) and many other clients to maintain transformed cells in an immortalized state. By buffering these mutations, Hsp90 acts as an anti-apoptotic and growth promoter contributing to tumor cell survival (Chiosis et al., 2004).

Because of Hsp90's role in so many different cancer progression pathways, it is hypothesized by some that inhibitors of this chaperone have the potential to induce a wide-range of anticancer effects (Vilenchik et al., 2004). The use of Hsp90 inhibitors as a means of cancer therapy, however, has revealed indiscriminate toxicity for normal and malignant cells as the chaperone is present in all cell types and is involved in so many cellular pathways (Supko et al., 1995). Structure-activity relationship studies on Hsp90 inhibitors, combined

with the fact that transformed cells express higher levels of Hsp90 than normal cells, has led to the identification of new drugs that are selective for cancer cells and display little target-associated toxicity (Schulte and Neckers, 1998). It is also believed that cancer cells exercising Hsp90-mediated mutant protein stabilization may be more sensitive to toxicity than normal cells (Chiosis et al., 2004). Furthermore, it was determined that some Hsp90 inhibitors exhibit dose limiting toxicity (Goetz et al., 2003). Inhibiting Hsp90 has been found to not only prevent stabilization, but also initiate ubiquitination of its clients, eventually leading to growth arrest (Xu, W. et al., 2002; McDonough and Patterson, 2003). Additionally, the signal transduction inhibitor hypericin causes ubiquitination of Hsp90 leading to the release and proteasome-independent degradation of clients such as mutant p53 (Blank et al., 2003).

#### iv. Hsp90 inhibition

Functional activation of Hsp90 as well as substrate and client binding are not permanent but require multiple ATP hydrolysis cycles to maintain. Therefore, targeting the ATP binding sites of Hsp90 is a widely accepted anticancer approach.

One class of compounds known to bind the N-terminus of Hsp90 is benzoquinone ansamycin antibiotics, including geldanamycin (GA) and herbimycin (Whitesell et al., 1994; Stebbins et al., 1997; Neckers et al., 1999; Roe et al., 1999). The most studied of these compounds is GA. Described as an ATP/ADP mimetic (Prodromou et al., 1997) or a competitive inhibitor of client-protein binding, GA interacts with the N-terminal ATP binding site, thus displacing the nucleotide (Grenert et al., 1997). This release of ATP disrupts the Hsp90 chaperone complexes on steroid hormones and results in the down-regulation of a number of oncogenic client proteins (Whitesell et al., 1994; Smith et al.,



1995). GA binding to Hsp90 also causes the release of p23 from the chaperone (Prodromou et al., 1997). This is believed to occur because GA seizes Hsp90 in its ADP-dependent state, which is incapable of binding p23 (Johnson and Toft, 1995; Grenert et al., 1997; Sullivan et al., 1997). In human telomerase, the same effect is seen as the Hsp90 interaction, but not the p23 interaction, is maintained with hTERT in the presence of GA (Holt et al., 1999). GA has also been shown to promote degradation of the heat shock protein's clientele prior to their complete activation by preventing Hsp90-client dissociation (Schneider et al., 1996; Schulte et al., 1997). Specifically, GA treatment of H1299 cells resulted in the perturbation of Hsp90 and the ubiquitination and degradation of hTERT in a proteasome-dependent fashion (Kim, J. H. et al., 2005). Besides being used to elucidate the roles of Hsp90, GA has also been tested as a potential therapeutic drug. In certain cell lines this ansamycin causes growth arrest, differentiation and apoptosis (Hostein et al., 2001; Munster et al., 2001). However, despite being a potent inhibitor of cellular Hsp90, clinical testing of GA and related compound has revealed a significant toxicity profile (Supko et al., 1995). Therefore, in terms of cancer therapy, new compounds had to be developed. One such compound is the GA derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG) (Schulte and Neckers, 1998). 17-AAG, now in phase II of FDA clinical trials, exhibits potent anti-Hsp90 activity at sub-toxic doses in various animal models (Kelland et al., 1999; Solit et al., 2002).

Aside from GA, another commonly used Hsp90 inhibitor employed to elucidate the cellular functions of Hsp90 is novobiocin (NB). NB is a coumarin-type antibiotic and a known inhibitor of DNA gyrase B. As opposed to GA, NB binds to the C-terminus of Hsp90 (Marcu et al., 2000a; Marcu et al., 2000b; Soti et al., 2002). Upon NB treatment, Hsp90 releases its interactions with both Hsp70, whose binding site overlaps the NB binding site,

and p23 (Young et al., 1998; Carrello et al., 1999; Marcu et al., 2000a). This fact had led to the hypothesis that NB may therefore provoke additional deleterious effects on Hsp90 function as compared to GA (Marcu et al., 2000a), which only induces the release of p23 from Hsp90 (Prodromou et al., 1997), though this premise has yet to be substantiated. Studies have also shown that NB binding to the carboxy terminus of Hsp90 prevents both nucleotide binding (Pratt and Toft, 2003) and GA binding (Marcu et al., 2000b) to the amino terminus, providing evidence for collaboration between the two termini. In cells, NB undermines a number of Hsp90-associated client proteins including Her2, Raf-1, v-src and mutant p53 and downregulates multiple downstream targets, though it binds to Hsp90 with poor affinity (Marcu et al., 2000a; Marcu et al., 2000b).

#### 6. The secondary role of telomerase in tumorigenesis

As previously discussed, telomerase aids in tumorigenesis by extending telomeric DNA and thus maintaining telomeric integrity (Masutomi and Hahn, 2003). Interestingly, however, recent evidence suggests that telomerase is involved in a secondary, anti-apoptotic role in tumorigenesis (Cao et al., 2002; Stewart et al., 2002). Cao et al. demonstrated that breast cancer cells undergoing apoptosis could be rescued by the expression of a mutant hTERT that lacked enzymatic activity and thus did not have the ability to elongate telomeres (Cao et al., 2002). The work by Stewart et al. also suggests that telomerase plays at least two roles in tumorigenesis (Stewart et al., 2002). Their research focused on an alternative mechanism of telomere maintenance (ALT – Alternative Lengthening of Telomeres) and its ability to substitute for telomerase expression. The ALT pathway, which is found in 7-10% of human cancers, elongates telomeres by inter-chromosomal recombination (Stewart et al., 2002; Reddel, 2003). They showed that ALT could not fully replace telomerase function in a

cell transformation assay (Stewart et al., 2002). Together, these independent reports suggest that telomerase has a secondary, anti-apoptotic role in tumorigenesis separate from its telomere-elongating activity (Saretzki, 2003). One proposed explanation for these results is that telomerase is involved in telomere capping (Blackburn, E., 1999; Blackburn, E. H., 2000; Blackburn, E. H., 2001; Masutomi et al., 2003). Therefore, the mere presence of telomerase, whether it is active or not, may elicit an anti-apoptotic effect as it interacts with and helps to maintain functional telomeres (Saretzki, 2003). We predict that inducing telomerase misassembly will alter these other roles of telomerase in cancer biology.

## 7. Regulation of telomerase

Telomerase regulation is a multi-faceted process taking place at the levels of transcription, hTERT maturation, subcellular localization and telomeric accessibility. Because hTERT expression is the rate-limiting factor in determining enzymatic activity, its regulation is of particular importance. In most normal cells hTERT is transcriptionally repressed. During immortalization, the gene is activated and upregulated allowing for unlimited cell proliferation.

### a. Transcriptional regulation

#### i. Positive regulators of hTERT transcription

Transcriptional regulation of hTERT at its promoter is thought to be a major determinant in protein expression (Meyerson et al., 1997; Nakamura et al., 1997). In fact, hTERT transient transfection with a promoter-luciferase reporter reveals that the hTERT promoter is active in immortalized cells, but inactive in normal cells (Cong et al., 1999; Takakura et al., 1999). The promoter contains many transcription factor binding sites that

have both positive and negative regulatory connotations (Cong et al., 1999). One such binding site is that for Myc/Mad, which contains an E-box and is a transcriptional target of the oncogene *c-myc* (Wang, J. et al., 1998; Greenberg et al., 1999; Wu et al., 1999). *c-Myc* has been linked to a number of different human cancers as it promotes proliferation, growth and apoptosis (DePinho et al., 1991; Grandori et al., 2000). In normal human mammary epithelial cells and primary fibroblasts, as well as other cell types, *c-myc* induction has been shown to correlate with hTERT expression (Wang, J. et al., 1998). Conversely, *c-myc* and hTERT are typically downregulated in non-proliferative cells. Furthermore, over-expression of the *c-Myc* antagonist Mad1 results in the down-regulation of hTERT (Gunes et al., 2000; Oh, S. et al., 2000). Although *c-myc* expression is an important aspect of hTERT regulation, additional transcription factors are usually required to account for the full transforming activity of *c-Myc*.

The general transcription factor Sp1 is also involved in hTERT up-regulation. It does so by binding GC-boxes within the core of the hTERT promoter (Kyo et al., 2000). In fact, these Sp1 binding sites are necessary components of the hTERT promoter as evidenced by the fact that mutations in the GC-boxes eradicate promoter activity (Cong and Bacchetti, 2000; Kyo et al., 2000). The reason for this requirement is because the hTERT promoter contains no TATA box. Sp1 therefore helps to initiate transcription of the hTERT promoter, as well as other TATA-less promoters, by binding general transcription machinery such as the TATA-box binding protein (TBP) and associated factors (Pugh and Tjian, 1991; Hoey et al., 1993; Emili et al., 1994). Sp1 has also been found to work in concert with *c-myc* in order to initiate hTERT transcription (Kyo et al., 2000).

One factor known to activate hTERT transcription independent of *c-myc* induction is the human papillomavirus 16 E6 protein (Gewin and Galloway, 2001; Oh, S. T. et al., 2001; Veldman et al., 2001). The protein's oncogenic variants have been found to induce telomerase activity in primary human keratinocytes and mammary epithelial cells (Klingelutz et al., 1996).

Several sex hormones have also been linked to telomerase activity regulation. For example, estrogen activates telomerase through direct and indirect transcriptional activation of hTERT expression in hormone-sensitive tissues such as mammary epithelial cells that express the estrogen receptor (Kyo et al., 1999; Misiti et al., 2000). Human ovary epithelial cells also show hTERT mRNA induction in the presence of the hormone, however, this transcriptional activation is dependent on the -950 estrogen response element of estrogen receptor  $\alpha$  and not estrogen receptor  $\beta$  (Misiti et al., 2000). The hTERT promoter can also be activated in MCF-7 breast cancer cells. Estrogen in these cells has been found to have an indirect effect on hTERT activation via *c-myc* induction (Kyo et al., 1999). Furthermore, the estrogen-specific initiation of hTERT transcription has also been confirmed by demonstrating that the anti-estrogen drug tamoxifen reduces telomerase activity in certain cell lines (Aldous et al., 1999; Nakayama, Y. et al., 2000). Progesterone, another sex hormone, also has an antagonistic effect on estrogen-induced hTERT expression (Wang, Z. et al., 2000). Though the mechanism of progesterone-mediated hTERT regulation is complex and still widely unknown, evidence suggests that progesterone targets the hTERT promoter and that the reversal of estrogen-mediated hTERT activation by progesterone may be indirect (Wang, Z. et al., 2000; Cong et al., 2002). Androgens also possess regulatory capabilities over hTERT, though, as is the case for progesterone, the mechanism of telomerase activation has yet to be

fully elucidated. Androgen depletion in androgen-dependent cell lines, but not androgen-independent cell lines, has been found to reduce telomerase activity. This activity in androgen-sensitive cells, however, can be restored by the addition of testosterone as androgen signaling upregulates hTERT expression (Guo et al., 2003).

## ii. Negative regulators of hTERT transcription

Transcriptional repression of hTERT is thought to be the main reason that most normal somatic cells do not express telomerase activity. Transformation of cancerous cells, therefore, is usually due to the loss of such repression. This hypothesis is supported by results from cell fusion experiments in which normal somatic cells repress telomerase activity in telomerase-positive immortalized cells (Wright et al., 1996a; Ishii et al., 1999). These results suggest that normal somatic telomerase-negative cells may express transcriptional repressors of hTERT (Shay, 1999; Cong et al., 2002).

Some normal human chromosomes contain transcriptional repressors of hTERT. Transfer of chromosomes 3 (Oshimura and Barrett, 1997) 6 (Steenbergen et al., 2001) and 10 (Nishimoto et al., 2001) into various human telomerase-positive cancer cell lines has been shown to downregulate telomerase activity and cause telomere shortening, suggesting the presence of alleged telomerase repressors on these chromosomes. Aside from these as yet undiscovered transcriptional repressors of hTERT, many transcription factors have already been identified as negative regulators of hTERT transcription.

One negative regulator already mentioned above is Mad 1. Mad, c-Myc and Max are transcription factors that dimerize in different combinations and bind to the E-box on the hTERT promoter to either upregulate or downregulate hTERT expression. While Max is expressed ubiquitously, c-Myc is usually upregulated in cancer cell lines and tumor cells and

Mad is upregulated in telomerase-negative normal somatic cells. The expression and dimerization of Myc and Mad with Max therefore results in increased or decreased hTERT transcription, respectively (Xu, D. et al., 2001).

Another negative regulator of hTERT transcription is the tumor suppressor p53. This 53-kDa protein is of central importance in terms of cellular transformation and immortality as over 50% of all human cancers contain dysfunctional p53 (Hollstein et al., 1994; Asker et al., 1999). p53 functions in part by inducing cell cycle arrest or apoptosis in order to keep uncontrolled growth in check (Levine, 1997). As a transcription factor, p53 perturbs telomerase activity by transcriptionally repressing hTERT, possibly independent of cell cycle arrest or apoptosis (Kusumoto et al., 1999; Kanaya et al., 2000; Xu, D. et al., 2000b). Counter intuitively, the transcription factor Sp1, an activator of hTERT transcription, is required for p53-dependent hTERT repression (Kanaya et al., 2000; Xu, D. et al., 2000b). Though it has been established that Sp1 binding to the hTERT promoter is impeded by p53 binding to Sp1, the precise mechanism of p53-mediated transcriptional repression of hTERT remains a conundrum (Xu, D. et al., 2000b). Several hypotheses have been proposed including the notion that p53 and Sp1 may impede the access of transcriptional activators (Avantaggiati et al., 1997) or recruit repressor complexes to the hTERT promoter (Murphy, M. et al., 1999).

p53 works closely with two other transcription factors during cell cycle regulation. pRB (Nguyen and Crowe, 1999; Crowe and Nguyen, 2001) and E2F (Henderson et al., 2000), when over expressed, have the potential to repress hTERT transcription in partnership with one another and possibly even independently. Despite the fact that there is no consensus mechanism of pRB/E2F-mediated hTERT repression, one established explanation

describes the involvement of histone deacetylase (HDAC) complexes (Harbour and Dean, 2000). Shown to be negative regulators of hTERT transcription (Cong and Bacchetti, 2000; Takakura et al., 2001; Xu, D. et al., 2001; Hou et al., 2002), histone deacetylases may be interacting with pRB and DNA-bound E2F at the promoter in order to stifle hTERT transcription.

Another tumor suppressor involved in hTERT repression is Wilms' tumor 1 (WT1). WT1 has been found to decrease levels of hTERT mRNA in 293 kidney cells as a result of direct interaction with the hTERT promoter (Oh, S. et al., 1999). Menin (Lin, S. Y. and Elledge, 2003), Interferon- $\alpha$  (Xu, D. et al., 2000a) and TGF- $\beta$  (Yang et al., 2001) are also negative regulators of telomerase activity, and the list continues. Transcriptional control of hTERT is undoubtedly a complex and cell cycle-dependent process involving transcription factors, hormones, oncogenic proteins, viral proteins and tumor suppressors, as well as many other known and unknown cell cycle regulators.

#### b. Posttranslational regulation

The suggestion of posttranslational hTERT modifications as a means of telomerase regulation arose when various cells lacking telomerase activity were found to express both hTR and hTERT mRNA (Liu et al., 1999; Tahara et al., 1999; Rohde et al., 2000; Ulaner et al., 2000; Minamino et al., 2001). This inconsistency supposes that telomerase activity does not always correlate with the presence of hTERT, but rather the presence of correctly modified and active hTERT. The most prevalent modification responsible for posttranslational regulation of a great deal of proteins including hTERT is reversible phosphorylation.



Activators and inhibitors of various kinases have been shown to be determinant factors in telomerase activity regulation. Protein kinase C (PKC) is a phospholipid-dependent kinase that functions to regulate growth, differentiation and carcinogenesis. In certain cells types, the PKC inhibitor phorbol myristate acetate upregulates telomerase activity and the PKC inhibitor bisindolylmaeimide I downregulates telomerase activity (Bodnar et al., 1996). Similarly, activators and inhibitors of phosphatases have the opposite effect on hTERT modification. Phosphatase 2A has been shown to downregulate telomerase activity in a breast cancer cell line. Conversely, okadaic acid upregulates telomerase activity in vivo by means of phosphatase 2A inhibition (Li et al., 1997). These reports, as well as others, demonstrate that phosphorylation of hTERT is a common and effective means of telomerase activity regulation, though the effectiveness is PKC isoform-dependent (Ku et al., 1997; Li et al., 1998; Yu et al., 2001).

A second kinase with significant involvement in hTERT regulation is Akt protein kinase, or protein kinase B. It has been suggested the hTERT protein contains two distinct phosphorylation sites, which, when phosphorylated by Akt kinase, increase telomerase's in vitro enzymatic activity (Kang et al., 1999). Accordingly, wortmannin downregulates cell-type specific telomerase activity via Akt kinase inhibition (Kang et al., 1999).

The tyrosine kinase c-Abl also possesses hTERT phosphorylation potential, however, its effects on telomerase activity oppose those of protein kinase C and Akt kinase. Via its SH3 domain, c-Abl directly interacts with and phosphorylates hTERT resulting in a decrease in telomerase activity (Kharbanda et al., 2000). Therefore, cells over-expressing c-Abl will typically have low levels of telomerase activity whereas cells lacking c-Abl will show signs of upregulated telomerase expression and increased telomere length.

Importantly, experiments studying T-lymphocyte activation revealed that the up-regulation of telomerase activity after hTERT phosphorylation may be the result of subcellular localization of hTERT from the cytoplasm into the nucleus (Liu et al., 2001). It is believed that phosphorylating hTERT tags it for nuclear import to bring it into close contact with the chromosomes and extend the telomeres.

### c. Telomeric accessibility

Telomere accessibility is the salient determinant in telomerase activity regulation. If the 3' overhang of the chromosome is not exposed and available for telomerase-mediated extension, then telomerase activity is not detectable. The formation of a t-loop at the end of the telomere dictates accessibility and controls telomere homeostasis. The accessibility of the 3' overhang of the telomere to telomerase is therefore the last line of regulation and is accomplished by the involvement and collaboration of a number of telomeric binding proteins (Evans and Lundblad, 2000; de Lange, 2002).

The telomeric repeat binding factors TRF1 and TRF2 are the chief components of telomere homeostasis (van Steensel and de Lange, 1997; Smogorzewska et al., 2000). As mentioned above, these two proteins bind duplex telomeric DNA. The over-expression of either factor causes telomere shortening in telomerase-positive cells as a result of preventing telomere-telomerase interaction (Smogorzewska et al., 2000). The role of TRF1 is to evaluate and regulate telomere length at each chromosome (van Steensel and de Lange, 1997; Smogorzewska et al., 2000; Ancelin et al., 2002). TRF1 is also implicated in parallel pairing of telomeric tracks as it has been shown by electron microscopy to seize and arrest telomeric repeat arrays and preclude the unwinding of DNA (Griffith, J. et al., 1998). Furthermore, not only does TRF1 prevent access to the G-strand of the telomere, but it also prevents DNA

polymerase-mediated C-strand synthesis, thus inhibiting telomere elongation by multiple mechanisms (Smucker and Turchi, 2001). Over-expression of a dominant negative TRF1 mutant in telomerase-positive cells therefore initiates telomere elongation as the endogenous TRF1 is displaced from the telomere (van Steensel and de Lange, 1997; Karlseder et al., 2002). TRF2, like TRF1, when over-expressed, results in telomere shortening (Smogorzewska et al., 2000; Karlseder et al., 2002). Interestingly, some data suggests that TRF2 also has the potential to initiate a telomeric DNA degradation pathway (Ancelin et al., 2002).

TRF1 and TRF2, however, are not the only proteins involved in telomerase regulation as Pot1 also plays a role in dictating telomeric accessibility. Pot1 regulates telomere length by binding to single-stranded TTAGGG repeats at the chromosome ends (Baumann and Cech, 2001), binding to TRF1 (Loayza and De Lange, 2003) and perhaps recruiting telomerase to the telomere (Evans and Lundblad, 1999; Evans and Lundblad, 2000). Pot1 itself is actually recruited to the telomere by TRF1 (Loayza and De Lange, 2003). Furthermore, the amount of Pot1 bound to the telomere, which depends on the length of single-stranded DNA, is regulated by TRF1 (Loayza and De Lange, 2003). TRF1, which binds to the double stranded region of the telomere, evaluates overall telomere length and, through its interaction with Pot1, relays this information to the telomere terminus (Loayza and De Lange, 2003). This collaboration therefore allows Pot1 to control telomerase-mediated telomere elongation (Loayza and De Lange, 2003). By over-expressing Pot1 splice variants in telomerase-positive human cell lines Colgin et al. first revealed that Pot1 acts as a positive regulator of telomere length (Colgin et al., 2003). Lei et al. further defined the role of Pot1 as they showed that Pot1 can actually act as both a positive and negative regulator of

telomere extension in vitro (Lei et al., 2005). Depending on the number of nucleotides that are free from Pot1 binding on the 3' overhang, telomere accessibility, and therefore telomerase activity, will either be promoted or hindered (Lei et al., 2005). Additionally, Loayza et al. have shown that Pot1( $\Delta$ OB), a Pot1 mutant that is deficient in single-stranded DNA binding but still able to be recruited to the telomere by TRF1, perturbs TRF1-mediated telomere length regulation and induces telomere elongation in human HTC75 cells (Loayza and De Lange, 2003). These results are expected as Pot1( $\Delta$ OB) is unable to cap the telomere or aid in t-loop formation, thus allowing telomerase unlimited access to the telomere. There exist many other telomeric binding proteins which are no doubt associated with telomere integrity, however, the roles described here for TRF1, TRF2 and Pot1 are the most well-defined and the most compelling in terms of telomerase accessibility and regulation.

#### B. Previously documented telomerase inhibitors

Since the majority of cancer cell types require telomerase-mediated telomere extension for survival (Masutomi and Hahn, 2003), the inhibition of telomerase should result in cancer cell mortality. In order for a drug to be considered an optimal telomerase inhibitor it must act through a telomere-dependent mechanism and accomplish a number of goals leading to the death of cancer cells (White et al., 2001). The inhibitor must first decrease telomerase activity and then, following a lag phase, progressively lead to telomere shortening after each cell division. Second, the addition of telomerase inhibitors should ultimately lead to the growth arrest or death of cancer cells. Finally, if a telomerase inhibitor is in fact acting through a telomere-dependent mechanism, then the amount of time it takes a cell to decrease proliferation after being given a telomerase inhibitor should vary depending on the cell's

initial telomere length (White et al., 2001). This last requirement, however, is changing and being redefined as the new secondary roles of telomerase in tumorigenesis described above are being discovered and elucidated.

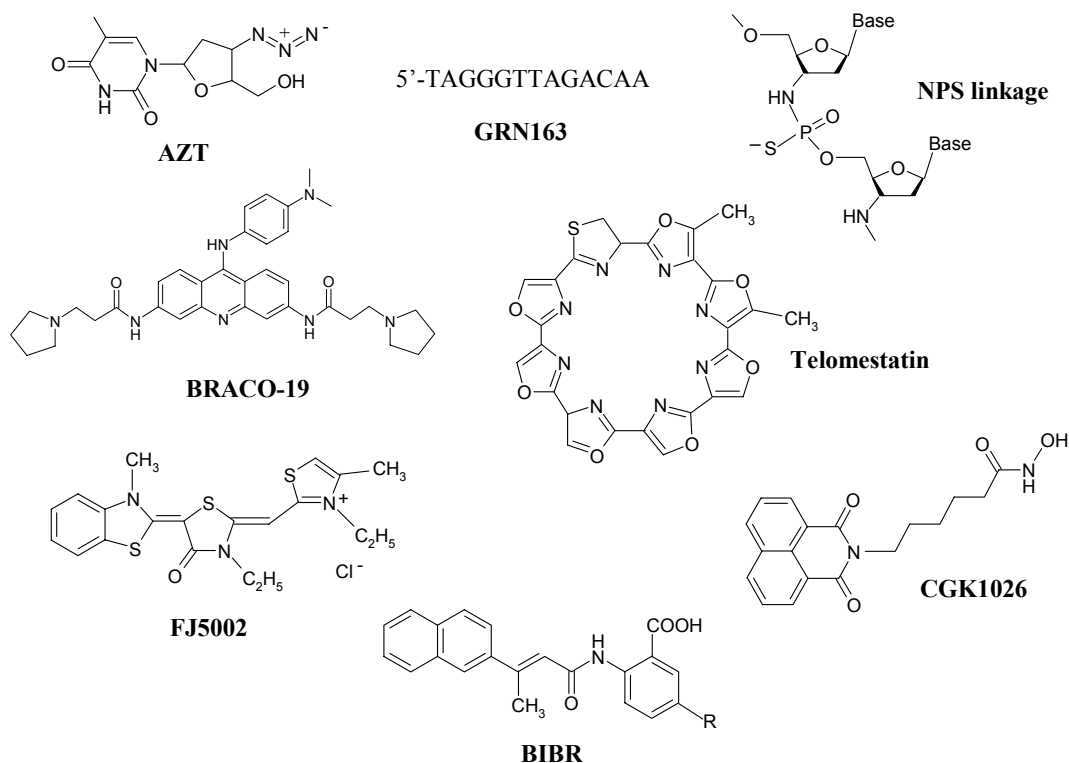
Several methods to affect telomerase inhibition have been documented (White et al., 2001; Saretzki, 2003). These include, but are not limited to, reverse transcriptase inhibitors, antisense oligonucleotides directed at the RNA subunit, particularly the template, G-quadruplex-stabilizing compounds, natural products and small molecules. These inhibitors generally target either the telomerase-catalyzed primer extension reaction or the telomerase primer (for some G-quadruplex-interacting molecules).

#### 1. Reverse transcriptase inhibitors

Reverse transcriptase inhibitors, used widely in the treatment of Human Immunodeficiency Virus (HIV), have been studied as anti-telomerase agents, though the results of these studies are inconsistent (White et al., 2001). The most extensively studied drug of this class of inhibitor in terms of telomerase inhibition is 3'-azido-3'-deoxythymidine (AZT) (Figure 1.4). Some studies using AZT revealed inhibition of telomerase activity and decreased proliferation of cells in culture, but no change in telomere length and no growth arrest (Strahl and Blackburn, 1996; Gomez et al., 1998; Melana et al., 1998; Murakami et al., 1999). Other studies using the reverse transcriptase inhibitor dideoxyguanosine did reveal telomere shortening in specific cell lines but no noticeable changes in cell viability were observed (Strahl and Blackburn, 1996). One possible explanation for these mixed results may be that these reverse transcriptase inhibitors are not acting through a selective inhibition of telomerase but are eliciting toxic effects on the cells by inhibiting mitochondrial DNA replication (White et al., 2001).

## 2. Antisense oligonucleotides directed against hTR

The use of antisense oligonucleotides targeting hTR became an attractive approach to inhibiting telomerase once Feng et al. identified the sequence of the RNA subunit in 1995 (Feng et al., 1995). The use of oligonucleotides as "drugs" has been difficult, however, because of their high cost and their low intracellular bioavailability caused by problems with cellular uptake and degradation. Recent advances in oligonucleotide modifications, though, have begun to address these problems. Several classes of modified oligonucleotides have been tested for their roles in telomerase inhibition including simple phosphodiester (Glukhov et al., 1998), 2-5A linked phosphodiester (Kondo et al., 1998), phosphorothioate-substituted molecules (Pitts and Corey, 1998; Elayadi et al., 2001), 2'-*O*-methyl molecules (Pitts and Corey, 1998), 2'-*O*-(2-methoxyethyl) (2'-MOE) molecules (Elayadi et al., 2001), N3'→P5' *thio*-phosphoramidate (NPS) molecules (Figure 1.4) (Asai et al., 2003), PNA molecules (Norton et al., 1996) and hammerhead ribozymes (Wan et al., 1998). The most promising results thus far have come from antisense oligonucleotides that target the template portion of hTR (Elayadi et al., 2001; Corey, 2002; Asai et al., 2003). By forming a stable duplex between an antisense molecule and the template, the telomeric primer is unable to interact with the telomerase complex and thus cannot be extended. Elayadi et al. used template-targeting 2'-MOE RNA molecules to inhibit telomerase and found that some had IC<sub>50</sub> values ranging from 5-10 nM in cell extracts (Elayadi et al., 2001). Asai et al. used a NPS oligonucleotide, GRN163, to target the template which yielded an IC<sub>50</sub> of ~1 nM in various tumor cell lines in the presence of carriers (e.g. Lipofectamine) (Figure 1.4) (Asai et al., 2003). GRN163, developed by Geron Corporation, not only inhibited telomerase activity in tumor cell culture but also caused telomere shortening and cellular



**Figure 1.4 Previously documented telomerase inhibitors.** GRN163 is a N3'→P5' *thio*-phosphoramidate (NPS) oligonucleotide which contains a 3-terminal amino group. BIBR1502, R=H.

senescence or apoptosis following a lag phase that correlated with initial telomere length (Asai et al., 2003). As evidence of the clinical usefulness of telomerase inhibition, Geron has filed an investigational new drug application and has begun clinical trials on GRN163. Furthermore, Djojosebroto et al. have revealed that GRN163L, a lipid-modified derivative of the parent compound also presently in human FDA clinical trials, is a more potent and efficacious inhibitor of telomerase activity and tumor growth in vitro and in vivo (Djojosebroto et al., 2005).

### 3. G-quadruplex stabilizing compounds

G-quadruplex-stabilizing compounds are promising telomerase inhibitors. G-rich, single-stranded DNA, such as that found at the end of the telomere, has been shown to form coplanar tetraplex structures. These structures, termed G-quadruplexes, are stabilized by pairs of Hoogsteen hydrogen bonds between adjacent guanine bases and are able to form both intra- and inter-molecular structures (Neidle and Read, 2000). Because telomeric DNA can fold into a quadruplex structure, quadruplex-stabilizing compounds can prevent telomerase from accessing the telomere and therefore inhibit telomere extension. A variety of G-quadruplex-interacting molecules have been tested and found to inhibit telomerase activity including anthraquinones (Sun et al., 1997; Read, M. A. et al., 1999; Read, M. et al., 2001), acridine derivatives (Harrison et al., 1999), porphyrin derivatives (Han et al., 2001; Shi et al., 2001), perylenes (Fedoroff et al., 1998; Han et al., 1999; Rangan et al., 2001), ethidium derivatives (Koeppel et al., 2001), fluoroquinophenoxazines (Duan et al., 2001) and fluorenone-based compounds (Perry et al., 1999). Though this class of telomerase inhibitor has been shown to inhibit telomerase activity and induce growth arrest, telomere shortening is often not an observed effect (Neidle and Read, 2000; White et al., 2001; Saretzki, 2003). In addition, some of these compounds have been found to exhibit nonspecific cytotoxicity and low relative affinities for quadruplex versus duplex DNA (Gowan et al., 2001). Despite these pitfalls, one potentially therapeutically relevant G-quadruplex-stabilizing compound is the natural product telomestatin (Figure 1.4), which selectively interacts with the human telomeric intramolecular G-quadruplex (Shin-ya et al., 2001; Kim, M. Y. et al., 2002). This fungal derivative interacts with the quadruplex in a 2:1 stacking complex, similar to the porphyrin-quadruplex interaction. Another promisingly potent G-quadruplex-stabilizing



compound is 9-[4-(*N,N*-dimethylamino)phenylamino]-3,6-bis(3-pyrrolidinopropionamido)acridine (BRACO-19) (Figure 1.4). This 3,6,9-trisubstituted acridine derived compound, discovered by computer modeling, has been shown to inhibit telomerase in the nanomolar range, decrease proliferation and induce senescence in a breast cancer cell line (Gowan et al., 2002). In human uterus carcinoma cells, BRACO-19 treatment results in a decrease in both hTERT expression and telomere length (Burger et al., 2005). Furthermore, this small molecule possesses low nonspecific acute cytotoxicity, enhances ubiquitin-mediated degradation of hTERT and exhibits both in vitro and in vivo antitumor activity (Gowan et al., 2002; Burger et al., 2005).

#### 4. Natural products

Aside from telomestatin mentioned above, other natural product telomerase inhibitors include tea catechins (Naasani et al., 1998), mistletoe extracts (Lyu et al., 2002) and curcumin (Ramachandran and You, 1999; Ramachandran et al., 2001; Ramachandran et al., 2002). The tea catechin epigallocatechin gallate has been found to inhibit telomerase activity, decrease proliferation and cause telomere shortening in leukemia and adenocarcinoma cells (Naasani et al., 1998). However, the precise mechanisms of these effects are speculative (Naasani et al., 1998) as tea catechins may also elicit a variety of other anticancer effects involving antioxidation (Yen and Chen, 1995) and interactions with a number of targets (Agarwal et al., 1992; Liao and Hiipakka, 1995; Suganuma et al., 1996; Hasaniya et al., 1997; Jankun et al., 1997; Lin, Y. L. and Lin, 1997). Korean mistletoe lectin downregulates telomerase activity and induces apoptosis (Lyu et al., 2002). This induction of apoptosis, though, is not solely due to telomerase inhibition due to the fact that this same extract also downregulates Bcl-2 and upregulates Bax (Lyu et al., 2002). Curcumin, the

yellow pigment found in the rhizomes of turmeric, has been found to inhibit telomerase activity in human mammary epithelial and breast carcinoma cell lines by down-regulating hTERT expression (Ramachandran et al., 2001; Ramachandran et al., 2002). Any effects on cell viability by curcumin, however, cannot be directly or completely linked with the down-regulation of hTERT because curcumin also downregulates Ki67, PCNA, and mutated p53 mRNAs in breast cancer cells (Ramachandran and You, 1999).

## 5. Small molecules

A number of telomerase inhibitors have been identified through the screening of large compound collections and combinatorial libraries. The use of large-scale screening models such as NCI-COMPARE has aided this approach. NCI-COMPARE is a bioinformatics screening model developed by Paull et al. which structurally categorizes molecules based on a given initial compound (Paull et al., 1989). Naasani et al. applied their disease-oriented screening database (Yamori, 1997) to COMPARE analysis and identified a number of compounds as potent telomerase inhibitors (Naasani et al., 1999). The most potent inhibitor found was rhodacyanine FJ5002, which yields an  $IC_{50}$  of  $\sim 2 \mu M$  (Figure 1.4). U937 human leukemia cells treated with FJ5002, which is believed to inhibit telomerase by a direct interaction, underwent telomere erosion and cellular senescence (Naasani et al., 1999).

Damm et al. describe a high-throughput endeavor to identify novel telomerase inhibitors using their non-peptide, non-nucleoside BIBR class of compounds (Damm et al., 2001). They found BIBR1532 (2-[(E)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid) to be the most potent inhibitor with an  $IC_{50}$  of 93 nM (Figure 1.4). BIBR1532 induced telomere erosion, a senescent phenotype and telomere dysfunction in multiple cell types (Damm et al., 2001). Furthermore, BIBR1532 is highly selective for telomerase as the compound had no

adverse effects on HIV-1 reverse transcriptase or various RNA polymerases or DNA polymerases (Damm et al., 2001). Additional studies revealed that BIBR1532 inhibited both recombinant and cellular telomerase activity by perturbing enzymatic processivity (Pascolo et al., 2002). Its mode of action was further defined as BIBR1532 does not exert its inhibitory effects by binding to the telomeric primer or the dNTP binding site. In fact, it is believed that this new class of telomerase inhibitor perturbs catalytic processivity by a mode similar to that for non-nucleoside HIV-1 reverse transcriptase inhibitors (Pascolo et al., 2002). The use of these and other compound collections and combinatorial libraries, combined with the development of new compound screening algorithms, will no doubt continue to expand the list of identified telomerase inhibitors.

The high-throughput screening of small synthetic organic molecules has also led to the identification of compounds that activate or derepress telomerase activity. This finding is of therapeutic relevance in the cancer field as it led to the identification of a previously unidentified mechanism of hTERT regulation. Won et al. set out to identify novel small molecules that possessed hTERT derepression capabilities. After screening a 20,000 member library from the Korea Advanced Institute of Science and Technology they discovered CGK1026 as a very promising prospect (Figure 1.4) (Won et al., 2004). Won et al. describe a mechanism of cellular hTERT repression in which E2F and pocket proteins interact with the hTERT promoter in a cell cycle-dependent manner while the recruitment of HDAC into the E2F-pocket protein complex suppresses promoter activity (Won et al., 2004). They therefore found that CGK1026 derepresses or activates hTERT expression via the disruption of HDAC-E2F-pocket protein complexes (Won et al., 2004). Accordingly,

adopting the anticancer approach of stabilizing HDAC-E2F-pocket protein interactions could prove to be a viable means to inhibit telomerase activity.

#### 6. Pitfalls of previous approaches to inhibit telomerase

Interestingly, some of these previously described telomerase inhibitors do not satisfy all of the criteria for acting through a telomere-dependent mechanism even though they inhibit telomerase activity. There are two possible explanations for these occurrences. First, the inhibition of telomerase could signal a cancer cell to initiate the ALT mechanism. The ALT mechanism could then stabilize telomere lengths and may lead to anti-telomerase resistance (Mokbel, 2003). Though it has been found that ALT cannot fully replace telomerase function (Stewart et al., 2002), the initiation of the ALT mechanism could still hamper the anti-tumorigenic effects of the telomerase inhibitor by maintaining telomere lengths. Secondly, some of these inhibitors may not be affecting telomerase's secondary role in tumorigenesis. Many of the previously described telomerase inhibitors knock out telomerase activity without significantly affecting the holoenzyme complex. Therefore, the physical presence of the intact telomerase complex may still be able to protect the telomere and thus delay, if not prevent, telomere shortening and entry into senescence or apoptosis. However, the precise mechanism of telomerase's role in preventing apoptosis has yet to be fully elucidated and may be telomere length-independent.

#### C. Targeting human telomerase assemblage

##### 1. Significance

Another aspect of telomerase biochemistry that has just recently been a major focus for drug discovery is assemblage of the ribonucleoprotein complex. Our hypothesis is that

targeting telomerase holoenzyme assemblage will create a misassembled complex that not only perturbs telomerase activity leading to telomere shortening, growth arrest and cell death, but will also interrupt the enzyme's secondary, anti-apoptotic property. If this is the case, then telomerase assemblage inhibitors could induce cancer cell death faster than the typical telomerase inhibitors discussed above (i.e. no lag phase).

#### D. Specific aims of this research

1. (Chapter II) Determine the feasibility of inhibiting human telomerase activity by affecting assemblage with hTR-targeting oligonucleotides in a direct assay.

2. (Chapter III) Test the hypothesis that known RNA-binding ligands will affect human telomerase assemblage and activity.

3. (Chapter IV) Evaluate the human telomerase assemblage inhibiting effects of the natural product tanshinone II-A and a group of novel derivatives.

4. (Chapter V) Characterize the roles of Hsp90 in human telomerase assemblage and activity.

5. (Chapter VI) Design and develop a high-throughput screen capable of identifying novel inhibitors of telomerase assemblage using scintillation proximity assay (SPA) technology.

## **Chapter II. Inhibiting telomerase assemblage with specific hTR-targeted oligonucleotides**

Adapted from: Keppler, B.R. and M.B. Jarstfer, *Inhibition of Telomerase Activity by Preventing Proper Assemblage*. Biochemistry, 2004. **43**(2): p. 334-43.

### **A. Introduction**

Several methods to affect telomerase inhibition have been documented (White et al., 2001; Saretzki, 2003). These include reverse transcriptase inhibitors (Strahl and Blackburn, 1996; Gomez et al., 1998; Melana et al., 1998; Murakami et al., 1999), G-quadruplex-stabilizing compounds (Neidle and Read, 2000), natural products (Naasani et al., 1998; Kim et al., 2002; Lyu et al., 2002), molecules screened from synthetic libraries (Hayakawa et al., 1999; Naasani et al., 1999; Damm et al., 2001) and antisense oligonucleotides directed at the RNA subunit, particularly the template (Elayadi et al., 2001). One aspect of telomerase biochemistry that has not been a major focus for drug discovery is assemblage of the ribonucleoprotein complex. We have therefore initiated a program to investigate the feasibility of inhibiting telomerase assemblage. In our initial efforts, we have specifically addressed the possibility of targeting hTR with oligonucleotides that block assemblage of the holoenzyme. Previous studies documented that non-templating portions of hTR can be targets for PNA-based inhibitors though inhibition of assemblage has not specifically been examined (Hamilton et al., 1999).

The telomerase RNA subunit has been identified from various organisms, including ciliates, mammals and yeast (Romero and Blackburn, 1991; Lingner et al., 1994; Singer and

Gottschling, 1994; Blasco et al., 1995; Feng et al., 1995; Tsao et al., 1998; Chen et al., 2000; Chen et al., 2002). A proposed secondary structure of hTR has been developed based on the sequencing work by Feng et al. (Feng et al., 1995) and the comparative phylogenetic analysis of various vertebrate telomerase RNAs by Chen et al. (Chen et al., 2000). hTR contains a number of distinct domains which are conserved among all vertebrates (see Figure 1.3). The conserved pseudoknot and CR4-CR5 domains of hTR, for example, have been shown to interact independently with hTERT and are required for telomerase activity (Tesmer et al., 1999; Beattie et al., 2000; Mitchell and Collins, 2000; Bachand and Autexier, 2001; Martin-Rivera and Blasco, 2001; Chen et al., 2002; Chen and Greider, 2003). Further evaluation of the CR4-CR5 domain soon revealed that the specific nucleotides responsible for binding to hTERT are those that constitute the P6.1 stem-loop (nucleotides 302-314) (Chen et al., 2002). hTR has also been implicated in telomerase dimerization via the P3 pairing region of the pseudoknot (Ly et al., 2003b) and the J7b/8a internal loop of the CR7 domain (Ren et al., 2003). Here, we confirm that the CR4-CR5 domain and the P3/P1 pairing region both interact autonomously with hTERT and report that targeted disruption of these interactions prior to holoenzyme assembly disrupts telomerase activity.

## B. Results

### 1. Identification of susceptible regions of hTR

To define regions of hTR that can be targeted for the disruption of telomerase assemblage, we tested a series of DNA oligonucleotides that were complementary to various regions of hTR for their ability to inhibit reconstituted telomerase activity *in vitro* (Table 2.1). Previously, the use of PNAs that were complementary to non-templating regions of

telomerase were shown to inhibit telomerase when added prior to assemblage (Hamilton et al., 1999). Because the PNA-RNA duplex is very stable, we thought that the PNAs could change the structure of the RNA subunit to inhibit telomerase as opposed to blocking hTR/hTERT interactions, which we were interested in. For example, a PNA that targeted nucleotides 1-12 was a strong inhibitor of telomerase prior to assemblage (Hamilton et al., 1999), even though these nucleotides are not required for telomerase activity (Ly et al., 2003a). This suggests that while the folded structure of hTR was perturbed, specific hTR/hTERT interactions were not inhibited. We chose to examine DNA oligonucleotides to

**Table 2.1 Summary of inhibition data with hTR-targeted oligonucleotides<sup>a</sup>**

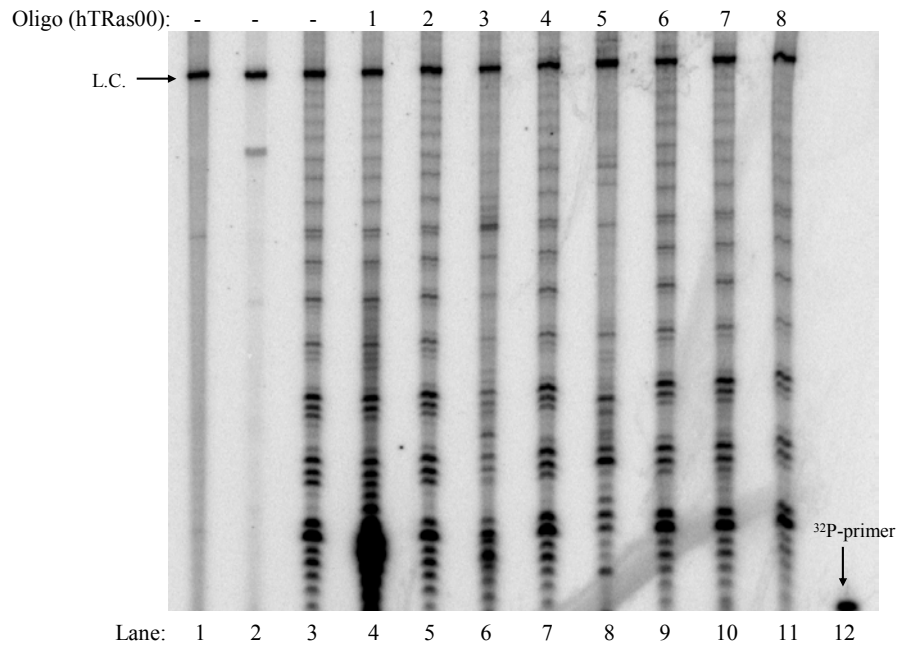
Name	Sequence	hTR region targeted	Nucleotides <sup>b</sup> targeted	% Activity <sup>c</sup>
hTRas001	5'-ATGGCAAGTCCGAATCGATCGT-3'	None	N/A	804
hTRas002	5'-TAGGGTTAGACAA-3'	Template (CR1)	42-54	97
hTRas003	5'-AAAGTCAGCGAGAAAAACAGCG-3'	Pseudoknot Domain (CR2/CR3)	94-115	97
hTRas004	5'-AACGGGCCAGCAGCTGACATT-3'	P3/P1 Pairing Region	174-195	37
hTRas005	5'-TGGGTGCCTCCGAGAAGCCCC-3'	L6 Loop	268-289	100
hTRas006	5'-CGGCTGACAGAGCCCAACTCT-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'- <u>AACGGGCCAGCAGCUGACA</u> UUU-3' <sup>d</sup>	P3/P1 Pairing Region	174-195	12
hTRas010	5'- <u>CGGCUGACAGAGCCCAACU</u> UU-3' <sup>d</sup>	CR4-CR5 Domain	301-322	18

<sup>a</sup> DNA oligonucleotides were added to hTERT and hTR prior to assemblage. Telomerase activity was determined as described in the Materials and methods. <sup>b</sup>Nucleotides within hTR that were targeted by the oligonucleotides. <sup>c</sup>"% Activity" indicates the amount of residual telomerase activity at a 1  $\mu$ M concentration compared to the primer-only control. <sup>d</sup>hTRas009 and hTRas010 are 2'-O-methyl oligonucleotides and underlined nucleotides indicate phosphorothioate linkages.

diminish this effect in an attempt to define regions of hTR/hTERT interactions that can be blocked. Eight DNA oligonucleotides (hTRas001-hTRas008) were screened for their ability to affect telomerase using an assemblage assay. All oligonucleotide inhibitors were maintained at a concentration of 1  $\mu$ M throughout the experiment. A positive control with no



oligonucleotide inhibitor was used to determine 100% activity and hTERT-only and hTR-only negative controls were used as controls for background activity (Figure 2.1). We found that only oligonucleotides hTRas004 and hTRas006, which target the P3/P1 pairing region and CR4-CR5 domain, respectively, significantly decreased telomerase activity, defined as <55% residual activity when compared to the positive control (Table 2.1 and Figure 2.1). Interestingly, hTRas008, which targets the CR7 domain, was slightly refractory towards telomerase activity (~70% residual activity). We also noted that the addition of a random



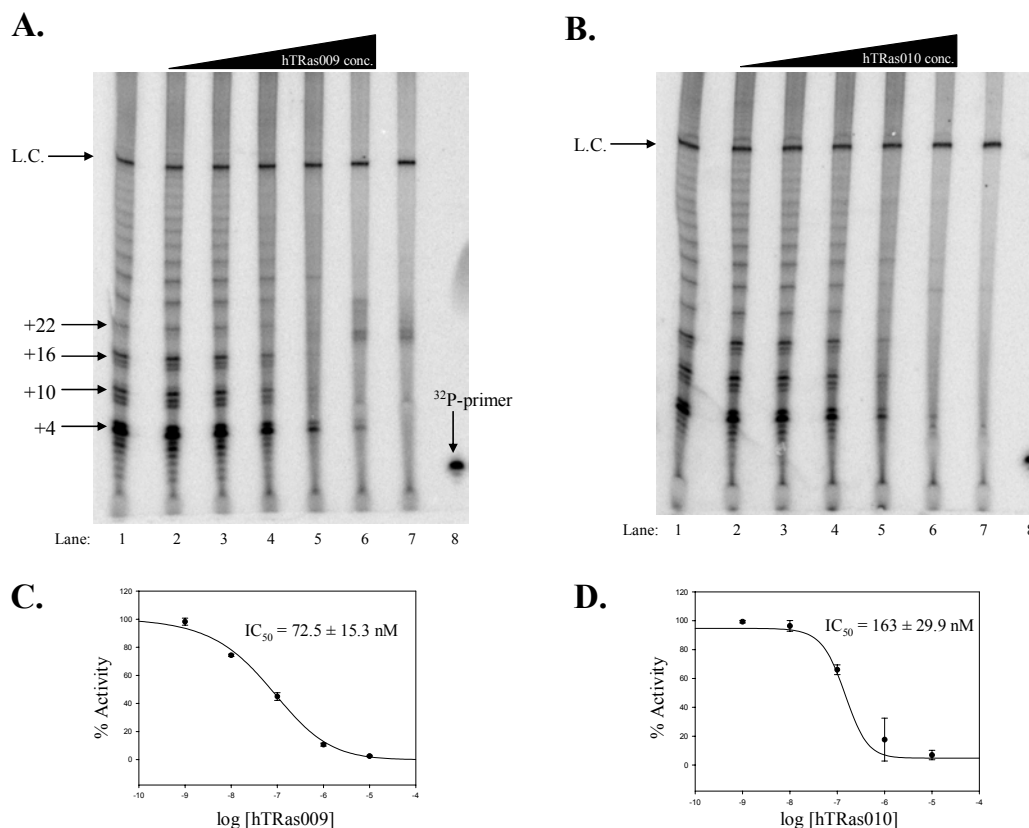
**Figure 2.1 Assemblage assay using hTR-targeted oligonucleotides.** DNA oligonucleotides were added to hTERT and hTR before assemblage and assayed for activity. Lane 1 is a protein-only control and lane 2 is a RNA-only control. Lane 12 is a <sup>32</sup>P-labeled primer to mark the beginning of primer extension. L.C. indicates loading control. Only telomerase-dependent bands were used for quantification (i.e. not bands seen in negative controls).

DNA molecule, hTRas001, resulted in increased telomerase activity (~800%). By contrast, a nonspecific PNA inhibited telomerase when added pre-assemblage with an  $IC_{50} = 30 \mu M$  (Hamilton et al., 1999), highlighting potential nonspecific effects of PNAs.

## 2. Pre- and post-assembly assays

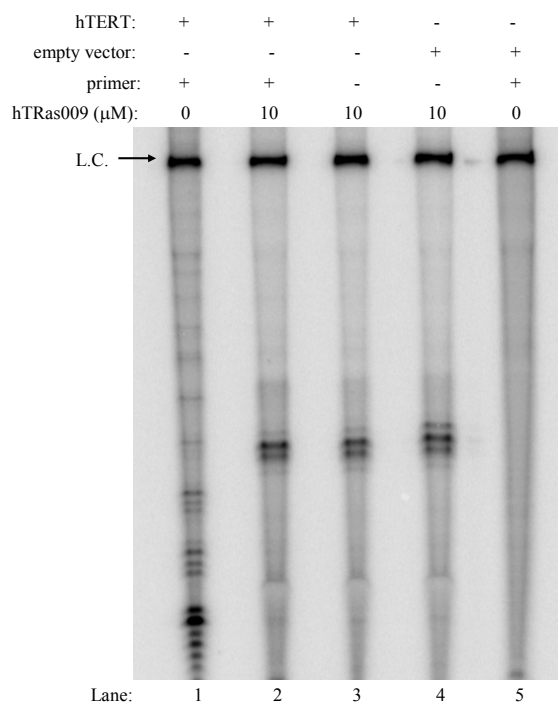
### a. Concentration dependence of hTRas009 and hTRas010 inhibition

To improve hybridization of the inhibitory oligonucleotides to hTR, 2'-*O*-methyl modifications were made at each nucleotide (Monia et al., 1993), and to afford increased nuclease resistance, phosphorothioate linkages were incorporated at the ends of each oligonucleotide (Eckstein, 2000) (Table 2.1). The modified oligonucleotides were tested for their ability to inhibit telomerase activity in an assemblage assay at a range of concentrations



**Figure 2.2 Concentration dependence of telomerase inhibition by hTRas009 and hTRas010 when added before assemblage.** hTRas009 (A) or hTRas010 (B) were added to an assemblage assay at concentrations of 1 nM, 10 nM, 100 nM, 1 μM and 10 μM (lanes 2-6 respectively). Lane 1 is a primer-only control. Lane 7 is a no-primer control. Lane 8 is a <sup>32</sup>P-labeled primer to mark the beginning of primer extension. The <sup>32</sup>P-labeled loading control (L.C.) and the human telomere primer-extension products (+4, +10, +16, +22, etc. nucleotides added) are indicated. IC<sub>50</sub> values were calculated and inhibition curves were plotted for hTRas009 (C) and hTRas010 (D) as described in the Materials and methods. IC<sub>50</sub> curves are based on results from duplicate experiments. Error bars in C and D indicate standard deviation and IC<sub>50</sub> error indicates standard error.

(Figures 2.2A and B). At high concentration (10  $\mu$ M) of hTRas009 or hTRas010 we observed a new product band in the analysis (lane 6, Figures 2.2A and 2.2B). We therefore carried out an assay with 10  $\mu$ M of oligonucleotide in the absence of a telomeric primer (lane 7, Figures 2.2A and 2.2B). These no-primer controls resulted in the production of the same products (compare lanes 6 and 7, Figures 2.2A and 2.2B). Furthermore, these artifacts were also produced in reactions catalyzed by reticulocyte lysate expressing an empty vector demonstrating that they are telomerase independent (Figure 2.3). Therefore, the bands in



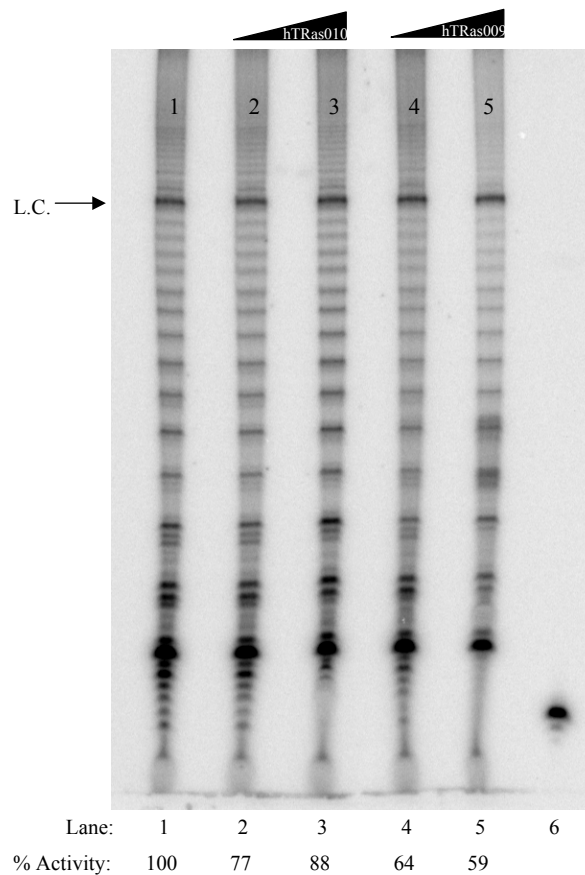
**Figure 2.3 High concentrations of hTRas009 in an assemblage assay produce telomerase-independent artifacts.** Lanes 4 and 5 contain no hTERT. L.C. indicates loading control.

lane 6 and lane 7 of Figure 2.2 not attributable to telomerase-catalyzed primer extension were excluded from the  $IC_{50}$  calculations. Both hTRas009 ( $IC_{50} = 72.5 \pm 15.3$  nM) and hTRas010 ( $163 \pm 29.9$  nM) were nanomolar inhibitors of telomerase (Figures 2.2C and 2.2D). By comparison, oligonucleotide inhibitors that target the template portion inhibit telomerase in

the low nanomolar range (Corey, 2002), though the concentration of hTR in these assays is much lower than in our assays, making a direct comparison impossible. Importantly, telomerase activity was almost completely eradicated (~5% residual activity) by either oligonucleotide at high concentration (10  $\mu$ M).

b. Direct telomerase assay in the presence of hTRas009 and hTRas010

Telomerase was pre-assembled *in vitro* and assayed in the presence of either hTRas009 or hTRas010. The direct telomerase assays were conducted in the presence of the



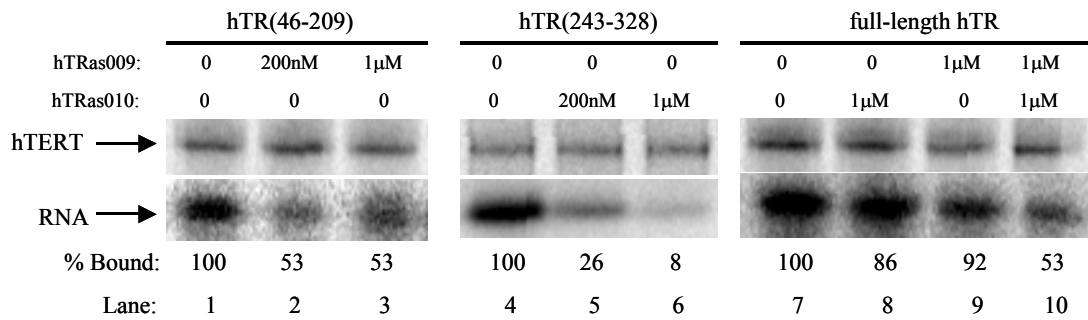
**Figure 2.4 Concentration dependence of telomerase inhibition by hTRas009 and hTRas010 when added after assemblage.** hTRas010 (lanes 2 and 3) or hTRas009 (lanes 4 and 5) were added to the direct telomerase assay at concentrations of 1  $\mu$ M (lanes 2 and 4) and 10  $\mu$ M (lanes 3 and 5). Lane 1 is a primer-only control. Lane 6 is a  $^{32}$ P-labeled primer to mark the beginning of primer extension. The  $^{32}$ P-labeled loading control (L.C.) is indicated. "% Activity" values indicate the amount of residual telomerase activity as compared to the primer-only control.

two highest oligonucleotide concentrations tested in the assemblage assay (Figure 2.4). Under these conditions, neither oligonucleotide significantly inhibited telomerase activity when compared to their inhibition in the assemblage assay (59% and 88% residual activity for hTRas009 and hTRas010, respectively, in the direct telomerase assay as compared to ~5% residual activity for either oligonucleotide in the assemblage assay).

### 3. hTRas009 and hTRas010 affect the association of hTR with hTERT

Since hTRas009 and hTRas010 only inhibit telomerase when added prior to assemblage, we hypothesized that they interfere with proper assemblage but do not directly inhibit enzymatic activity. We therefore studied the ability of these antisense molecules to affect binding of hTR to hTERT using a co-immunoprecipitation assay. Because the pseudoknot and CR4-CR5 domains have previously been shown to interact independently with hTERT (Tesmer et al., 1999; Mitchell and Collins, 2000; Chen et al., 2002; Chen and Greider, 2003), we tested each oligonucleotide for their ability to block binding of their respective targeted domains to hTERT (Figure 2.5). T7-tagged hTERT was translated in the presence of [<sup>35</sup>S]-methionine, which allowed normalization of the co-immunoprecipitation data, and was incubated with <sup>32</sup>P-labeled RNA fragments to allow for RNA/hTERT binding. The resulting complexes were then immunoprecipitated. Controls with no oligonucleotide were performed along with two different concentrations of oligonucleotide (200 nM and 1 μM). We found that, in the absence of inhibitory oligonucleotides, the pseudoknot domain (nucleotides 46-209) and the CR4-CR5 domain (nucleotides 243-328) of hTR both independently interact with hTERT, consistent with previous reports (Tesmer et al., 1999; Mitchell and Collins, 2000; Chen et al., 2002; Chen and Greider, 2003) (lanes 1 and 4, Figure 2.5). When the pseudoknot RNA fragment was incubated with hTERT in the presence of

hTRas009, co-immunoprecipitation of the RNA with hTERT was inhibited by ~50% (lanes 2 and 3, Figure 2.5). Conversely, hTRas010 was able to inhibit binding of the CR4-CR5 RNA fragment by 92% of the maximum binding (lanes 5 and 6, Figure 2.5).

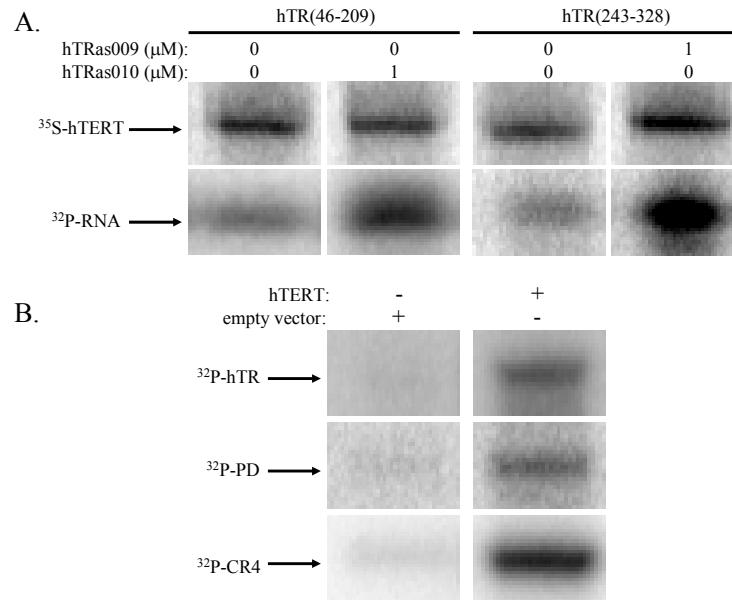


**Figure 2.5 Inhibition of hTR/hTERT interactions by hTRas009 and hTRas010.** <sup>32</sup>P-labeled pseudoknot RNA fragment (lanes 1-3), <sup>32</sup>P-labeled CR4-CR5 RNA fragment (lanes 4-6) or <sup>32</sup>P-labeled full-length hTR (lanes 7-10) were incubated with <sup>35</sup>S-hTERT either in the presence or absence of oligonucleotide inhibitor(s). The resulting complexes were co-immunoprecipitated and resolved on a denaturing Bis-Tris SDS gel. The [<sup>35</sup>S]-labeled hTERT bands were used as a loading control. hTRas009 was added to an assembly reaction at a concentration of 200 nM (lane 2) or 1 μM (lane 3) to inhibit binding of the pseudoknot RNA fragment. Lane 1 is a control reaction with no oligonucleotide. hTRas010 was added to an assembly reaction at a concentration of 200 nM (lane 5) or 1 μM (lane 6) to inhibit binding of the CR4-CR5 RNA fragment. A control with no oligonucleotide was also performed (lane 4). 1 μM hTRas010 (lane 8), 1 μM hTRas009 (lane 9) or 1 μM each of hTRas009 and hTRas010 (lane 10) was added to an assembly reaction to inhibit binding of full-length RNA. Lane 7 is a control reaction with no oligonucleotide. "% Bound" values indicate the amount of RNA bound to the protein when compared to control reactions with no oligonucleotide. The "% Bound" values in lanes 1-3 are average values from duplicate experiments. Note: The pseudoknot RNA fragment (nucleotides 46-209), CR4-CR5 RNA fragment (nucleotides 243-328), and full-length RNA (451 nucleotides) are different lengths despite the depiction in the figure.

We also tested the effect of hTRas009 and hTRas010 on the association between hTERT and full-length hTR (lanes 7-10, Figure 2.5). <sup>32</sup>P-labeled hTR (451 nt) was incubated with <sup>35</sup>S-labeled hTERT either alone, with 1 μM hTRas009, with 1 μM hTRas010, or with both 1 μM hTRas009 and 1 μM hTRas010. Compared to the control with no oligonucleotide, hTRas009 and hTRas010 had little effect on the association of hTR and hTERT (compare lanes 8 and 9 to lane 7, Figure 2.5). However, when both hTRas009 and

hTRas010 were present, hTR binding was decreased by ~50% compared to the control with no oligonucleotide (compare lane 10 to lane 7, Figure 2.5).

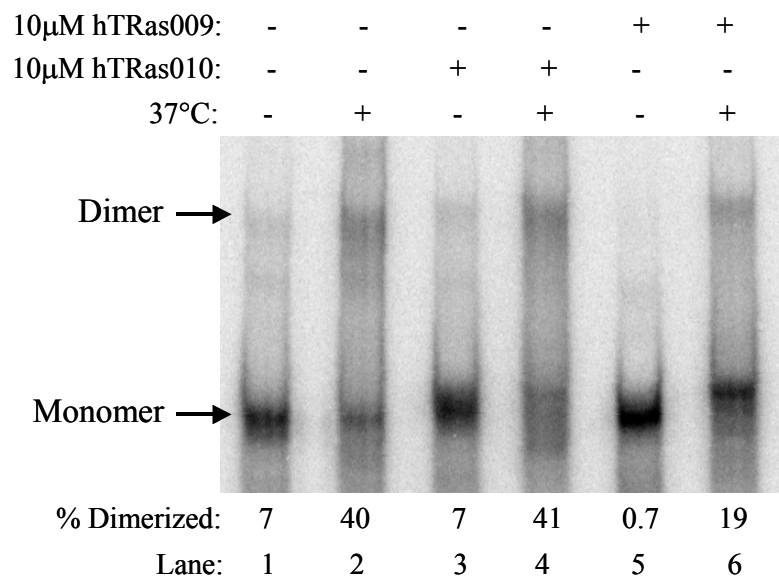
To demonstrate the specificity of the oligonucleotides for their respective  $^{32}\text{P}$ -labeled RNA fragments, hTRas009 was incubated with  $^{35}\text{S}$ -labeled hTERT and the CR4-CR5 RNA fragment, and hTRas010 was incubated with  $^{35}\text{S}$ -labeled hTERT and the pseudoknot RNA fragment. We found that neither oligonucleotide was able to block the binding between the RNA and protein compared to the controls with no oligonucleotide (Figure 2.6A). Instead, we saw an increase in the association of the RNA fragments with hTERT when the non-targeting oligonucleotides were present. Nonspecific binding was analyzed using an empty vector control. In these samples, less than 5% of the RNAs bound to beads compared to samples containing hTERT (Figure 2.6B).



**Figure 2.6 Oligonucleotide and RNA specificity** (A) Non-targeting oligonucleotides cause no decrease in pull-down of RNA.  $^{35}\text{S}$ -hTERT bands were used to normalize  $^{32}\text{P}$ -RNA bands. (B) Nonspecific binding of RNA to beads in the absence of hTERT is minimal. hTR designates full-length telomerase RNA, PD designates hTR(46-209) and CR4 designates hTR(243-328).

#### 4. hTRas009 affects hTR homo-dimerization

Recently, hTR was shown to dimerize through an intermolecular interaction between stems of the hTR pseudoknot (Ly et al., 2003b). To determine if hTRas009 or hTRas010 affect hTR homo-dimerization,  $^{32}\text{P}$ -labeled hTR was incubated either in the presence or absence of hTRas009 or hTRas010 at a final concentration of 10  $\mu\text{M}$  and resolved on a nondenaturing gel (Figure 2.7). As expected, we found that the addition of hTRas010 (lane 4) had no effect on hTR homo-dimerization as compared to the control (lane 2), however, the addition of hTRas009 (lane 6) decreased dimerization by about 50% as compared to the control (Figure 2.7). A similar trend in dimerization values is seen for the non-incubated controls (lanes 1, 3, and 5).



**Figure 2.7 hTRas009 inhibits hTR homo-dimerization.**  $^{32}\text{P}$ -labeled full-length hTR was allowed to dimerize either alone (lanes 1-2) or in the presence of 10 $\mu\text{M}$  hTRas010 (lanes 3-4) or 10 $\mu\text{M}$  hTRas009 (lanes 5-6). Samples were either incubated at 37 °C to allow for dimerization (+) or were kept on ice to prevent dimerization (-). "% Dimerized" values indicate the amount of hTR found in the dimer form compared to the total amount of hTR in the sample.



### C. Discussion

The involvement of telomerase in a wide range of cancers has made it an attractive target for anticancer drug discovery. Though a complete description of human telomerase and all of its subunits awaits further elucidation, the minimally active complex, a dimer of hTR and hTERT, provides several conceivable approaches for inhibition. In particular, the identification and proposed secondary structure of hTR (Chen et al., 2000) has opened a number of doors for specific antisense inhibition. Oligonucleotides that are complementary to the template of hTR have been extensively studied (Elayadi et al., 2001), and oligonucleotides that target non-templating portions have received some attention (Hamilton et al., 1999; Pruzan et al., 2002). Here, we investigated the ability to inhibit telomerase activity by abrogating assemblage of the holoenzyme complex. We identified two 2'-*O*-methyl RNA oligonucleotides that bind to specific regions of hTR and inhibit telomerase activity with IC<sub>50</sub> values in the nanomolar range.

We used eight DNA oligonucleotides that are complimentary to separate regions of hTR (Table 2.1) and discovered that targeting the pseudoknot domain at the P3/P1 intersection and the CR4-CR5 domain at the P6.1 stem-loop resulted in the greatest inhibitory effects. A third oligonucleotide, hTRas008, which targets the CR7 domain, also decreased telomerase activity, however, we chose not to follow-up on this result at this time. Interestingly, hTRas001, an oligonucleotide that is not specific for any region of hTR, increased telomerase activity. This unexpected result suggests that a nucleic acid-binding protein or proteins in the reticulocyte lysate are refractory towards telomerase activity and/or assemblage. Because this oligonucleotide does not target any region of the hTR, it was free

to interact with other entities in the reticulocyte lysate. This suggests that the addition of random DNA to telomerase reconstitution assays performed in reticulocyte lysates can increase the yield of active complexes. PNAs that are complimentary to a wide variety of hTR regions inhibit telomerase when added prior to assemblage (Hamilton et al., 1999). By contrast, DNA oligonucleotides were only inhibitory when targeting three specific regions of hTR. This is presumably because DNA oligonucleotides form less stable duplexes that can be out competed by native structures. For example, hTRas002, which is complementary to the template, did not inhibit telomerase even though corresponding PNAs are excellent inhibitors (Hamilton et al., 1999). In the case of the DNA oligonucleotide, hTRas002, the inhibitor was unable to compete with the telomeric primer for the template. We therefore concluded that the DNA oligonucleotides we identified in our screen block interactions between hTR and hTERT as opposed to disrupting more stable native hTR structures, though we cannot rule this out.

Ultimately, we decided to conduct further studies on the oligonucleotides targeting the P3/P1 pairing region (hTRas004) and the CR4-CR5 domain (hTRas006), two regions of hTR that were previously shown to be required for both telomerase activity and the association of hTR with hTERT (Tesmer et al., 1999; Beattie et al., 2000; Mitchell and Collins, 2000; Bachand and Autexier, 2001; Martin-Rivera and Blasco, 2001; Chen et al., 2002; Chen and Greider, 2003). For these experiments we re-synthesized the DNA oligonucleotides with 2'-*O*-methyl-modified RNA and phosphorothioate linkages on their 5'- and 3'-ends (hTRas009 and hTRas010, Table 2.1). These alterations increased the inhibitory effects of the oligonucleotides. The increased inhibition can be attributed to the fact that 2'-*O*-methyl RNA oligonucleotides hybridize to RNA better than DNA oligonucleotides (Monia

et al., 1993). The phosphorothioate modifications were designed for future cell culture experiments in order to decrease oligonucleotide degradation.

In assemblage assays, hTRas009 ( $IC_{50} = 72.5 \pm 15.3$  nM) was found to be a more potent inhibitor of telomerase activity than hTRas010 ( $IC_{50} = 163 \pm 29.9$  nM) (Figure 2.2). The banding pattern observed is characteristic of human telomerase activity with the first major extension product being four nucleotides longer than the primer and each successive major product having six additional nucleotides. We also noted the presence of unexpected bands in the no-primer controls revealing that hTRas009 and hTR010, at concentrations of 10  $\mu$ M, act as substrates for  $^{32}$ P-dGTP addition by some component of the reticulocyte lysate (lane 7, Figures 2.2A and 2.2B). This observation, however, should not influence the inhibitory effects of the oligonucleotides as the unexpected bands were not seen at lower concentrations. Furthermore, although hTRas009 has a lower  $IC_{50}$  value than hTRas010, both oligonucleotides were able to completely inhibit (~5% residual activity) telomerase activity at the highest concentration tested (10  $\mu$ M). Notably, the  $IC_{50}$  values for both hTRas009 and hTRas010 are in the same range as the hTR concentration (95 nM) used in the assemblage reactions.

Neither hTRas009 nor hTRas010 significantly inhibited telomerase activity when added after telomerase was allowed to assemble (Figure 2.4), though hTRas009 inhibited slightly, with 59% residual activity. Perhaps a high concentration of hTRas009 can disrupt interactions within the intact telomerase complex. Similar results were found for several PNAs that target non-templating regions of hTR (Hamilton et al., 1999). These results suggest that enzyme activity was inhibited by impeding essential interactions between hTR and hTERT or by disrupting the folding of hTR. Further, our results indicate that the P3/P1

pairing region and the CR4-CR5 domain are not exposed once proper holoenzyme formation is complete. Alternatively, if these domains remain exposed in the holoenzyme, then oligonucleotide interactions after holoenzyme formation do not interfere with activity. Chemical and enzymatic foot-printing of hTR revealed that the P6.1 stem-loop, within the CR4-CR5 domain, and the P3/P1 pairing region are protected from cleavage reagents when complexed with hTERT (Antal et al., 2002). This is consistent with the idea that hTRas009 and hTRas010 cannot hybridize to hTR once the telomerase complex has completely and properly formed.

We tested the hypothesis that hTRas009 and hTRas010 inhibit telomerase by specifically inhibiting interactions between hTERT and the targeted hTR domains. Independent fragments of hTR that are specifically targeted by their respective oligonucleotides were co-immunoprecipitated with hTERT. As expected, we observed that the pseudoknot and CR4-CR5 domains of hTR independently bind hTERT in the absence of oligonucleotide (lanes 1 and 4, Figure 2.5). hTRas010, which targets hTR at nucleotides 301-322, hybridizes with the entire P6.1 stem-loop (nucleotides 302-314) and blocks 92% of the binding between hTERT and the CR4-CR5 domain at 1  $\mu$ M (lane 6, Figure 2.5). This is the expected result if the interaction between the CR4-CR5 domain and hTERT is dependent on interactions with the P6.1 stem-loop, as previously observed by the Greider lab (Chen et al., 2002). Interestingly, 1  $\mu$ M hTRas009 did not exhibit the same degree of inhibition for the interaction between the pseudoknot fragment and hTERT (lane 3, Figure 2.5). The fact that hTRas009 was able to decrease the ability of hTERT to bind the pseudoknot fragment by ~50% of the maximum suggests that the region it targets (nucleotides 174-195) is important

for protein binding. However, it appears that other regions in the pseudoknot domain (nucleotides 46-209) also contribute to interactions with hTERT.

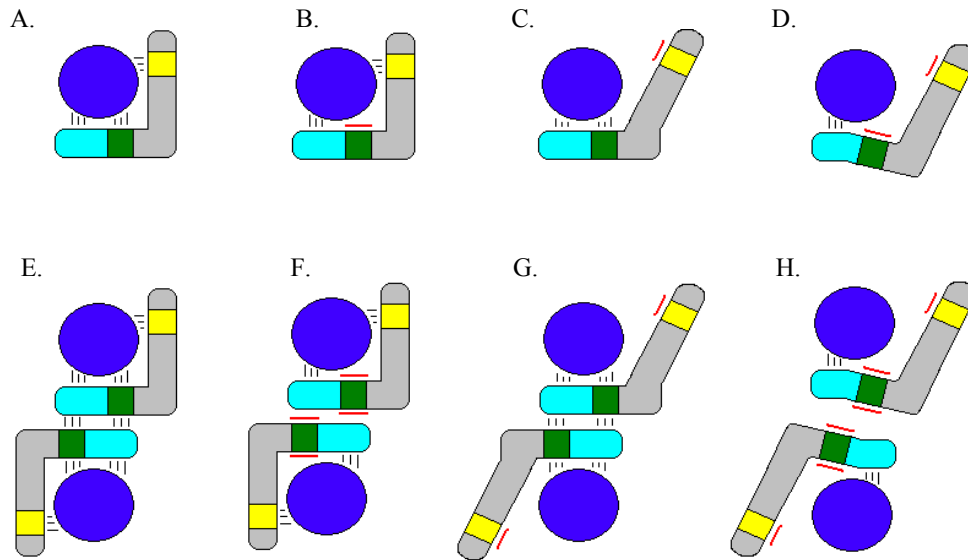
If hTRas009 does not completely prevent binding of hTERT to the pseudoknot domain, what is its mechanism of inhibition? One intriguing possibility is that hTRas009, which hybridizes to the P3/P1 pairing region, perturbs telomerase activity by interrupting hTR homo-dimerization. Ly et al. proposed that hTR homo-dimerization involving contacts in the P3 pairing region results in an essential *trans*-pseudoknot structure (Ly et al., 2003b). They demonstrated that mutants incapable of forming the P3-*trans* interaction were unable to reconstitute telomerase activity but were able to bind to hTERT and suggested that hTR dimerization may provide a foundation for holoenzyme assembly. We tested the ability of hTRas009 to inhibit hTR homo-dimerization by nondenaturing PAGE (Figure 2.7). The addition of hTRas009 (lane 6) blocked ~50% of hTR homo-dimerization as compared to the control (lane 2), whereas the addition of hTRas010 (lane 4) had no effect, as expected (Figure 2.7). We therefore propose that the mechanism of inhibition of telomerase activity by hTRas009 includes disrupting both RNA-protein interactions and hTR homo-dimerization. Based on this, it is surprising that hTRas003, which targets nucleotides 94-115 in the P3 pairing region of the pseudoknot domain, was not an inhibitor of assemblage (see Table 2.1). Interestingly, a PNA that targeted nucleotides 93-105 was an inhibitor ( $IC_{50} = 0.1 \mu M$ ) of telomerase when added post-assemblage, and a PNA that targeted the other portion of the dimerization domain, nucleotides 180-194, was a more potent inhibitor ( $IC_{50} = 0.01 \mu M$ ) (Hamilton et al., 1999). One explanation is that the PNA targeting nucleotides 180-194 and hTRas009, which targets nucleotides 174-195, either form more stable duplex structures compared to the native RNA structure or disrupt important interactions between hTR and

hTERT. It is notable that hTR homo-dimerization was not entirely disrupted. This may be explained by the recent observation that a second RNA/RNA interaction site within the CR7 domain can contribute to homo-dimerization (Ren et al., 2003), and may be related to the observed inhibition by hTRas008. In support of this, Ren et al. showed that an oligonucleotide that targets a similar sequence in hTR inhibited hTR multimerization (Ren et al., 2003).

In order to further characterize the effects of hTRas009 and hTRas010 on hTR binding to hTERT, each oligonucleotide alone or both oligonucleotides together were incubated with full-length hTR (451 nt) and hTERT. We found that hTRas009 or hTRas010 alone had little effect on hTR/hTERT binding (lanes 8 and 9, Figure 2.5). When both oligonucleotides were used in combination, however, 53% of the RNA remained bound as compared to the control suggesting that other parts of the full-length RNA continued to interact with hTERT (lane 10, Figure 2.5). Interestingly, the percentage of RNA still bound to hTERT is the same as that in the experiment using hTRas009 alone to inhibit binding of the pseudoknot RNA fragment to hTERT (compare % Bound values in lanes 3 and 10, Figure 2.5). This suggests that the elements of hTR that continue to bind hTERT in the presence of both oligonucleotides reside in the pseudoknot domain (nucleotides 46-209). One explanation for this result is that hTERT interacts with hTR at a minimum of three specific positions and hTRas009 blocks one, hTRas010 blocks another, and the third resides between nucleotides 46-159 as suggested by our work and the work of Bachand et al. (Bachand and Autexier, 2001) (Figure 2.8).

Our results demonstrate that targeting telomerase holoenzyme assemblage is an effective approach towards inhibiting telomerase activity. Both hTRas009 and hTRas010

had a significant inhibitory effect on telomerase activity in the assemblage assay (Figures 2.2A and 2.2B). However, neither appreciably decreased full-length hTR binding alone (lanes 8-9, Figure 2.5) suggesting that this inhibitory approach not only prevents proper telomerase assembly but also sequesters the telomerase subunits in an inactive state. A



**Figure 2.8 hTRas009 and hTR010 prevent proper telomerase assemblage by blocking essential interactions between hTR and hTERT.** (A) In the absence of oligonucleotide inhibitor, hTERT (dark blue circle) interacts directly with hTR via the CR4-CR5 domain (yellow), the P3/P1 pairing region (green) and other portions of the pseudoknot domain (light blue). (B) hTRas009, targeting the P3/P1 pairing region, partially blocks hTR/hTERT binding. (C) hTRas010, targeting the CR4-CR5 domain, partially blocks hTR/hTERT binding. (D) hTRas009 and hTRas010 partially block hTR/hTERT binding. (E) Proposed model for telomerase complex dimerization via the P3 pairing regions in the absence of oligonucleotide inhibitor. (F) hTRas009 partially blocks hTR/hTERT binding and blocks dimerization. (G) hTRas010 partially blocks hTR/hTERT binding while dimerization remains intact. (H) hTRas009 and hTRas010 partially block hTR/hTERT binding and block dimerization. Oligonucleotides are depicted by the red lines.

model consistent with this data is that hTRas009 and hTR010 prevent proper telomerase assemblage by blocking essential interactions between hTR and hTERT (Figure 2.8).

Although hTRas009 and hTRas010 were unable to reverse the interaction between pre-assembled hTR and hTERT in order to inhibit telomerase activity (Figure 2.4), this mode

of inhibition should be relevant to cancer therapy. In cancer cells, hTR has an extended half-life concomitant with a high transcription rate (Yi et al., 1999). This suggests that the telomerase complex itself has a high turnover rate. In addition, as cancer cells divide, more telomerase must be generated. Molecules that sequester hTR and induce misassembly could then have an opportunity to elicit their effects on rapidly reproducing cells. In fact, it has been demonstrated that PNAs targeting non-templating regions of hTR can inhibit intracellular telomerase (Hamilton et al., 1999). The approach described here to block telomerase assembly could also provide a chemogenetic method to study telomerase assembly in cultured cells.

#### D. Materials and methods

##### 1. Oligonucleotides.

All oligonucleotides were purchased from and purified by Integrated DNA Technologies (Coralville, Iowa). Oligonucleotide concentrations were determined by UV absorbance at 260 nm using the molar extinction coefficient provided by Integrated DNA Technologies.

##### 2. pET-28c-hTERT and phTR+HH expression plasmids.

An hTERT plasmid (pCI-neo-hTERT) was a gift from Dr. Lorel Colgin (Children's Medical Research Institute, Westmead Australia) and CAMBIA (Canberra Australia) (Colgin et al., 2000). The hTERT-containing insert from this plasmid was subcloned into the *EcoRI* and *SaII* sites of the T7-tag-containing plasmid pET-28c (Novagen) to give the construct pET-28c-hTERT. phTR+HH, a plasmid designed for in vitro transcription of human



telomerase RNA with a self-cleaving hammerhead ribozyme that generates the natural 5' end, was a gift from Dr. Jamie Sperger (University of Colorado, Boulder).

### 3. In vitro transcription and purification of hTR.

15 µg of phTR+HH was linearized by digestion with *Fok*-1 followed by extraction with phenol/chloroform/isoamyl alcohol, precipitation with ethanol and resuspension in a suitable volume of TE (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA). In vitro transcription was carried out at 37 °C for 18 h using the linearized DNA, 0.32 units/µL T7 RNA polymerase (Promega), 1× transcription-optimized buffer (Promega), 10 mM DTT, 0.8 units/µL RNasin (Fisher Scientific), 5 mM MgCl<sub>2</sub> and 1 mM each NTP. After transcription, the magnesium-inorganic diphosphate complexes were removed by centrifugation for 2 min at 22 000g. Cleavage by the hammerhead ribozyme was initiated by the addition of MgCl<sub>2</sub> to a final concentration of 12 mM followed by incubation at 45 °C for 1 h. The entire reaction was then treated with 15 units of RQ1-DNase (Promega) for 20 min at 37 °C followed by extraction with phenol/chloroform/ isoamyl alcohol and ethanol precipitation. The RNA was resuspended in a suitable volume of denaturing loading buffer (7 M urea/10% glycerol/1× TBE) and purified on a 4% denaturing polyacrylamide gel. The RNA was recovered using a modified version of the "crush and soak" method (Maxam and Gilbert, 1977). Briefly, the RNA was located in the gel by UV shadowing, cut out, crushed by passing through a sterile plastic syringe and extracted into 2 volumes of 1× TEN (10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 200 mM NaCl) at 4 °C for 16 h. After centrifugation, the supernatant was collected and a second extraction was performed with 2 volumes of 1× TEN at room temperature for 1 h. The combined supernatants were filtered through a Whatman® GF/C filter, precipitated with ethanol and resuspended in a suitable volume of TE.

#### 4. Synthesis of hTERT.

hTERT was transcribed and translated using the TNT<sup>®</sup> Coupled Reticulocyte Lysate Systems kit (Promega). A 400  $\mu$ L reaction contained 8  $\mu$ g of pET-28c-hTERT, 16  $\mu$ L of [<sup>35</sup>S]-methionine (1175 Ci/mmol, 10  $\mu$ Ci/ $\mu$ L; Perkin Elmer) and other components provided in the kit as described by the manufacturer. The reaction was incubated at 30 °C for 90 min, flash-frozen in a dry ice/ethanol bath and stored at –80 °C. hTERT used in the direct telomerase assay and the assemblage assay was made without [<sup>35</sup>S]-methionine as described by the manufacturer. A reticulocyte lysate reaction with pET-28c (empty vector control) in place of pET-28c-hTERT served as a negative control in several experiments.

#### 5. Reconstitution of telomerase.

Pre-assembled telomerase was prepared by adding 4  $\mu$ g of in vitro transcribed hTR to the 400  $\mu$ L reticulocyte lysate reaction prior to incubation for 90 min at 30 °C. Direct telomerase assays contained 10  $\mu$ L of pre-assembled telomerase. Reconstitution of telomerase in the assemblage assay was initiated by combining 10  $\mu$ L (~50 fmol) of hTERT from a reticulocyte lysate reaction, 212.5 ng of in vitro transcribed hTR and the appropriate amount of water or oligonucleotide inhibitor to a final volume of 15  $\mu$ l followed by incubation at 30 °C for 90 min. The pre-assembled telomerase complex and the hTERT protein were used in assays without further purification from the reticulocyte lysate.

#### 6. Direct telomerase assay.

Telomerase activity was measured using a modification of a previously described direct assay (Chen and Greider, 2003). Each 25  $\mu$ L reaction contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM  $\beta$ -mercaptoethanol, 1 mM spermidine, 1  $\mu$ M human

telomere primer (5'-TTAGGGTTAGGGTTAGGG), 0.5 mM dATP, 0.5 mM dTTP, 2.9  $\mu$ M dGTP, 0.33  $\mu$ M [ $\alpha$ - $^{32}$ P]-dGTP (3000 Ci/mmol, 10  $\mu$ Ci/ $\mu$ L; Perkin Elmer) and 10  $\mu$ L of pre-assembled telomerase. Inhibition studies also included varying amounts of oligonucleotide inhibitor. Primer extension was carried out at 30 °C for 90 min. After the addition of a  $^{32}$ P-labeled loading control (114 nucleotide, 5'-end labeled DNA oligonucleotide, 1000 cpm per reaction), the primer extension products were extracted with phenol/chloroform/isoamyl alcohol and ethanol precipitated in the presence of 0.6 M NH<sub>4</sub>OAc and 35 ng/ $\mu$ L glycogen. Products were precipitated at -80 °C in 2.5 volumes of absolute ethanol for 30 min followed by centrifugation at 22 000g at 4 °C for 25 min and washing with 2 volumes of 70% ethanol. Pellets were resuspended in a suitable volume of TE and ethanol precipitation was repeated to ensure the removal of all unincorporated [ $\alpha$ - $^{32}$ P]-dGTP. The final pellets were dissolved in a formamide loading buffer containing 40% formamide, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue. The products were heated at 95 °C for 5 min and resolved on a pre-warmed, 0.4 mm thick, 20 x 20 cm, 10% polyacrylamide/7 M urea/1 $\times$  TBE gel. A small amount of the human telomere primer was labeled with [ $\gamma$ - $^{32}$ P]-ATP and T4 Polynucleotide Kinase (Fisher) and loaded in a separate lane to be used as a marker for the start of primer elongation. The gel was run at 800 V for 1 h in 1 $\times$  TBE. After drying the gel and exposing it to a phosphorimager screen (Molecular Dynamics) overnight, telomerase activity was imaged using a phosphorimager (Molecular Dynamics Storm 860) and quantified with ImageQuant (version 5.2). The intensities of each band in each sample were summed and normalized to the loading control.

#### 7. Assemblage assay.

Each 25  $\mu$ L reaction contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM  $\beta$ -mercaptoethanol, 1 mM spermidine, 1  $\mu$ M human telomere primer (5'-TTAGGGTTAGGGTTAGGG), 0.5 mM dATP, 0.5 mM dTTP, 2.9  $\mu$ M dGTP, 0.33  $\mu$ M [ $\alpha$ -<sup>32</sup>P]-dGTP (3000 Ci/mmol, 10  $\mu$ Ci/ $\mu$ L; Perkin Elmer) and 15  $\mu$ L of reconstituted telomerase (see Reconstitution of Telomerase above). Inhibition studies also included varying amounts of oligonucleotide inhibitor. Primer extension was carried out at 30 °C for 90 min and the samples were processed as described for the direct telomerase assay. Notably, the inhibitor concentration was maintained during both the assembly reaction and extension reaction.

#### 8. Synthesis of <sup>32</sup>P-labeled CR4-CR5 and pseudoknot RNA fragments and full-length hTR.

RNA fragments were generated using a modification of the protocol described by Chen et al. (Chen and Greider, 2003). The desired DNA templates were amplified by PCR using the phTR+HH plasmid described above. The pseudoknot PCR fragment (nucleotides 46-209) was synthesized using the T7 promoter-containing forward primer 5'-GGGTACCTAATACGACTCACTATAGGCTAACCCTAACTGAGAAGGGCGTAGGCGCCGTG-3' and the reverse primer 5'-CCCCGGGAGGGGCGAACGGGCCA-3'. The CR4-CR5 PCR fragment (nucleotides 243-328) was synthesized using the T7 promoter-containing forward primer 5'-GCGGGAATTCTAATACGACTCACTATAGGCCCGCCTGGAGGCCGC-3' and the reverse primer 5'-GACCCGCGGCTGACAGAGC-3'. Both RNA fragments start with two guanosine residues on their 5' end in order to aid in T7 transcription efficiency. The PCR products were resolved on a 0.8% agarose gel and purified using a Wizard<sup>®</sup> PCR Preps DNA Purification System (Promega). In vitro transcription was carried out at 37 °C

for 18 h using the purified PCR products or linearized phTR+HH, 1 unit/ $\mu$ L T7 RNA polymerase (Promega), 1 $\times$  transcription optimized buffer (Promega), 10 mM DTT, 1 unit/ $\mu$ L RNasin (Fisher Scientific), 5 mM  $\text{MgCl}_2$ , 1 mM ATP, 1 mM GTP, 1 mM UTP, 20  $\mu$ M CTP and 0.75  $\mu$ M [ $\alpha$ - $^{32}\text{P}$ ]-CTP (800 Ci/mmol, 10  $\mu$ Ci/ $\mu$ L; Perkin Elmer). After transcription, the magnesium-inorganic diphosphate complexes were removed by centrifugation for 2 min at 22 000g. The addition of  $\text{MgCl}_2$  to the hTR+HH transcription reaction was not required in this protocol as gel analysis revealed that the hammerhead had cleaved during the 18 h incubation at 37 °C. The reaction was then treated with 7 units of RQ1-DNase (Promega) for 20 min at 37 °C followed by extraction with phenol/chloroform/isoamyl alcohol. The RNA was precipitated with ethanol in the presence of 2 M  $\text{NH}_4\text{OAc}$  and resuspended in a suitable volume of TE. A second ethanol precipitation was performed to decrease the amount of unincorporated NTPs. The samples were then passed through Microspin<sup>TM</sup> G-25 columns (Amersham) to ensure the removal of all unincorporated [ $\alpha$ - $^{32}\text{P}$ ]-CTP.

#### 9. Immunoprecipitation/RNA pull-down.

A 75  $\mu$ L reaction contained 50  $\mu$ L (~250 fmol) of  $^{35}\text{S}$ -labeled hTERT from a reticulocyte lysate reaction and 400-900 fmol of  $^{32}\text{P}$ -labeled pseudoknot or CR4-CR5 RNA fragment or full-length hTR ( $1.3\text{-}1.9 \times 10^6$  cpm per reaction). Inhibition studies also included varying amounts of oligonucleotide inhibitor(s). Reactions were incubated at 30 °C for 90 min to allow binding between the RNA and the protein. Each reaction was immunoprecipitated using 25  $\mu$ L of anti-T7 antibody agarose beads (Novagen). Before use, the anti-T7 beads were washed four times with 375  $\mu$ L of wash buffer #1 (20 mM Tris-acetate, pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM  $\text{MgCl}_2$ , 100 mM potassium glutamate,

0.1% IGEPAL and 1 mM DTT) and blocked twice with 250  $\mu$ L of blocking buffer (20 mM Tris-acetate, pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM  $MgCl_2$ , 100 mM potassium glutamate, 0.1% IGEPAL, 1 mM DTT, 0.5 mg/mL lysozyme, 0.5 mg/mL BSA, 0.05 mg/mL glycogen and 0.1 mg/mL yeast RNA) for 15 min at 4 °C. In between each washing and blocking step the beads were precipitated by centrifugation at 1500g and the supernatant was removed. 75  $\mu$ L of blocking buffer was then mixed with the 75  $\mu$ L RNA/protein sample and centrifuged at 17 000g for 10 min at 4 °C to remove any precipitates. This supernatant was then added to the blocked beads and the samples were mixed on a rotary platform for 2 h at 4 °C. Following mixing, the beads were washed three times with 325  $\mu$ L of Wash Buffer #2 (20 mM Tris-acetate, pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM  $MgCl_2$ , 300 mM potassium glutamate, 0.1% IGEPAL and 1 mM DTT) and twice with 325  $\mu$ L of TMG (10 mM Tris-acetate, pH 7.5, 1 mM  $MgCl_2$  and 10% glycerol). The beads were precipitated by centrifugation at 1500g in between each wash and the supernatant was removed. The beads were then resuspended in 1 $\times$  SDS gel loading buffer containing 10 mM DTT. Samples were heated for 5 min at 95 °C and the supernatant was loaded onto a 4-12% Bis-Tris SDS gel (Invitrogen). The gel was run at 130 V for 1 h, dried and exposed to a phosphorimager screen overnight. RNA band intensities were quantified, normalized to the  $^{35}S$ -hTERT protein bands and compared to the positive control.

#### 10. Inhibition studies.

Direct telomerase or assemblage assays were conducted as described above with varying concentrations of inhibitors.  $IC_{50}$  values were calculated using the Regression Wizard program of SigmaPlot (version 7.0). Six data points were used with oligonucleotide

concentrations ranging from 0 to 10  $\mu$ M. The following four-parameter logistic curve equation was used:

$$y = D + [(A-D)/(1 + (x/C)^B)]$$

where  $x$  is the concentration of inhibitor,  $y$  is the % Activity relative to the primer-only control,  $A$  is the maximal activity,  $B$  is the slope factor,  $C$  is the  $IC_{50}$  (i.e. the concentration required for 50% inhibition) and  $D$  is the minimal activity. Inhibition data were plotted as % Activity vs. log oligonucleotide concentration and fit using the same equation.

#### 11. Nondenaturing polyacrylamide gel electrophoresis of $^{32}$ P-hTR.

Nondenaturing PAGE was performed using a modification of the protocol described by Ly et al. (Ly et al., 2003b). Briefly, each 10  $\mu$ L reaction contained 0.6  $\mu$ g of unlabeled hTR and about 11000 cpm of tracer  $^{32}$ P-hTR. The samples were heated at 95  $^{\circ}$ C for 5 min, immediately placed on ice and adjusted to 50 mM NaCl, 25 mM Tris-Cl, pH 7.0, 10 mM  $MgCl_2$ . hTRas009 or hTR010 was then added to a final concentration of 10  $\mu$ M. Samples were then either kept on ice or incubated at 37  $^{\circ}$ C for 2 hrs to allow for dimerization. Sample products were analyzed on a 4% nondenaturing gel in 90 mM Tris-borate, 0.1 mM  $MgCl_2$ . The gel was run at 100 V for 6 hrs in a cold room (4  $^{\circ}$ C). After drying the gel and exposing it to a phosphorimager screen overnight, the extent of dimerization was quantified.

### Chapter III. RNA-binding ligands affect telomerase assemblage

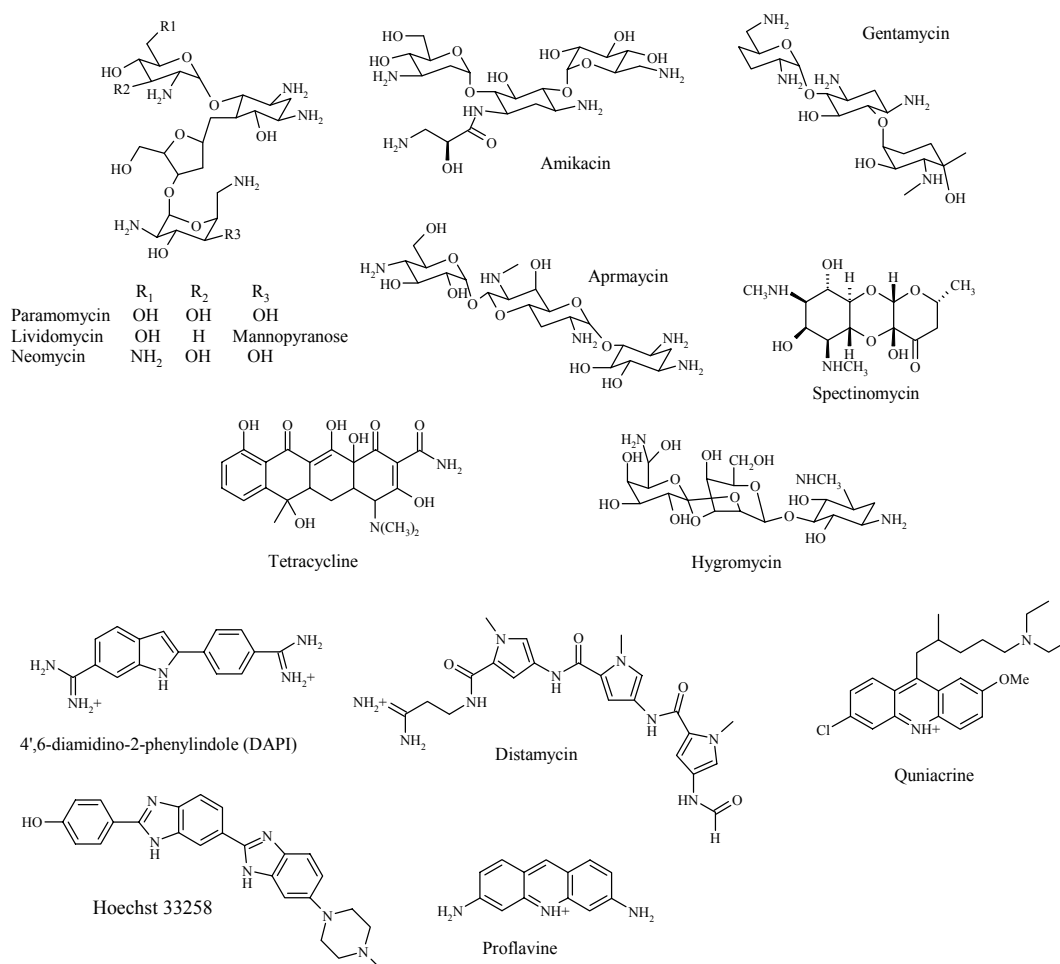
Adapted from: Dominick, P. K., Keppler, B. R., Legassie, J. D., Moon, I. K. and M.B. Jarstfer, *Nucleic-Acid Binding Ligands Identify New Mechanisms to Inhibit Telomerase*. Bioorganic and Medicinal Chemistry Letters, 2004. 14, 3467-3471

#### A. Introduction

In devising a new approach towards telomerase inhibition, we considered the available targets and the past approaches taken to mediate telomerase activity. Telomerase is a multi-subunit ribonucleoprotein complex, but the minimal requirements to establish telomerase activity in vitro include only the catalytic protein subunit, hTERT (human telomere reverse transcriptase) and the RNA subunit, hTR (human telomerase RNA), which contains the template for reverse transcription (Beattie et al., 1997; Weinrich et al., 1997). Several approaches to inhibit telomerase have been investigated and can be broken down into four major categories: G-quadruplex stabilizers (Cuesta et al., 2003), antisense oligonucleotides (Corey, 2002), reverse transcriptase inhibitors (Strahl and Blackburn, 1996) and non-reverse transcriptase inhibitors (Damm et al., 2001; Beltz, 2002). The most effective of these to date are phosphoramidate, locked nucleic acid (LNA) and 2'-methoxyethyl oligonucleotides that are complementary to the template portion of hTR (Corey, 2002). In fact, the only FDA-approved telomerase inhibitors presently in clinical trials are GRN163, a template-targeting *thio*-phosphoramidate oligonucleotide, and GRN163L, a lipid-modified version of GRN163. Given the current drawbacks of oligonucleotide-based therapeutics, however, small molecule telomerase inhibitors continue



to be an area of great interest. In order to explore novel mechanisms for the inhibition of telomerase, we have developed a parallel screen to identify molecules that inhibit telomerase by affecting proper assemblage of the holoenzyme complex (see Chapter II) (Keppler and Jarstfer, 2004). Here, we describe the use of this screen to sieve through a small library of nucleic acid-binding ligands. Potential inhibitors include several aminoglycosides, DNA intercalators including the known telomerase inhibitor proflavine as a control and DNA



**Figure 3.1** Compounds tested as telomerase inhibitors.

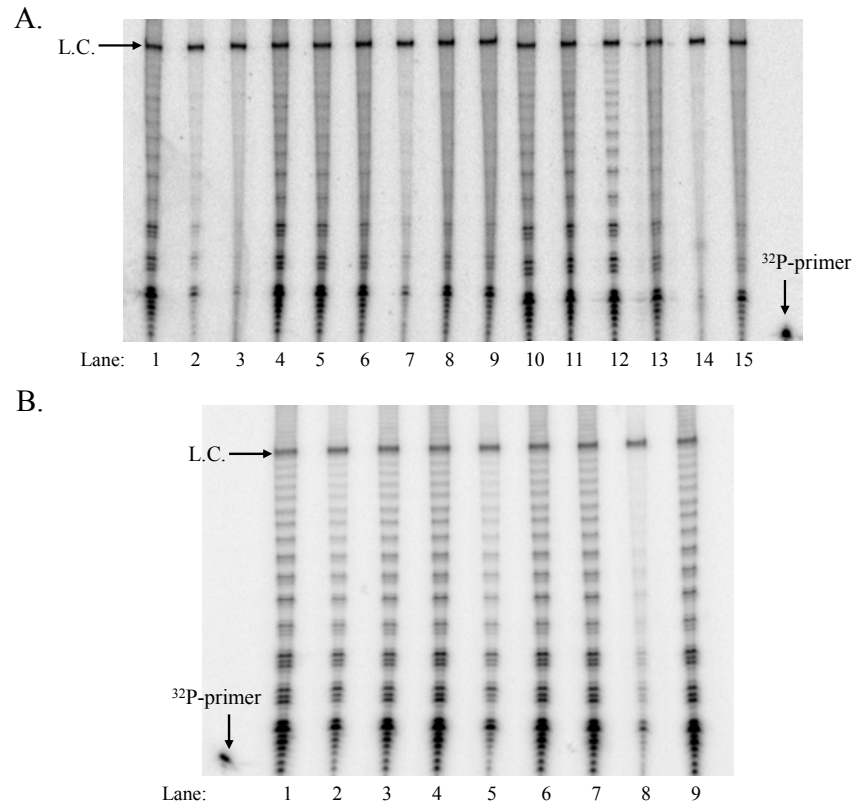
minor groove binders (Figure 3.1). Our main objective in this study was to identify small molecules that inhibit telomerase by interacting with hTR, leading to a dysfunctional telomerase complex.

## B. Results

### 1. Pre- and post-assembly assays

To determine if the selected small molecules were inhibitors of human telomerase activity each compound was first tested in an assembly assay. Compounds were added at a final concentration of 50  $\mu$ M prior to hTERT/hTR association. After allowing assembly, telomerase activity was assayed using a direct assay (Figure 3.2A). In the direct assay, the concentration of inhibitor present in the assembly portion was maintained. The most potent inhibitors (defined as  $\geq 50\%$  inhibited) were quinacrine, Hoechst 33258, lividomycin, 4',6-diamidino-2-phenylindole (DAPI), gentamycin, neomycin, proflavine and paromomycin. These inhibitory compounds were then tested at the same concentration in direct assays using pre-assembled telomerase (Figure 3.2B). Compounds that were inhibitory were divided into two groups: compounds in Group I inhibited telomerase to a greater extent ( $> 2$  fold difference) when added prior to assembly whereas those in Group II inhibited to a similar extent ( $< 2$  fold difference) irrespective of the order of addition (Table 3.1). This group of compounds was also assayed for their ability to inhibit telomerase from other organisms. All fourteen small molecules were tested in turn using recombinant telomerase from the ciliated protozoan *Tetrahymena thermophila* both before and after assembly of its protein (tTERT) and RNA (tTR) counterparts. We found that only

proflavine, quinacrine, Hoechst 33258 and DAPI had an inhibitory effect (Table 3.1). We also determined the inhibitory potential of our small library against endogenous telomerase



**Figure 3.2 RNA binding ligand-induced inhibition of telomerase pre- and post-assembly** (A) Inhibition of telomerase by nucleic-acid ligands added before telomerase assembly. Lane 1, no inhibitor; 2, quinacrine; 3, Hoechst 33258; 4, spectinomycin; 5, amikacin; 6, lividomycin; 7, DAPI; 8, gentamycin; 9, neomycin; 10, hygromycin; 11, tetracycline; 12, distamycin A; 13, apramycin; 14, proflavine; 15, paromomycin. Ligand concentration was maintained at 50  $\mu$ M throughout the assay. (B) Inhibition of assembled telomerase by nucleic acid-binding ligands. Lane 1, no inhibitor; 2, quinacrine; 3, Hoechst 33258; 4, lividomycin; 5, DAPI; 6, gentamycin; 7, neomycin; 8, proflavine; 9, paromomycin. Ligand concentration was maintained at 50  $\mu$ M throughout the assay. L.C. is a loading control and  $^{32}$ P-labeled primer indicates the start of primer elongation.

purified from the ciliated protozoan *Euplotes aediculatus*. In this organism, proflavine, quinacrine, Hoechst 33258 and distamycin A were the only compounds found to significantly perturb telomerase activity (Table 3.1).

**Table 3.1 Percent inhibition of telomerase by selected nucleic acid-binding ligands<sup>a</sup>**

	<i>Inhibitor Group<sup>b</sup></i>	<i>Human Pre<sup>c</sup></i>	<i>Human Post<sup>c</sup></i>	<i>Tetrahymena Pre<sup>d</sup></i>	<i>Tetrahymena Post<sup>d</sup></i>	<i>Euplotes<sup>e</sup></i>
<i>Intercalators</i>						
Proflavine	II	95%	90%	90%	90%	52%
Quinacrine	II	82%	50%	93%	80%	75%
<i>DNA minor groove binders</i>						
Hoechst 33258	I	81%	34%	50%	15%	86%
Diamidino-2-phenylindole	II	90%	72%	70%	70%	0%
Distamycin A	n/a	10%	n.d. <sup>f</sup>	0%	0%	75%
<i>Aminoglycosides</i>						
Neomycin	I	70%	23%	0%	0%	0%
Gentamycin	I	64%	19%	0%	0%	0%
Paromomycin	I	60%	29%	0%	0%	0%
Lividomycin	I	50%	12%	0%	0%	10%
Apramycin	n/a	30%	n.d.	0%	0%	0%
Amikacin	n/a	25%	n.d.	0%	0%	0%
Spectinomycin	n/a	0%	n.d.	0%	0%	0%
Hygromycin	n/a	0%	n.d.	0%	0%	0%
<i>Other classes</i>						
Tetracycline	n/a	10%	n.d.	0%	0%	0%

<sup>a</sup>Telomerase from several sources was assayed in the presence of 50  $\mu$ M of the indicated nucleic-acid binding ligand. 100% inhibition indicates no activity observed, and 0% inhibition indicates wild type activity.

<sup>b</sup>Inhibitors were designated as Group I or Group II as defined in the text and n/a indicates that the designation is not applicable.

<sup>c</sup>Determined using recombinant hTR and hTERT in a direct assay as described in Chapter II.

<sup>d</sup>Determined using recombinant *T. thermophila* TERT and TR in a direct assay (Bryan et al., 2000b).

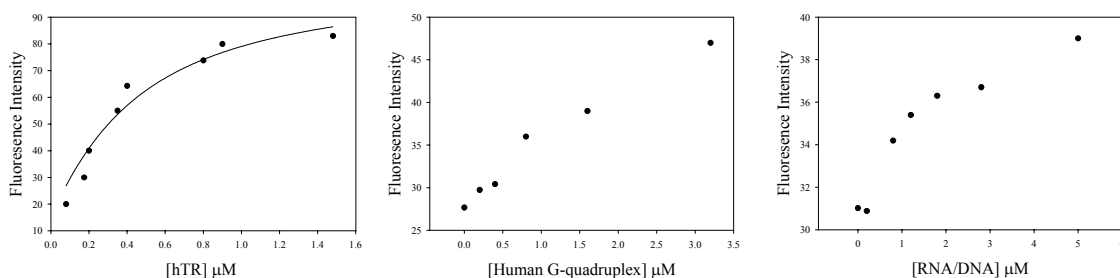
<sup>e</sup>Determined using purified endogenous telomerase from *E. aediculatus* in a direct assay (Jarstfer and Cech, 2002).

<sup>f</sup>Not determined.

## 2. Binding studies of Hoechst 33258 to hTR

Turning our attention toward elucidating the modes of inhibition of the compounds, we next determined if they hampered telomerase activity by binding to hTR. Because Hoechst 33258 was previously identified as an RNA-binding ligand that exhibits an easily monitored change in fluorescence upon binding to nucleic acids, we used fluorescence spectroscopy to directly test our hypothesis (Dassonneville et al., 1997; Cho and Rando, 2000). We observed the change in fluorescence of 0.2  $\mu$ M Hoechst 33258 in the presence of full-length hTR, a G-quadruplex formed from the human telomeric DNA sequence 5'-

(GGGATT)<sub>3</sub>GGG and the RNA-DNA duplex representing the *E. aediculatus* telomerase-primer duplex 5'-dGGTTTTGGGGTTTTG/r3'-CCUUUCCCCUUUUC (Figure 3.3), which was chosen because it forms the most stable duplex of the three telomerase complexes in



**Figure 3.3 Hoechst 33258 binds to hTR but not to other nucleic acid components present in the telomerase reaction.** The fluorescence intensity of Hoechst 33258 (0.2 μM) was monitored as a function of the concentration of three different nucleic acid components as indicated. The hTR curve was fit to the quadratic equation for a binding isotherm (see Materials and methods).

these studies. Of these, only hTR binding to Hoechst 33258 led to a saturated increase in fluorescence (Figure 3.3). Plotting Hoechst 33258 fluorescence versus hTR concentration, we obtained a dissociation constant ( $K_D$ ) of 0.36 μM.

### C. Discussion

Our initial effort to determine if hTR can be a small molecule drug target was based on the premise that nucleic acid-binding ligands can interact with hTR either before or after telomerase assemblage, resulting in a non-functional telomerase complex. To test this proposal, we screened a small library of known nucleic acid-binding ligands as inhibitors of recombinant telomerase. Potential inhibitors (Figure 3.1) were added either before assemblage of the telomerase complex or after assemblage (Figure 3.2). To determine if these compounds were specific inhibitors of human telomerase, we examined their ability to affect telomerase from two other organisms. An inhibition study with recombinant

telomerase from *Tetrahymena thermophila* (Bryan et al., 2000) conducted by adding inhibitors either before or after assemblage revealed a similar distribution of inhibition as observed for human telomerase (Table 3.1). One notable exception was that none of the aminoglycosides tested were inhibitors of the *T. thermophila* enzyme suggesting a specific interaction between the inhibitory aminoglycosides and hTR. The small library was also tested against endogenous *Euplotes aediculatus* telomerase (Jarstfer and Cech, 2002). With purified *E. aediculatus* telomerase we found that the DNA minor groove-binding drugs distamycin A and Hoechst 33258 were excellent inhibitors as were the intercalators quinacrine and proflavine. Other drugs tested had no significant affect on *E. aediculatus* telomerase activity.

After identifying several new telomerase inhibitors, we desired to elucidate the modes of inhibition of the compounds that significantly inhibited telomerase from all three organisms. The only compounds that meet this criterion are proflavine, quinacrine and Hoechst 33258. We chose to focus on Hoechst 33258 over the intercalators due to the fact that minor groove binders are typically less promiscuous in terms of specificity for nucleic acids. We examined the change in fluorescence of Hoechst 33258 in the presence of several nucleic acid components involved in the telomerase reaction cycle and found that only hTR produced a large and saturable increase in the fluorescence intensity of Hoechst 33258, indicating specificity for hTR (Figure 3.3). A plot of fluorescence versus concentration of hTR gave an approximate dissociation constant of 0.36  $\mu$ M assuming a single binding site or multiple non-cooperative binding sites. We conclude from this study that hTR is the target for inhibition by this ligand. Because the other Group I inhibitors are known to bind RNA in general and we directly observed a specific interaction between Hoechst 33258 and hTR, we

suggest that all Group I inhibitors act by interacting with hTR leading to formation of a dysfunctional telomerase complex after assemblage. Additionally, the fact that the reticulocyte lysate used to reconstitute telomerase contains large amounts of tRNA, rRNA and mRNA further suggests a high specificity of Group I inhibitors for hTR versus other RNA molecules.

The mechanisms of inhibition of the remaining molecules await further investigation. However, several points can be highlighted. The intercalators quinacrine and proflavine appear to be telomerase inhibitors that lack species specificity. Because the telomeric repeats produced by telomerase from each of these species can fold into G-quadruplexes, one possible mode of action for these molecules is quadruplex stabilization. Proflavine has been reported as an inhibitor of human telomerase ( $IC_{50} = 3.9 \mu M$ ), and a variety of proflavine derivatives have been shown to stabilize G-quadruplex structures (Perry and Jenkins, 2001). In our direct assays, the banding patterns of the telomerase products generated in the presence of quinacrine and proflavine were not consistent with typical quadruplex stabilizing compounds, which tend to cause enrichment of products associated with four repeats of the telomeric sequence (Sun et al., 1997). However, we cannot rule out this mode of inhibition at this time. Interestingly, the minor groove binder distamycin A was a strong inhibitor of *E. aediculatus* telomerase, but did not inhibit either recombinant human or *T. thermophila* telomerase. This was a surprise since a distamycin derivative was previously shown to inhibit human telomerase (Zaffaroni et al., 2002). By contrast, the minor groove binder DAPI, which does not inhibit viral reverse transcriptases (Filipowsky et al., 1996), did inhibit both recombinant human and *T. thermophila* telomerase, but did not inhibit *E. aediculatus*

telomerase. This suggests that the DAPI skeleton would be a useful starting point for the synthesis of selective telomerase inhibitors.

In conclusion, we have utilized a two-part parallel screen to identify inhibitors from a small library of known nucleic acid-binding ligands. Two general types of inhibitors were identified. Several of the compounds, the Group I inhibitors, appear to inhibit telomerase by specifically interacting with unbound hTR. Spectroscopic studies of the interaction between Hoechst 33258 and hTR, increased inhibition of human telomerase compared to inhibition of telomerase from other species and the increased inhibition of Group I inhibitors when added before assemblage of the telomerase complex support this conclusion. Group II inhibitors were similarly effective when added before or after telomerase assemblage. The most likely explanation for the differences in Group I and Group II inhibitors is that the Group I binding sites are present in hTR and are only available in unbound hTR. By contrast, Group II binding sites, which may be protein, RNA, quadruplex DNA or the RNA–DNA duplex, are available in the holoenzyme. These results provide an impetus to further examine hTR as a target for small-molecule drugs. Furthermore, these studies confirm that telomerase assemblage can be targeted with small molecules and plants the seed for future studies, which will include the screening of large compound libraries.

#### D. Materials and methods

See Chapter II, section D for information on the pET-28c-hTERT and pHTR+HH expression plasmids and methods for in vitro transcription and purification of hTR, synthesis of hTERT, reconstitution of human telomerase, direct human telomerase assays and human assemblage assays.



### 1. Chemical reagents

4',6-diamidino-2-phenylindole (DAPI), apramycin sulfate, distamycin A hydrochloride, gentamycin sulfate, Hoechst 332558 (bisbenzimidazole H 33258), hygromycin B, lividomycin A sulfate salt, neomycin, proflavine hemisulfate salt, spectinomycin dihydrochloride and tetracycline hydrochloride were purchased from Sigma. Amikacin sulfate and quinacrine dihydrochloride were purchased from Fluka. Paromomycin sulfate monohydrate was purchased from INC Biomedicals Inc. Each compound was solubilized and diluted in ddH<sub>2</sub>O and stored at -20 °C.

### 2. *T. thermophila* telomerase assays

These experiments were performed by Jason D. Legassie using a previously described protocol (Bryan et al., 2000).

### 3. *E. aediculatus* telomerase assays

These experiments were performed by Ian K. Moon using a previously described protocol (Jarstfer and Cech, 2002).

### 4. Fluorescence spectroscopy

The fluorescence intensity of 0.2 μM Hoechst 33258 was monitored as a function of the concentration of either hTR, (GGGTTA)<sub>3</sub>GGG or 5'-dGGTTTTGGGGTTTTG/r3'-CCUUUCCCCUUUUC as indicated. Hoechst was excited at 350 nm and monitored at 460 nm. Graphs were plotted using SigmaPlot 2000. The hTR curve was fit to the quadratic equation for a binding isotherm:  $F = F_0 + \Delta F \left( \frac{([Hoechst]_0 + [L]_0 + K_D) - (([hTR]_0 + [Hoechst]_0 + K_D)^2 - 4[hTR]_0[Hoechst]_0)^{1/2}}{2} \right)$  where  $F_0$  is the fluorescence intensity in the absence of hTR,  $F$  is the fluorescence intensity in the presence of hTR and  $\Delta F$  is the

difference between the fluorescence of 0.2  $\mu\text{M}$  Hoechst in the presence of saturating hTR and the absence of hTR.  $[\text{hTR}]_0$  and  $[\text{Hoechst}]_0$  are initial concentrations. These experiments were performed by Dr. Pamela K. Dominick.

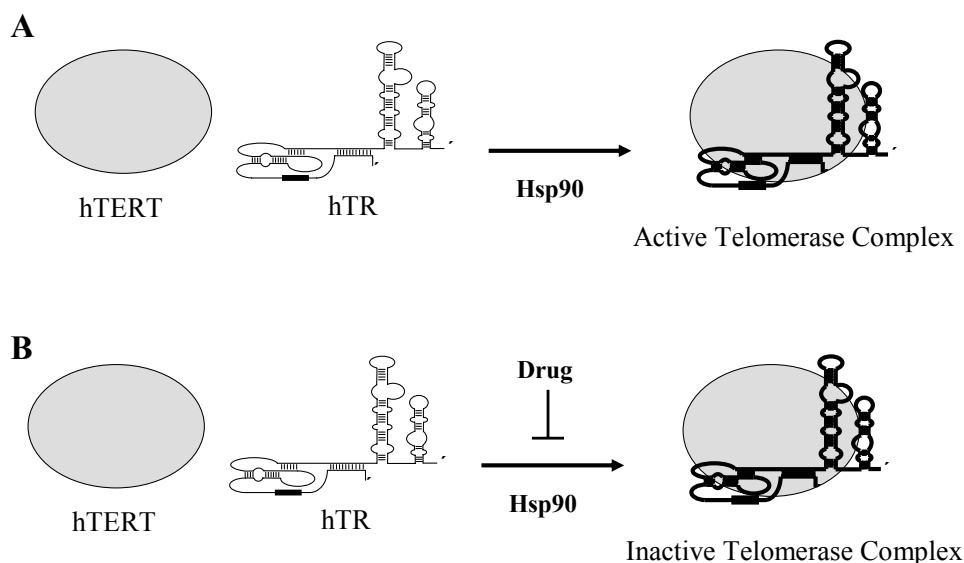
#### 5. Calculation of dissociation constant

This experiment was performed by Dr. Pamela K. Dominick.

## Chapter IV. The natural product tanshinone II-A and novel derivatives inhibit telomerase assemblage

### A. Introduction

We have initiated a new platform to identify chemical entities that disrupt the assemblage of the telomerase holoenzyme structure (Chapters II and III) (Dominick et al., 2004; Keppler and Jarstfer, 2004). This approach is diverse in that it can identify molecules that inhibit hsp90, bind the catalytic subunit hTERT or bind the RNA subunit hTR (Figure 4.1). Thus, drugs that affect the assemblage of telomerase can do so through several mechanisms including inhibiting essential chaperones (Holt et al., 1999; Villa et al., 2003),



**Figure 4.1 Effectors of telomerase assemblage.** (A) Telomerase holoenzyme components required in order to attain enzymatic activity. (B) A screen for molecules that perturb the assemblage of telomerase will identify compounds that bind hTR, hTERT or inhibit hsp90. The end result of telomerase assemblage modulators is a dysfunctional telomerase complex.

disrupting critical protein-protein interactions or preventing pertinent protein-RNA interactions required for telomerase function (Dominick et al., 2004; Keppler and Jarstfer, 2004). The end result of perturbing telomerase holoenzyme assemblage is the in situ generation of a dominant negative complex that may be deficient in all telomerase-dependent functions. Previously, we established that blocking specific hTR-hTERT interactions prevents proper telomerase assemblage (Chapter II) (Keppler and Jarstfer, 2004), and we have shown that small molecules that bind hTR can initiate the formation of a dysfunctional telomerase complex (Chapter III) (Dominick et al., 2004). Here, we examine a class of natural products for their ability to produce a dominant negative telomerase complex in an in vitro assemblage assay.

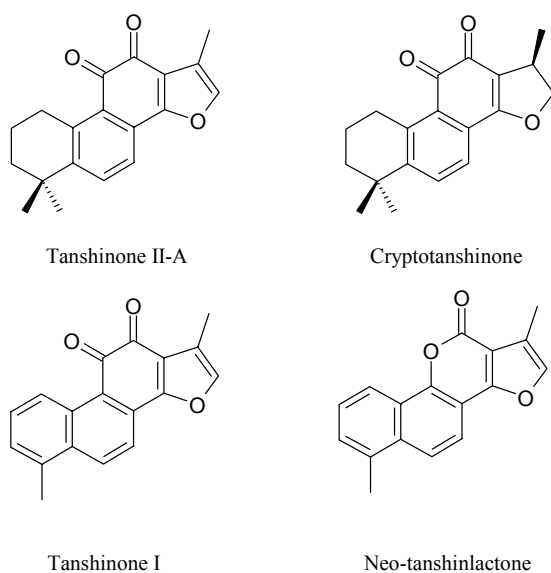
Tanshinones are diterpene pigments isolated from the root of *Salvia miltiorrhiza* BUNGE, which has been used in numerous traditional Chinese folk remedies (Baillie and Thomson, 1968; Lee et al., 1987). Several tanshinones have demonstrated cytotoxic effects and are considered prospective leads for developing new anticancer drugs (Wu et al., 1991; Ryu et al., 1997). The best studied and most abundant tanshinone, tanshinone II-A (Tan II-A), induces apoptosis in a wide variety of cancer cell lines (Sung et al., 1999; Yuan et al., 2004). The mechanism of Tan II-A-induced apoptosis, however, is not understood. Given the recent evidence that telomerase directly affects apoptosis and the general interest in new telomerase inhibitors, we hypothesized that telomerase may be one of the molecular targets of Tan II-A. We examined the effect of several natural tanshinones on telomerase activity to test this hypothesis. Here, we show that the diketone tanshinones Tan II-A, tanshinone I and cryptotanshinone inhibit telomerase activity in an in vitro assemblage assay, suggesting that they perturb the assemblage of telomerase or are time dependent inhibitors of telomerase.

Furthermore, we demonstrate that their cytotoxicity coincides with telomerase activity. Thus, tanshinones provide a new structural class of small molecules to explore as telomerase inhibitors.

## B. Results

### 1. Tanshinone II-A is a potent and specific inhibitor of telomerase assemblage

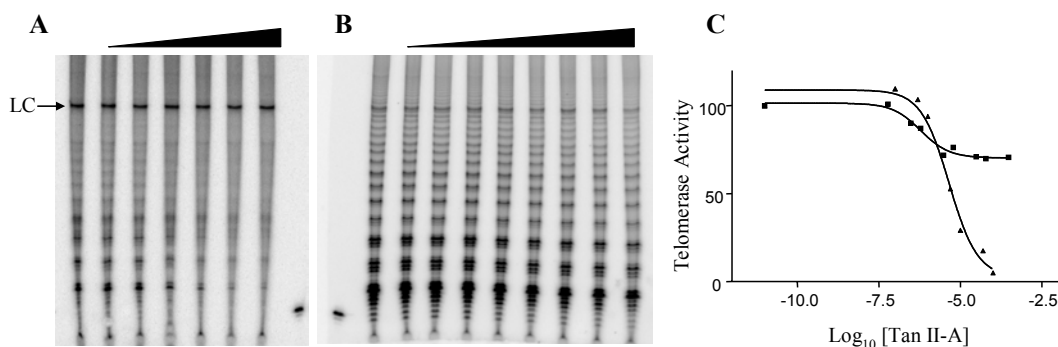
We recently developed a telomerase assemblage assay that allows one to identify small molecules that affect telomerase activity through several mechanisms including direct inhibition of its enzymatic activity, blocking specific protein-RNA interactions required for assemblage of active telomerase and inhibiting the chaperone hsp90, which is required for telomerase activity (Holt et al., 1999). The assay takes advantage of the ability to assemble recombinant telomerase in rabbit reticulocyte lysates (RRL) (Beattie et al., 1997; Weinrich et al., 1997) followed by a direct assay for telomerase activity using a telomeric primer and [ $\alpha$ - $^{32}$ P]-dGTP as one of the substrates. In this study, we focused on the tanshinone class of



**Figure 4.2 Tanshinones tested in this study**

natural products because they have broad anti-proliferative effects that we reasoned could be related to inhibition of telomerase-mediated activities (Figure 4.2).

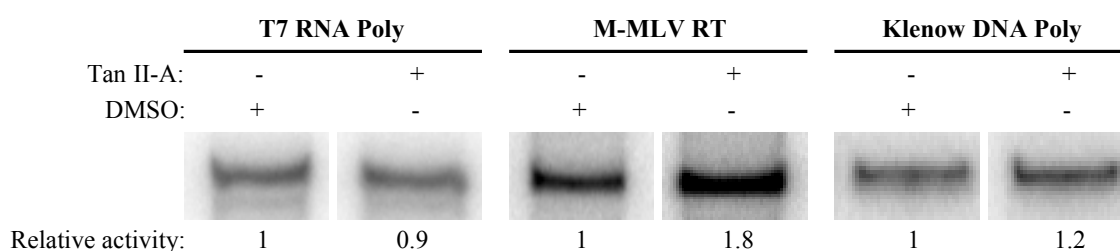
We evaluated the affect of Tan II-A on telomerase activity by adding varying concentrations of Tan II-A to a reaction mixture containing in vitro translated hTERT in RRL and in vitro transcribed hTR followed sequentially by an assemblage reaction and a primer extension assay to evaluate telomerase activity. Tan II-A was a potent inhibitor of telomerase in this assemblage assay with an  $IC_{50} = 4.8 \mu M$  (Figure 4.3, curve with  $\blacktriangle$  symbols). In a separate experiment, varying concentrations of Tan II-A were added to telomerase after it was allowed to assemble (a post-assemblage assay). Again, Tan II-A was an inhibitor of telomerase with an  $IC_{50} = 0.5 \mu M$  (Figure 4.3, curve with  $\blacksquare$  symbols), however, the maximum amount of inhibition was only 30% when Tan II-A was added to assembled telomerase, compared to >95% when Tan II-A was added before assemblage.



**Figure 4.3 Inhibition of telomerase by tanshinone II-A.** (A) Assemblage assay using varying concentrations of Tan II-A (from 0.5 to 100  $\mu M$ ). (B) Direct assay using varying concentrations of Tan II-A (from 0.1 to 100  $\mu M$ ). The wedge indicates increasing concentrations of Tan II-A. LC is a loading control used to normalize the telomerase products present in each lane. (C) Quantification of telomerase assays. % Activity of experimental samples was determined by comparison with a positive control containing DMSO as a carrier.

## 2. Tanshinone II-A inhibition of various nucleic acid polymerases

The selectivity of Tan II-A for telomerase was assessed using the Klenow fragment of DNA polymerase I, T7 RNA polymerase and MMLV reverse transcriptase as examples of other nucleic acid processing enzymes. At 50  $\mu\text{M}$ , over ten times the  $\text{IC}_{50}$  for telomerase, Tan II-A did not inhibit the other nucleic acid polymerases, though we did observe a noteworthy increase in the activity of M-MLV RT (Figure 4.4).



**Figure 4.4 Effects of Tanshinone II-A on different nucleic acid processing enzymes.** Positive controls contained an equal volume of DMSO in place of the tanshinone. “Relative activity” refers to the amount of enzymatic activity as compared to the DMSO-only control. The concentration of Tan II-A was 50  $\mu\text{M}$  in each experiment.

## 3. The tanshinone quinone moiety is essential for inhibition

To determine the salient features of the tanshinone structure required for inhibition, we examined several natural tanshinones (Figure 4.2) for their ability to affect telomerase activity when added prior to assemblage. Tan II-A, Tan I and cryptotanshinone, which all have a 1,2-diketone C ring, inhibited telomerase, while neo-tanshinlactone, which has a lactone C ring, was a less potent inhibitor (Table 4.1). Thus, the 1,2-diketone moiety present in the tanshinone structure is essential for maximum activity. Our results also showed that an aromatic A ring (compare tanshinone I to Tan II-A) and a heteroaromatic D ring (compare Tan II-A to cryptotanshinone) are not required for telomerase inhibition.

**Table 4.1 Natural tanshinones affect telomerase activity**

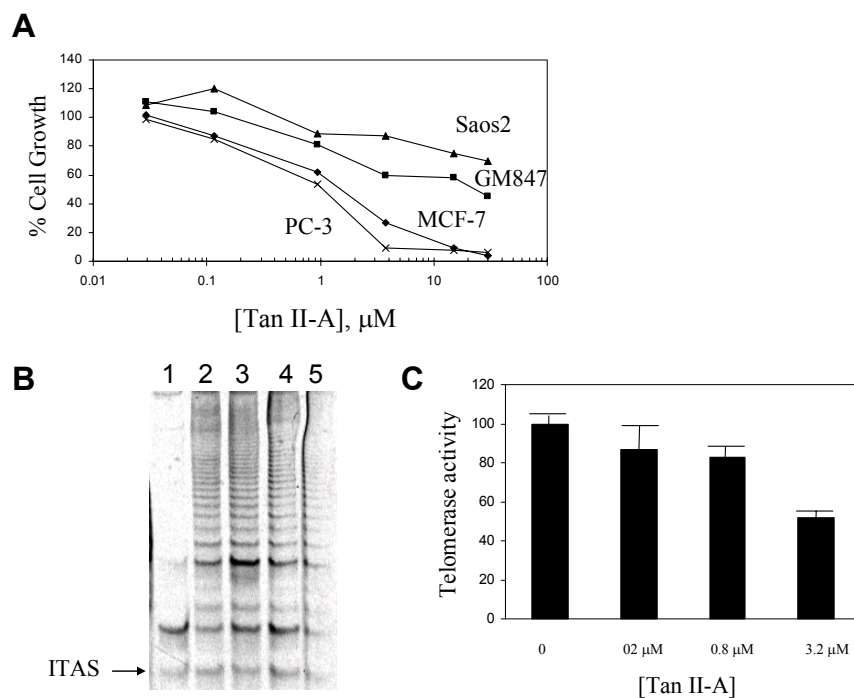
Compound	Percent Telomerase Activity at 50 $\mu$ M
Tan II-A	26
Tanshinone I	35
Cryptotanshinone	22
Neo-tanshinlactone	75

Compounds were added to telomerase assemblage assays at a final concentration of 50  $\mu$ M. Telomerase activity was quantified by comparison with a DMSO-only control. Values are the average of at least two separate experiments and have standard errors < 2%.

4. Tanshinone II-A inhibits telomerase in cultured cells and is selectively cytotoxic towards telomerase positive cells

We next tested the cytotoxicity of Tan II-A in telomerase-positive and telomerase-negative cells lines to determine if telomerase inhibition coincided with observed cytotoxicity. Tan II-A was a potent cytotoxin of the telomerase-positive breast cancer cell line MCF-7 ( $EC_{50}$  0.90  $\mu$ M) and prostate cancer cell line PC-3 ( $EC_{50}$  1.2  $\mu$ M), consistent with previous reports using a variety of cancer cell lines (Figure 4.5A) (Wu et al., 1991; Ryu et al., 1997; Wang, X. et al., 2004b; Yuan et al., 2004). Furthermore, telomerase activity was reduced by 50% in MCF-7 cells after 24 hr exposure to cytotoxic concentrations of Tan II-A (Figures 4.5B and C). By contrast, in telomerase-negative GM847 and Saos-2 cells, we found that Tan II-A was much less toxic with an  $EC_{50}$  of >20  $\mu$ M against both cell lines (Figure 4.5A).





**Figure 4.5 Consequences of treating immortalized cells with tanshinone II-A.** (A) Cytotoxicity assays of Tan II-A. (B) Residual telomerase activity of MCF-7 cells 24 hr after Tan II-A exposure. Lane 1, heat inactivated MCF-7 extracts; lane 2, extract from untreated MCF-7 cells; lane 3 extract from MCF-7 cells treated with 0.2  $\mu$ M Tan II-A; lane 4 extract from MCF-7 cells treated with 0.8  $\mu$ M Tan II-A; lane 5 extract from MCF-7 cells treated with 3.2  $\mu$ M Tan II-A. ITAS, internal amplification standard. (C) Graphical representation of telomerase assays. Telomerase activity is the percent of residual telomerase activity in treated cells relative to untreated control cells and is the average of two independent calculations. Error bars indicate standard deviation.

## C. Discussion

Telomerase has the potential to be a universal anticancer drug target, and many approaches to target telomerase have been explored (Saretzki, 2003). Currently the most promising telomerase inhibitors are oligonucleotide-based template antagonists that inhibit the enzymatic activity of telomerase (Corey, 2002). Because telomerase is involved in several pathways that promote cancer cell proliferation, targeting only the enzymatic activity of telomerase might not achieve the maximum potential of telomerase-based therapeutics. We have therefore initiated a concerted effort to discover telomerase inhibitors that affect

assemblage of the telomerase holoenzyme structure, as opposed to simply inhibiting its enzymatic activity. The hypothesis behind our approach is that disrupting telomerase assemblage has the potential of creating, in situ, a dominant negative telomerase complex that is dysfunctional in all telomerase-related activities. In the current study, we investigated the ability of the tanshinone natural products to affect telomerase activity. This compound class was examined because the underlying mechanism of tanshinone-induced cytotoxicity has not been identified, and because the broad cytotoxicity of tanshinones against cancer cells suggests they target a pathway shared by many cell types (Wu et al., 1991).

We found that tanshinones containing a 1,2 di-ketone were inhibitors of telomerase (Table 4.1) and were demonstrably more active when present during telomerase assemblage. This latter result suggests that they perturb telomerase assemblage resulting in a dysfunctional complex. Previous reports suggest that hsp90 inhibitors also inhibit telomerase (Holt et al., 1999; Villa et al., 2003) by perturbing telomerase assemblage leading to the hypothesis that Tan II-A is an hsp90 inhibitor. Recent studies in our lab, however, have shown that hsp90 inhibitors geldanamycin and novobiocin completely inhibit telomerase even after it is assembled, suggesting that the role of hsp90 is more complex than simply mediating assemblage. In contrast to geldanamycin and novobiocin, Tan II-A only partially inhibited pre-assembled telomerase resulting in a 30% decrease in activity and an  $IC_{50}$  that was comparable to the  $IC_{50}$  obtained when Tan II-A was added during the assemblage process (Figure 4.3). This result suggests that Tan II-A is only a partial antagonist of the telomerase holoenzyme, or is a time dependent inhibitor. If hsp90 were the molecular target for Tan II-A, we would expect complete inhibition of pre-assembled telomerase, by comparison to geldanamycin and novobiocin. Inhibition of telomerase by Tan II-A

resembles the inhibition of telomerase by nucleic acid-binding ligands Hoechst 33258 and neomycin, which have been proposed to inhibit telomerase by targeting its RNA subunit (Chapter III) (Dominick et al., 2004). Based on the similarities of its pattern of inhibition, we speculate that Tan II-A also targets hTR. Alternatively, Tan-IIA could utilize its ortho-quinone moiety to engage in a REDOX cycling reaction that causes oxidation of hTR or hTERT. This possibility is currently being investigated.

We hypothesize that the ability of Tan II-A to inhibit nucleic acid processing enzymes is specific for telomerase, an RNA-dependent DNA Polymerase, as the tanshinones failed to significantly affect the activity of T7 RNA Polymerase, M-MLV RT and Klenow DNA Polymerase (Figure 4.4). We did observe a notable increase in activity when Tan II-A was added to an M-MLV RT reaction, however, this increase in activity does not influence our hypothesis that the compound has inhibitory specificity for telomerase.

Our cell-based assays demonstrated that Tan II-A is cytotoxic to both telomerase-positive and telomerase-negative cells, but that telomerase-positive cell lines were more sensitive to Tan II-A than telomerase-negative cells (Figure 4.5A). We also showed that Tan II-A decreases telomerase activity in telomerase-positive MCF-7 cells (Figures 4.5B and C). Thus, disrupting telomerase assemblage in telomerase-positive cells likely contributes to the overall mechanism of Tan II-A-mediated cytotoxicity. Perhaps in rapidly dividing cells, the telomerase complex actively participates in an anti-apoptotic process. Whereas in telomerase-negative cells that are sensitive to Tan II-A, the apoptotic block served by telomerase is replaced by other factors. Tan II-A has several biological effects that are consistent with this model. In cell-based assays, Tan II-A inhibited differentiation of precursor cells to osteoclasts and inhibited bone reabsorption by differentiated osteoclasts in

cultured mouse cells (Takahashi et al., 2002). In this case, Tan II-A affected the gene expression of proteins required for osteoclast differentiation and activity but did not induce apoptosis. While the telomerase activity of these cells was not examined, human osteoblasts and osteoclasts are generally considered telomerase negative (Yudoh et al., 2001; Darimont et al., 2002). Tan II-A-induced apoptosis of human hepatocellular carcinoma cells coincided with upregulation of the apoptosis-related genes *fas*, *bax* and *p53* coincidental with downregulation of *bcl2* and *c-myc* (Yuan et al., 2004). Downregulation of *c-myc* may also contribute to the observed inhibition of telomerase in cultured cells as *c-myc* appears to participate in the regulation of hTERT expression (Horikawa et al., 1999). These results suggest that Tan II-A has variable effects depending on cell type and can affect multiple signaling pathways. These multiple effects of Tan II-A presumably arise from its ability to affect several targets, which now includes telomerase.

In summary, our results indicate that the tanshinones containing a 1,2-diketone provide a unique structural class of telomerase inhibitors that function through a unique mechanism, perturbing telomerase assemblage. Our results demonstrate that cytotoxicity of Tan II-A in part correlates with telomerase expression. Results suggest that the ortho-quinone moiety of Tan II-A is the important pharmacophore for inhibition of telomerase. This suggests that Tan II-A is involved in REDOX cycling that could generate reactive oxygen species leading to oxidative damage of telomerase, similar to the polyaromatic quinine-mediated catalytic inactivation of protein tyrosine phosphatases observed by Wang et al. (Wang, Q. et al., 2004a). If true, this would explain the broad pharmacological activity of Tan II-A and shed light on the REDOX regulation of telomerase (Borras et al., 2004).

## D. Materials and methods

See Chapter II, section D for information on pET-28c-hTERT and pHTR+HH expression plasmids and methods for in vitro transcription and purification of hTR, synthesis of hTERT, reconstitution of human telomerase, direct telomerase assays and assemblage assays.

### 1. Chemical reagents

Tanshinones were made available from a previous study and were stored as 5 mM stocks in sterile DMSO (Wang, X. et al., 2004b).

### 2. Cells and cell culture

The human breast cancer cell line MCF-7 (telomerase-positive adenocarcinoma, from ATCC, Manassas, VA) was cultured in  $\alpha$ MEM medium (Gibco, Carlsbad, Calif.) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, Mo.), 10  $\mu$ g/ml insulin (Gibco) and sodium pyruvate (Gibco). The human prostate cancer cell line PC-3 (telomerase-positive adenocarcinoma, from ATCC) was cultured in Dulbecco's modified Eagle/F12 medium (DMEM/F12) (Gibco) supplemented with 10% FBS. The human osteosarcoma cell line Saos-2 (telomerase-negative, from ATCC) was cultured in McCoy's 5A medium supplemented with 15% FBS. The immortalized fibroblast cell line GM847 (telomerase-negative) was a gift from Shelia Stewart and Robert Weinberg at the Massachusetts Institute of Technology (Cambridge, Mass.) and was cultured in DMEM supplemented with 10% FBS (Sigma, St. Louis, Mo.) and 50  $\mu$ g/ml of gentamycin (Sigma).

### 3. Telomerase activity of cell extracts

Telomerase activity in cell extracts was determined by a modified telomeric repeat amplification protocol (TRAP assay) (Kim and Wu, 1997). In short, cell lysates were prepared from  $4 \times 10^4$  cells in 100  $\mu$ L of lysis buffer (10 mM Tris, pH 7.5, 1 mM  $\text{MgCl}_2$ , 1 mM EGTA, 0.1 mM benzamidine, 0.5 mM  $\beta$ -mercaptoethanol, 0.5% CHAPS, 10% glycerol) by incubating the suspended cells on ice for 30 min followed by three freeze thaw cycles using liquid  $\text{N}_2$  to freeze the samples. Cell lysates were clarified by centrifugation (10,000g, 30 min at 4 °C) and protein levels in the cell extracts were determined by the method of Bradford (Bradford, 1976). Cell extracts (100 ng of total protein) were incubated with 0.1  $\mu$ g TS primer (5'-AATCCGTCGAGCAGAGTT) in a 25  $\mu$ L reaction containing TRAP buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM  $\text{MgCl}_2$ , 63 mM KCl, 0.05% Tween20 and 1 mM EGTA) and 50  $\mu$ M of each dNTP at 30 °C for 30 min. Telomerase extension reactions were then heated to 95 °C for 5 min before the products were amplified by adding 0.1  $\mu$ g ACX primer (5'-GCGCGG(CTTACC)<sub>3</sub>CTAACC), 0.1  $\mu$ g NT primer (5'-ATCGGCTTCTCGGCCTTTT), 0.001 amol TSNT template (5'-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAA-GCGAT, used as a PCR and loading control), 0.5 units HotMaster *Taq* DNA polymerase (Eppendorf), 5  $\mu$ L of 10 $\times$  *Taq* buffer and dNTPs to a final concentration of 50  $\mu$ M. The reactions were then subjected to 33 PCR cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s. Reaction products were separated on a 12.5% nondenaturing polyacrylamide gel, stained with SYBR Green I (Molecular Probes) and imaged on a phosphorimager. Reactions were quantified using ImageQuant and the product intensity for each reaction was normalized to the TSNT internal control.

#### 4. Cytotoxicity assay

Acute cell growth inhibition by drugs was determined using a sulforhodamine B (SRB) assay (Papazisis et al., 1997). Between 3,000 and 6,000 cells/well were seeded into 96-well microtiter plates in 0.2 ml of media and allowed to attach overnight. A range of concentrations of Tan II-A was added from a 5 mM stock in DMSO to quadruplicate wells and cells were incubated for 96 hr. Cell numbers of treated and untreated cells were compared. First, cells were fixed by adding 50  $\mu$ L of cold 50% trichloroacetic acid to the top of the 200  $\mu$ L culture medium and incubated for 30 min at 4 °C. Fixed cells were washed extensively with deionized water and dried for 2 hr at room temperature. Cells were stained for 20 min in 0.4% SRB, 1% acetic acid, rinsed with 1% acetic acid and air dried. The bound SRB was solubilized with 200  $\mu$ L 10 mM Tris-base and the absorbance at 562 nm was measured.

#### 5. Testing the effects of Tanshinone II-A on T7 RNA polymerase, M-MLV reverse transcriptase and the Klenow fragment of DNA polymerase I

To test the effect of 50  $\mu$ M Tan II-A on T7 RNA Polymerase, the compound was added to a reaction transcribing  $^{32}$ P-labeled pseudoknot RNA fragment (see Chapter II). The RNA was resolved on a denaturing gel and compared to RNA transcribed in the presence of DMSO as a carrier.

To determine if 50  $\mu$ M Tan II-A influences M-MLV reverse transcription, the compound was added to a reaction containing 0.5 mM dNTPs, 0.33  $\mu$ M [ $\alpha$ - $^{32}$ P]-dGTP (3000 Ci/mmol, 10  $\mu$ Ci/ $\mu$ L; Perkin Elmer), 20 units RNasin (Promega), 10 ng RNA template, 1  $\mu$ M primer, 100 units M-MLV reverse transcriptase (Promega) and 1 $\times$  M-MLV buffer (Promega). The dNTPs, RNA, primer, Tan II-A and buffer were first combined and

incubated at 95 °C for 5 min followed by 0 °C for 3 min. The [ $\alpha$ -<sup>32</sup>P]-dGTP and enzymes were then added and the reaction was incubated at 37 °C for 2 h followed by 95 °C for 5 min. After passing through a Microspin G-25 column (Amersham), the sample was resolved on a denaturing gel and compared to a reaction containing DMSO in place of Tan II-A.

To test the effect of 50  $\mu$ M Tan II-A on the Klenow Fragment of DNA Polymerase I, we first performed a double digestion on pET-28c using Sal I and Xho I in order to generate a 21 base-pair piece of DNA with sticky ends. About 2  $\mu$ g pET-28c was combined with 20 units Sal I (New England Biolabs, NEB), 20 units Xho I (NEB), 100  $\mu$ g/mL BSA and 1 $\times$  Sal I buffer (NEB) and incubated at 37 °C for 90 min, followed by 65 °C for 20 min to heat inactivate the enzymes. ~600 ng of the digested DNA was then combined with 50  $\mu$ M dNTPs, 0.165  $\mu$ M [ $\alpha$ -<sup>32</sup>P]-dGTP (3000 Ci/mmol, 10  $\mu$ Ci/ $\mu$ L; Perkin Elmer), 5 units Large (Klenow) Fragment of DNA Polymerase I (NEB), 1 $\times$  NEB buffer #2 and Tan II-A to a final concentration of 50  $\mu$ M. The reaction was incubated at 25 °C for 30 min followed by 75 °C for 20 min and passed through a Microspin G-25 column. The degree of Tan II-A-induced Klenow inhibition was quantified by resolving the final product on a native gel along side a positive control reaction containing DMSO instead of Tan II-A.

## 6. Natural product isolation and derivative synthesis

These experiments were performed by Xihong Wang in the laboratory of Dr. Kuo-Hsiung Lee.



## **Chapter V. Hsp90 is required to maintain telomerase in an active conformation**

### **A. Introduction**

The complete subunit composition of the human telomerase ribonucleoprotein (RNP) complex has yet to be fully elucidated, but the minimally active enzyme requires the reverse transcriptase catalytic subunit (hTERT) and an RNA subunit (hTR), which contains the template (Feng et al., 1995; Kilian et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997). Recent findings revealed that chaperone proteins such as Hsp90, p23, Hsp70, p60 and Hsp40/ydj are also part of the telomerase RNP, at least during part of the cell cycle (Holt et al., 1999; Forsythe et al., 2001). In fact, these chaperones are required to obtain a functionally active telomerase RNP in vitro, though their precise role in telomerase assemblage remains enigmatic (Holt et al., 1999; Forsythe et al., 2001).

Hsp90 is a highly conserved and abundant protein found in all eukaryotic cells (Lai et al., 1984; Jolly and Morimoto, 2000) that functions as part of a “foldosome” complex (Pratt, 1993; Hutchison et al., 1994). Together with other chaperones, Hsp90 facilitates the accurate arrangement of numerous proteins. Hsp90 possesses a number of previously identified functional domains. Important for pharmacological concerns are the two ATP-binding sites located in both the N-terminus (Grenert et al., 1997; Prodromou et al., 1997) and C-terminus (Csermely and Kahn, 1991; Marcu et al., 2000) of the protein. These sites are essential for Hsp90 function because ATP binding and hydrolysis are crucial in the conformational regulation of Hsp90 and therefore its effects on client proteins (Prodromou et al., 2000). It

has been suggested that the two ATP-binding sites act cooperatively, allowing cross-talk mediated by a central charged domain between the two termini (Marcu et al., 2000). Hsp90 is a functional homodimer with one homo-dimerization domain in the C-terminus (Nemoto et al., 1995) and a second located within the N-terminus (Prodromou et al., 2000). There are also a number of co-chaperone binding domains including one for the acidic phosphoprotein p23, which binds to the amino-terminal and central region of Hsp90 in an ATP-dependent manner (Johnson and Toft, 1994; Grenert et al., 1997; Sullivan et al., 1997; Bohen, 1998; Fang et al., 1998), and one for Hsp70, which binds to the carboxyl terminus of Hsp90 (Freitag et al., 1997; Young et al., 1998; Carrello et al., 1999; Marcu et al., 2000; Murphy et al., 2001).

The best characterized domain of Hsp90 is undoubtedly the highly conserved, N-terminal, nucleotide-binding pocket (Grenert et al., 1997; Prodromou et al., 1997; Stebbins et al., 1997), which also serves as the binding site for the Hsp90 inhibitor geldanamycin (GA) (Whitesell et al., 1994; Grenert et al., 1997). GA, a benzoquinone ansamycin antibiotic, exerts its inhibitory effect by blocking the ATP-dependent binding of p23 to Hsp90 (Grenert et al., 1997; Prodromou et al., 1997). Another Hsp90 inhibitor, the coumarin-type antibiotic novobiocin (NB), binds to the C-terminus of Hsp90 and hinders chaperone complex formation by blocking its association with both Hsp70 and p23 (Marcu et al., 2000). Due to their antagonizing effects on Hsp90 function, both GA and NB are also inhibitors of proper telomerase holoenzyme assembly and therefore telomerase activity (Holt et al., 1999; Haendeler et al., 2003).

The Hsp90 client list, which is constantly being updated, includes various transcription factors, polymerases, signaling protein kinases and steroid receptors, as well as

other proteins (Pratt and Toft, 2003). Besides telomerase, Hsp90 has been implicated in activating another reverse transcriptase (RT), duck hepatitis B virus (DHBV) RT (Hu and Seeger, 1996). Interestingly, Hu and Seeger demonstrated that the role of Hsp90 in DHBV RT activity is to maintain the RT in a “protein-priming” conformation capable of binding to the viral pregenomic RNA, which serves as the template for reverse transcription.

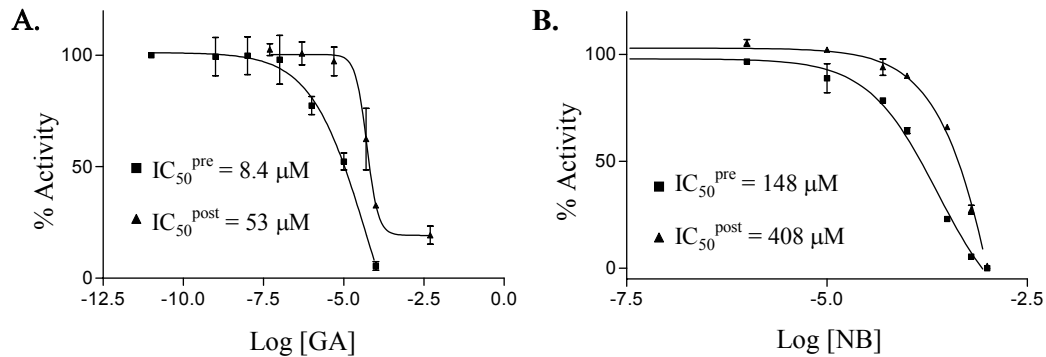
Because we are interested in targeting telomerase assemblage as an anticancer approach, it is important to elucidate and understand the role of Hsp90 in telomerase RNP assemblage and function. Here, we show that the addition of GA or NB to a direct telomerase assay inhibits telomerase activity independent of the order of inhibitor addition, i.e. before or after telomerase assembly. We also report that hTERT and hTR are capable of interacting with each other in the absence of a functional Hsp90-p23 complex, though such a complex is inactive. In these experiments, GA inhibition could only be overcome if telomerase was pre-incubated with its primer. This leads us to propose a model in which the maturation of the human telomerase RNP into its final “primer-accepting” conformation requires Hsp90 and its co-chaperones. Hsp90 may therefore play a role in human telomerase RNP maturation and ligand binding similar to its role in establishing active DHBV RT (Hu and Seeger, 1996) and steroid receptors (Bledsoe et al., 2002).

## B. Results

### 1. Hsp90 inhibitors affect in vitro reconstituted human telomerase activity both before and after assembly

To examine the role of Hsp90 in telomerase activation we used two Hsp90 inhibitors: GA, which binds to the N-terminal ATP-binding site, and NB, which binds to the C-terminal ATP-binding site. Despite their separate binding sites, both compounds have been reported

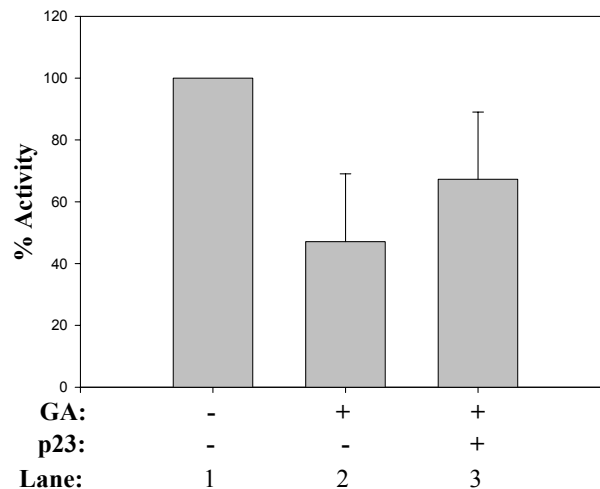
to inhibit human telomerase (Holt et al., 1999; Haendeler et al., 2003). In our hands, GA inhibited the in vitro reconstitution of telomerase activity when added before assembly with an  $IC_{50}^{pre}$  of 8.4  $\mu$ M and NB inhibited with an  $IC_{50}^{pre}$  of 148  $\mu$ M (Figure 5.1). Notably, the concentrations of GA and NB were maintained throughout the assays, including both the assemblage and the primer extension reactions. These findings are consistent with the hypothesis that Hsp90 inhibitors exert their effect by preventing Hsp90-dependent association between hTR and hTERT. We further tested the role of Hsp90 by adding the Hsp90 inhibitors to telomerase that was pre-assembled. Surprisingly, we found that both



**Figure 5.1 GA and NB inhibit telomerase when added before or after assembly.** (A) Concentration dependence of GA on telomerase activity in an assemblage assay (pre-assembly, ■) and a direct assay (post-assembly, ▲). (B) Concentration dependence of NB on telomerase activity in an assemblage assay and a direct assay.  $IC_{50}$  values were calculated and inhibition curves were plotted as described in the Materials and methods. Error bars indicate standard deviation.

GA and NB inhibited telomerase activity with  $IC_{50}^{post}$  values of 53  $\mu$ M and 408  $\mu$ M, respectively (Figure 5.1). Although the  $IC_{50}$  values for GA and NB were slightly higher when added post-assemblage when compared to their addition pre-assemblage, both drugs

were full antagonists of assembled telomerase. Therefore, we predicted that the effect of the Hsp90 inhibitors was not caused by explicitly preventing hTR-hTERT interaction, but resulted from an affect on some other aspect of telomerase. To rule out direct inhibition of telomerase, we showed that the addition of p23 suppressed inhibition by GA (Figure 5.2).



**Figure 5.2 p23 has a slight rescue effect on GA-induced telomerase inhibition.**

Telomerase activity was inhibited in an assembly assay by the addition of 75  $\mu$ M GA (lanes 2 and 3). p23 was added to the reaction for 30 min prior to the addition of GA and hTR at a final concentration of 20  $\mu$ M (lane 3). % Activity indicates the amount of residual telomerase activity as compared to the positive control (DMSO). Error bars indicate standard deviation.

## 2. Telomerase maintains hTR-hTERT interactions in the presence of geldanamycin and novobiocin after hTERT translation

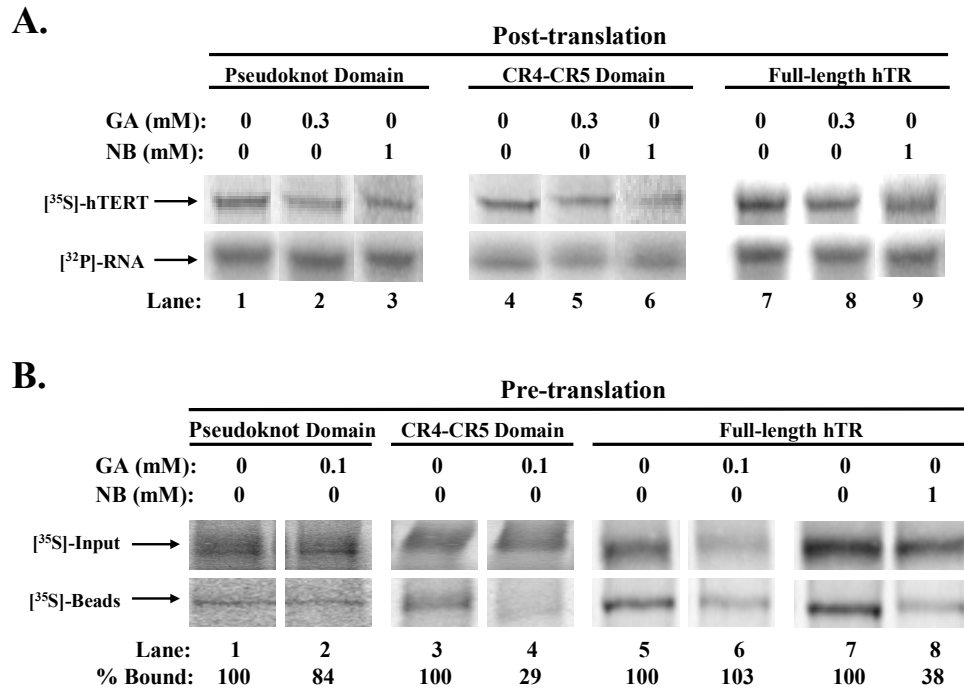
Hsp90 was previously shown to be required for reconstituting active recombinant telomerase and to be involved in maintaining telomerase activity in cultured human cells (Holt et al., 1999; Akalin et al., 2001). The precise role of Hsp90 in establishing active telomerase, however, is not clear from these studies. To determine if Hsp90 is involved in establishing the association of hTR and hTERT during telomerase assembly, we performed co-immunoprecipitation assays using T7-tagged [ $^{35}$ S]-hTERT and [ $^{32}$ P]-RNA in the presence

of 300  $\mu$ M GA or 1 mM NB. We tested the ability of GA and NB to inhibit hTERT binding to full-length hTR as well as the CR4-CR5 and pseudoknot domains, which are two regions of hTR previously shown to interact independently with hTERT (Tesmer et al., 1999; Mitchell and Collins, 2000; Chen et al., 2002; Chen and Greider, 2003; Keppler and Jarstfer, 2004). The CR4-CR5 domain is a structurally conserved domain found in vertebrate telomerase RNAs. In humans, the CR4-CR5 domain is well defined and has been found to contain a stem-loop termed P6.1, whose direct interaction with hTERT is required for telomerase activity (Tesmer et al., 1999; Mitchell and Collins, 2000; Chen et al., 2002; Chen and Greider, 2003). The pseudoknot domain, which contains the template, also interacts with hTERT in a telomerase activity-dependent manner (Tesmer et al., 1999; Mitchell and Collins, 2000). After allowing assembly between the two subunits, hTERT was immunopurified, along with bound RNA, and the resulting complexes were analyzed by SDS PAGE. We found that Hsp90 inhibition does not affect the association of hTERT with hTR or the two domains of hTR we tested (Figure 5.3A). This suggests that Hsp90 does not facilitate the association of hTR and hTERT. Clearly, however, the telomerase complex that is assembled when Hsp90 is inhibited is dysfunctional.

Hsp90 and other chaperones function in part to stabilize peptides during translation to prevent improper folding (Wegele et al., 2004). In the previous experiments, GA and NB were added after hTERT had been translated while Hsp90 was presumably active. To determine if Hsp90 activity is required to allow the nascent hTERT transcript to assume an hTR-binding competent state, we inhibited Hsp90 during hTERT translation. Because inhibition of Hsp90 during translation appeared to affect immunoprecipitation efficiency in

preliminary studies, we used affinity purification of the RNA subunit to assess hTR-hTERT association.

To analyze the association of full-length hTR with hTERT, telomerase complexes were affinity purified using a biotinylated oligonucleotide that was complimentary to the RNA template (Schnapp et al., 1998). To analyze the association of the CR4-CR5 and pseudoknot domains with hTERT, [<sup>35</sup>S]-hTERT was translated in the presence of biotin-



**Figure 5.3 hTR/hTERT interactions are unaffected by the presence of GA and NB after translation of hTERT, but hTR/hTERT interactions are effected by the presence of GA and NB during translation.** (A) [<sup>32</sup>P]-Pseudoknot RNA fragment (lanes 1-3), [<sup>32</sup>P]-CR4-CR5 RNA fragment (lanes 4-6) or [<sup>32</sup>P]-full-length hTR (lanes 7-9) were incubated with T7-tagged [<sup>35</sup>S]-hTERT either in the presence or absence of 300  $\mu$ M GA or 1 mM NB. The complexes were immunopurified and resolved on an SDS gel. [<sup>35</sup>S]-hTERT bands were used to normalize [<sup>32</sup>P]-RNA bands. (B) Pseudoknot RNA fragment (lanes 1-2), CR4-CR5 RNA fragment (lanes 3-4) or full-length hTR (lanes 5-8) were added to TNT Coupled Reticulocyte Lysate reactions, which translated T7-tagged [<sup>35</sup>S]-hTERT in the presence of 100  $\mu$ M GA or 1 mM NB. The complexes were affinity purified and resolved on an SDS gel. “% Bound” values indicate the amount of protein bound to the RNA when compared to control reactions with no GA or NB. Gels shown are representative of multiple (2-4) experiments. Independent experiments revealed no significant nonspecific binding (< 5%).

labeled RNAs and GA (Keppler and Jarstfer, 2004). Interestingly, only the interaction of the CR4-CR5 domain was dramatically affected by the presence of GA during hTERT translation (Figure 5.3B, lanes 3-4), though there was a small decrease in the amount of pseudoknot RNA that co-purified with hTERT (Figure 5.3B, lanes 1-2). The interaction between hTERT and full-length hTR was not affected by the presence of GA during translation (Figure 5.3B, lanes 5-6). NB, on the other hand, was found to prevent over 60% of the full-length hTR/hTERT interaction when present during translation (Figure 5.3B, lanes 7-8).

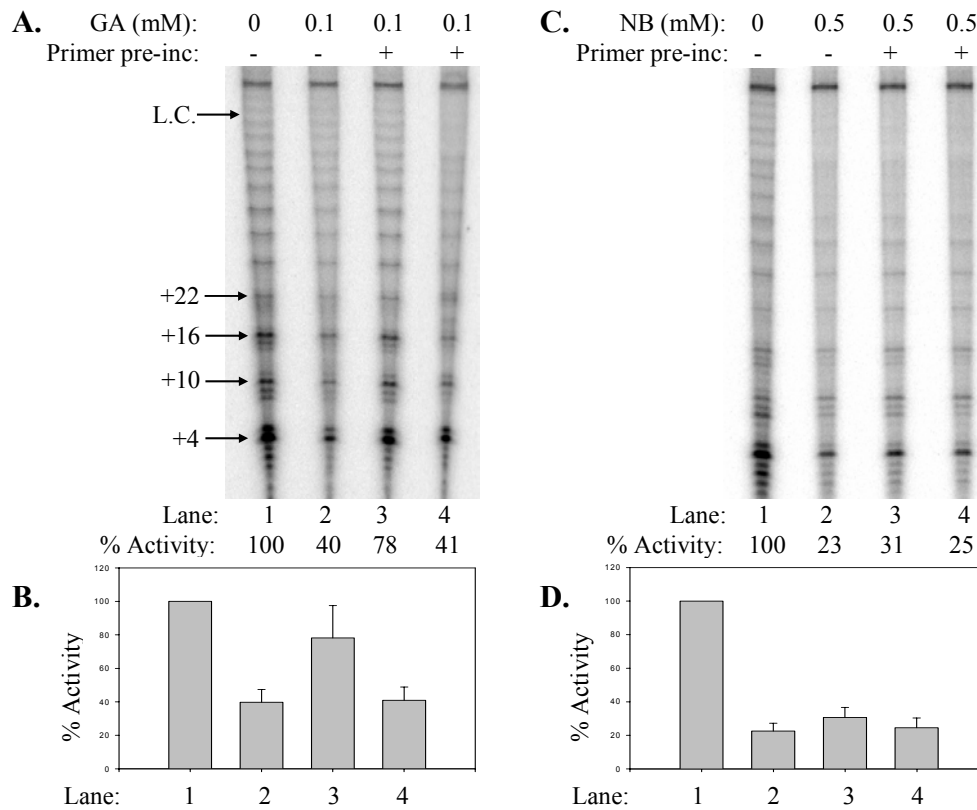
### 3. Primer binding overcomes GA, but not NB, inhibition of human telomerase

If GA does not affect telomerase by preventing the association of hTR with hTERT, then how does it inhibit its enzymatic activity? One possibility is that Hsp90 is involved in loading telomerase onto the chromosome end. That is, Hsp90 might be involved in telomerase-primer docking. Hu and Seeger found that Hsp90 maintains the DHBV RT in a “protein-priming” conformation capable of binding the RNA template used for reverse transcription (Hu and Seeger, 1996). Similarly, Hsp90 promotes binding of steroid receptor complexes to their ligands (Tanenbaum et al., 1998; Williams and Sigler, 1998; Bledsoe et al., 2002; Koide et al., 2002). Thus, it seems that Hsp90 can participate in preparing its clients for substrate and ligand binding. Based on this precedence, we determined if Hsp90 plays a direct role in telomerase-primer loading.

We performed direct extension assays in the presence of either GA or NB and telomerase or telomerase pre-incubated with a telomeric primer. We found that pre-incubation of a telomeric primer with telomerase overcame GA-induced inhibition (Figures 5.4A and B). This effect was dependent on a telomeric primer, since a non-telomeric primer



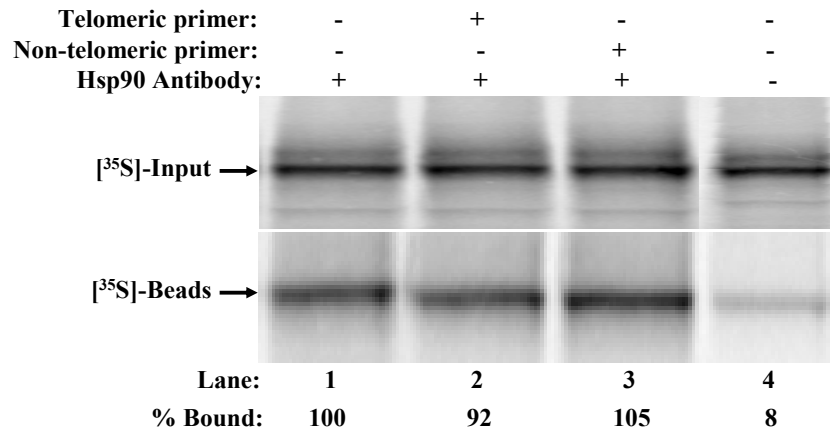
did not elicit the rescue effect (Figures 5.4A and B, lane 4). Because NB, like GA, is an Hsp90 inhibitor, we would expect to see a similar rescue effect. Surprisingly, pre-incubation of a telomeric primer with telomerase did not overcome NB inhibition (Figures 5.4C and D). This suggests that the different ATP binding sites confer separate telomerase-specific functions to Hsp90.



**Figure 5.4 Pre-Incubation with primer rescues GA-induced but not NB-induced telomerase inhibition.** Pre-assembled telomerase was assayed for activity in the absence (lane 1) or presence (lanes 2-4) of GA (A) or NB (C). Pre-assembled telomerase was pre-incubated with primer (primer pre-inc), followed by the addition of GA or NB (A and C, lanes 3 and 4). Lane 3 included a telomeric primer while lane 4 included a non-telomeric primer. Quantified telomerase activity is displayed in (B) and (D). “% Activity” is the amount of residual telomerase activity when compared to the positive control (lane 1). The [ $^{32}$ P]-loading control (L.C.) and the human telomere primer-extension products (+4, +10, +16, +22, etc. nucleotides added) are indicated. Lane numbers for (B) and (D) correlate with (A) and (C), respectively. Error bars indicate standard deviation.

#### 4. Hsp90 is associated with hTERT after telomeric primer binding

If Hsp90 is involved in primer loading, then it might be expected to release the telomerase complex once docking has occurred, similar to the behavior of Hsp90 in facilitating ligand binding to steroid receptors (Bledsoe et al., 2002). To test this possibility, we examined the association of [<sup>35</sup>S]-hTERT with Hsp90 in the presence or absence of a telomeric primer. The holoenzyme complexes were pre-incubated with a telomeric primer, a non-telomeric primer, or in the absence of added DNA. The resulting complexes were immunoprecipitated with an anti-Hsp90 antibody. We found that the presence of the telomeric primer had no significant effect on the interaction between Hsp90 and hTERT when compared to the positive control without primer (Figure 5.5). Similarly, a non-telomeric primer, which served as a control for specificity, exhibited little effect (Figure 5.5,

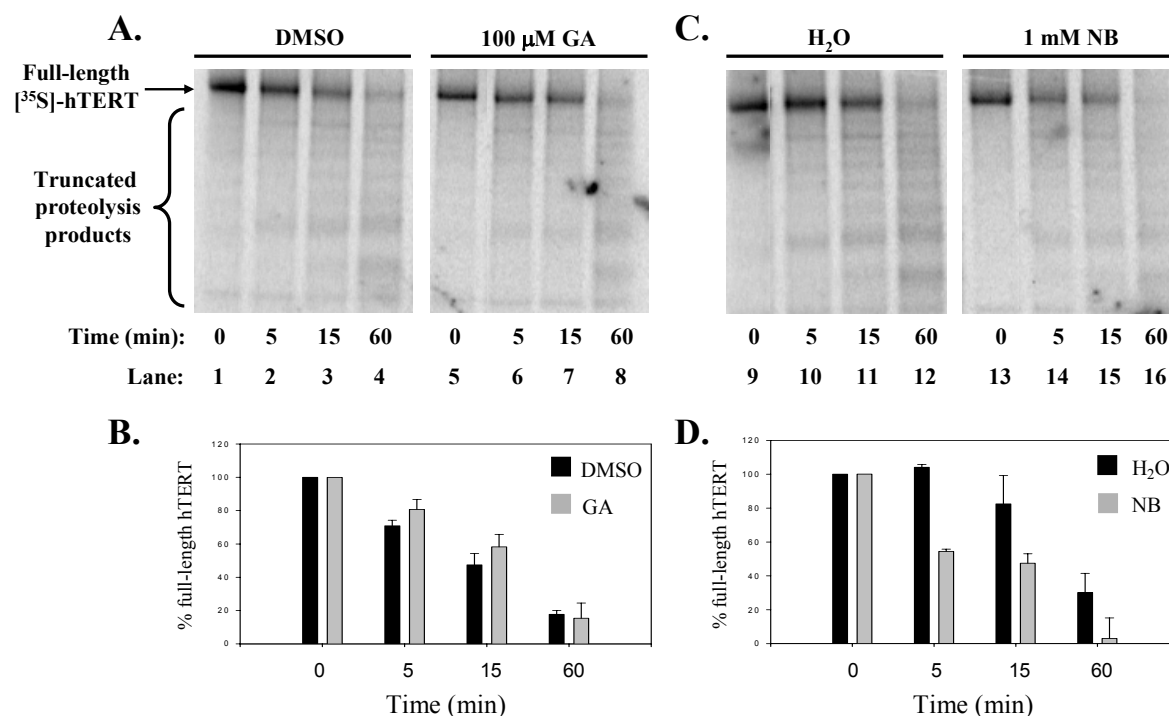


**Figure 5.5 Hsp90 remains associated with hTERT after primer loading.** [<sup>35</sup>S]-labeled telomerase was incubated alone (lane 1), in the presence of telomeric primer (lane 2) or in the presence of non-telomeric primer (lane 3) and immunopurified with an Hsp90 antibody. “% Bound” values indicate the amount of [<sup>35</sup>S]-labeled telomerase bound to Hsp90 when compared to the positive control (lane 1). Lane 4 is a negative control performed with no primer or antibody. All immunoprecipitations were performed in duplicate and representative results are shown.

lane 3). This result is consistent with a previous report that demonstrated the association of Hsp90 with active human telomerase (Holt et al., 1999). A negative control with no primer or antibody yielded  $\leq 8\%$  nonspecific binding of [ $^{35}\text{S}$ ]-hTERT to the beads (Figure 5.5, lane 4).

#### 5. Hsp90 inhibition alters telomerase holoenzyme structure

One possible explanation for the effects of GA and NB on telomerase is that Hsp90 is involved in stabilizing hTERT and therefore the telomerase complex. For example, hTERT stability or its conformation could depend on Hsp90 activity. We used partial proteolysis to examine the global structure of hTERT. Partial proteolysis is a powerful technique used to identify substructures and functional domains in various proteins (Konigsberg, 1995) and has been used to examine the global structure of *Tetrahymena thermophila* TERT (Bryan et al., 2000). To determine if GA and NB affect hTERT stability, we treated Hsp90-inhibited assembly reactions with Lys-C, an endoproteinase that cleaves at K-X bonds. Following proteolysis, the rate of full-length hTERT degradation in the absence and presence of Hsp90 inhibitors was analyzed over time. In the absence of Hsp90 inhibitors, hTERT degraded with a half-life of  $\sim 15$  min (Figure 5.6). Further, several prominent hTERT digestion products were observed. In the presence of GA, the hTERT half-life was similar, and the same degradation banding pattern was observed. In contrast, we found that NB significantly affected the rate of full-length hTERT proteolysis by decreasing the half-life to  $\sim 5$  min. The most obvious difference in hTERT degradation between NB and its control is seen at the earliest time point in which there is  $\sim 100\%$  hTERT remaining in the control reaction but only  $\sim 50\%$  left when NB is present (Figure 5.6, compare lane 10 to lane 14). Furthermore, when comparing the amounts of truncated proteolysis products at the 60 min time point,

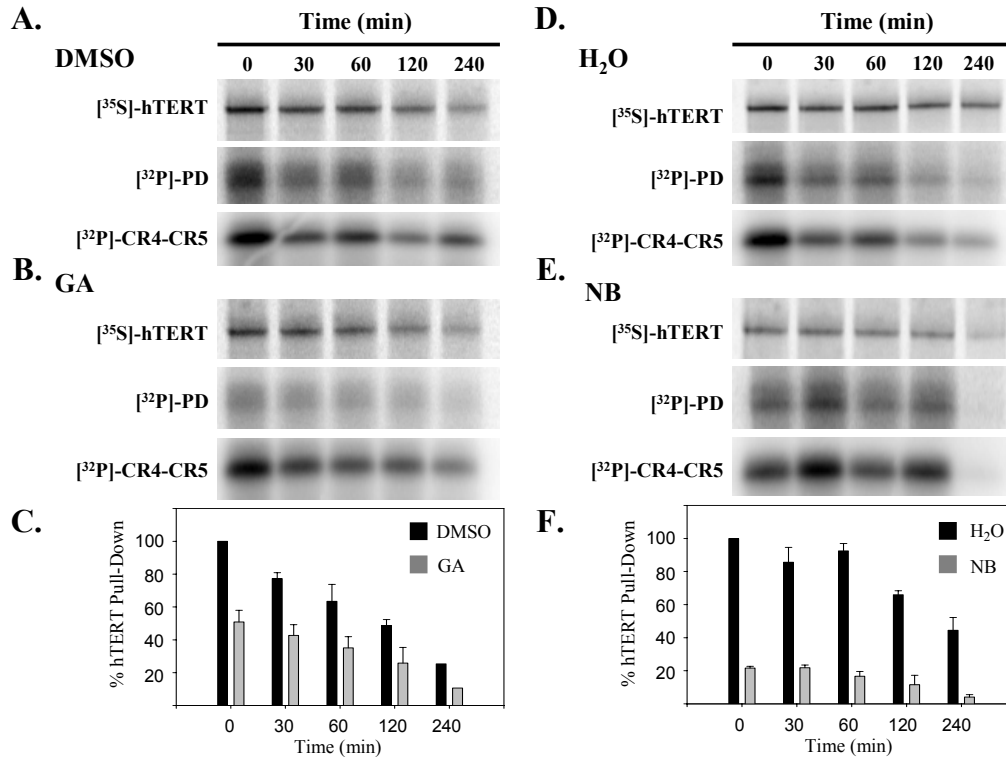


**Figure 5.6 NB, but not GA, increases the proteolysis rate of hTERT.**  $[^{35}\text{S}]$ -hTERT and hTR were assembled in the presence or absence of Hsp90 inhibitor. Following co-immunopurification, the complexes were digested with Lys-C and aliquots were quenched at the indicated time points.  $[^{35}\text{S}]$ -hTERT fragments digested after GA (A) or NB (C) treatment were resolved by SDS PAGE. The amount of full-length  $[^{35}\text{S}]$ -hTERT proteolysis was quantified and is displayed in (B) and (D). Error bars indicate standard deviation.

the NB treated sample has a significantly decreased level of truncated products when compared to both the  $\text{H}_2\text{O}$  control and the GA-treated sample (Figure 5.6, compare lane 16 to lanes 8 and 12). This presumably resulted from increased Lys-C digestion of the already truncated proteolysis products due to decreased hTERT stability. Further experiments will need to be performed in order to determine precisely what domains of hTERT have become vulnerable to proteolysis. The increase in Lys-C degradation in the presence of NB, but not GA, may explain why primer binding does not overcome NB inhibition.

## 6. NB destabilizes hTERT

A possible consequence of decreased hTERT stability is a commensurate decrease in telomerase complex stability. Using a pulse-chase experiment, we determined if telomerase stability was affected by GA or NB by examining the dissociation rate of the CR4-CR5 and pseudoknot RNA domains from hTERT. We did not detect any change in the dissociation rate of the RNA-protein complexes in our assay (Figure 5.7). However, we consistently

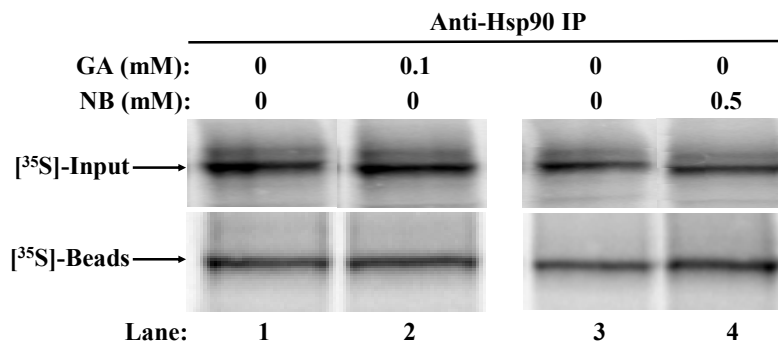


**Figure 5.7 hTERT stability, but not RNA/protein complex half-life, is affected by NB.** T7-tagged [<sup>35</sup>S]-hTERT was incubated with [<sup>32</sup>P]-CR4-CR5 RNA, [<sup>32</sup>P]-PD RNA and either 100  $\mu$ M GA (B) or 1 mM NB (E). Positive controls contained DMSO (A) or H<sub>2</sub>O (D). Following a 90 min incubation at 30  $^{\circ}$ C, 70-fold excess CR4-CR5 and PD RNA were added to the reaction and a 50  $\mu$ L aliquot was taken immediately and placed on ice (Time = 0). The reaction was incubated at 30  $^{\circ}$ C for 4 h and additional 50  $\mu$ L aliquots were taken at 30, 60, 120 and 240 min and placed on ice. Following immunoprecipitation, gel analysis and phosphorimaging, the RNA band intensities were quantified, normalized to the [<sup>35</sup>S]-hTERT protein bands and compared to the positive control. The amount of hTERT pulled-down in the presence of GA (C) or NB (F) is displayed. “% hTERT Pull-Down” indicates the amount of [<sup>35</sup>S]-hTERT immunoprecipitated as compared to Time = 0 for the positive controls. PD indicates the pseudoknot domain and CR4-CR5 indicates the CR4-CR5 domain of hTR. All immunoprecipitations were performed at least twice. Error bars indicate standard deviation.

observed a time-dependent decrease in the ability to immunoprecipitate hTERT in the presence of NB as compared to the positive control, suggesting that the hTERT structure is not stable in the presence of NB owing to the inhibition of Hsp90. GA also had a slight effect on the immunoprecipitation though the effect was much less severe than the NB effect. These results are consistent with the conclusion that NB destabilizes hTERT.

#### 7. Hsp90 is associated with hTERT following geldanamycin or novobiocin treatment

Yun et al. showed that NB binding to Hsp90 induces a conformational change that induces dissociation from client proteins (Yun et al., 2004). They specifically showed that this “client-release” conformation promotes the dissociation of Hsp90 from the heme-regulated eIF2 $\alpha$  kinase. A similar phenomenon would explain our finding that NB caused increased Lys-C proteolysis of hTERT and explain why pre-loading primer does not overcome NB inhibition. Previously, Holt et al. showed that GA has no effect on Hsp90/hTERT interaction (Holt et al., 1999). Using an anti-Hsp90 antibody and co-



**Figure 5.8 Hsp90/hTERT interactions are unaffected by the presence of GA and NB after hTERT translation.** Pre-assembled telomerase was combined with GA or NB and immunoprecipitated with an anti-Hsp90 antibody. Negative controls using DMSO (lane 1) or H<sub>2</sub>O (lane 3) were also performed. [<sup>35</sup>S]-hTERT protein band intensities were quantified and [<sup>35</sup>S]-Input values were used to normalize [<sup>35</sup>S]-Bead values. All immunoprecipitations were performed in duplicate and representative results are shown.

immunoprecipitating [<sup>35</sup>S]-hTERT we confirm that GA has no negative affect on Hsp90/hTERT interactions and report that NB also caused no decrease in the co-immunoprecipitation efficiency of hTERT with Hsp90, even at a concentration that caused 80% inhibition in telomerase activity (Figure 5.8). Apparently NB-induced dissociation of Hsp90 from its clients is dependent on the particular client, and hTERT remains bound to Hsp90 even in the presence of NB.

### C. Discussion

Human telomerase must be properly assembled to produce an active complex. The biogenesis of telomerase has received much attention and appears to be a complex process that requires specific pathways for maturation of its RNA subunit (Mitchell et al., 1999; Fu and Collins, 2003) as well as assemblage of the holoenzyme complex (Holt et al., 1999; Forsythe et al., 2001; Collins and Mitchell, 2002). It was previously revealed that the Hsp90 chaperone complex interacts with hTERT and that Hsp90 activity is required for establishing active telomerase in both in vitro reconstitution assays and in cultured human cells. In this work, we set out to further expand our understanding of the biochemical role of Hsp90 in maintaining telomerase activity and established that the role of Hsp90 in activating telomerase is more complex than previously thought.

We confirmed that Hsp90 inhibitors GA and NB prevent reconstitution of active human telomerase (Figure 5.1). Surprisingly, we found that this inhibition was not the result of preventing the interaction between hTERT and hTR or specific domains of hTR (Figure 5.3A). On the contrary, we found that hTR and hTERT are capable of interacting in the

presence of either GA or NB, even though the RNP complex that is assembled in the presence of these Hsp90 inhibitors is inactive. Interestingly, when added to an assembly reaction prior to hTERT translation, NB caused a decrease in the amount of hTERT that co-immunoprecipitated with full-length hTR, whereas GA only affected the association of the CR4-CR5 domain with hTERT (Figure 5.3B). This suggests that the nascent hTERT transcript requires Hsp90 activity to achieve a structure that is fully competent in hTR binding and that the NB-targeted C-terminus of Hsp90 may be more important in maintaining RNP stability than the GA-targeted N-terminus. In summary, our results indicate that Hsp90 does not chaperone the marriage of hTERT to hTR, per se, but may instead be involved in fine-tuning and stabilizing the assembled structure and ensuring that the nascent hTERT transcript folds properly.

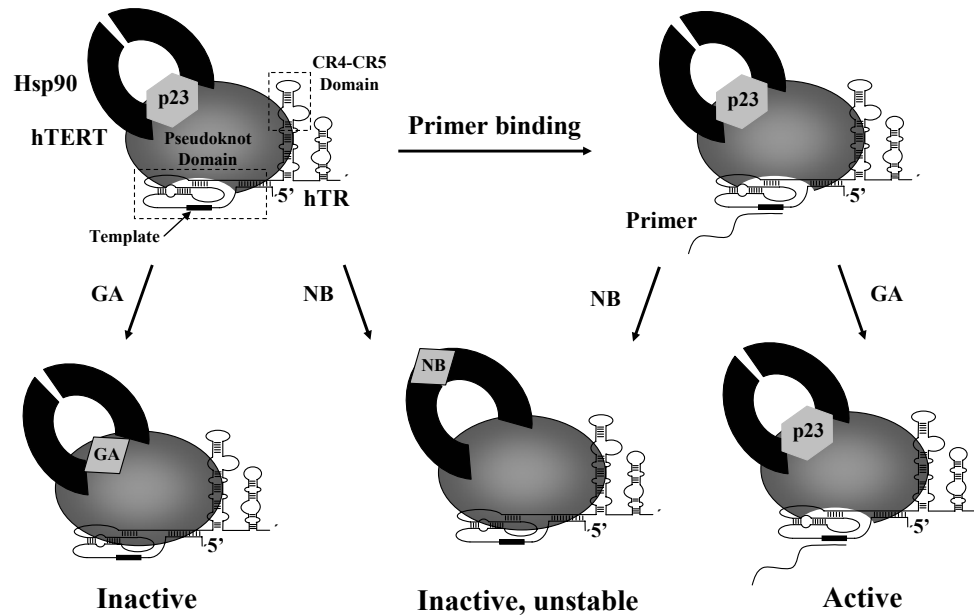
When GA or NB was added to pre-assembled telomerase, we observed complete inhibition of telomerase activity, albeit with a higher  $IC_{50}$  than when the inhibitors were added before assemblage (Figure 5.1). There are several possible explanations for this result. During early work on the mechanism of GA action, it was noted that GA is an inhibitor of several nucleotide transferases including DNA Pol  $\alpha$  (Yamaki et al., 1982), though it is not clear from these studies if the effect is related to Hsp90 inhibition or specific polymerase inhibition. More recently, it was found that while GA inhibits hepatitis B RT, it does so by binding Hsp90 and is not an RT inhibitor as GA inhibition could be overcome using a synthetic mutant that was Hsp90 independent (Hu and Seeger, 1996; Wang et al., 2003). While we cannot entirely rule out direct inhibition of telomerase by GA and NB, the history of GA and NB suggests that Hsp90 is the more likely candidate in our studies. Several observations are consistent with this conclusion. First, GA and NB have different effects on



telomerase and are known to bind two distinct sites on Hsp90 that have distinct functions in the Hsp90 reaction cycle. If telomerase were the target, we might expect GA and NB to generate more similar effects on telomerase. Furthermore, the addition of p23, which has an overlapping binding site with GA on Hsp90, partially rescued telomerase activity from GA inhibition in an assemblage assay (Figure 5.2). We also showed that NB affects the stability of hTERT, consistent with an Hsp90-dependent activity. Together, the known pharmacological effects of GA and NB on Hsp90 and the observations we describe here are more consistent with an effect on Hsp90 than a direct inhibitory effect on telomerase.

Since Hsp90 does not seem to be explicitly required to promote the association of hTERT and hTR, we speculated that it might be involved in primer loading. This hypothesis was based on work with DHBV RT, for which Hsp90 removes an autoinhibitory domain away from the RT active site to allow primer loading and RNA binding (Hu and Seeger, 1996; Wang et al., 2003). We found that pre-incubation of a telomerase primer with pre-assembled telomerase, prior to the addition of GA, rescued GA-induced telomerase inhibition (Figures 5.4A and B). However, NB-induced telomerase inhibition was not overcome by pre-incubating primer (Figures 5.4C and D). Though it has been suggested that the two termini of Hsp90 engage in cooperativity and cross-talk (Marcu et al., 2000), our results indicate that GA and NB dissociate different facets of Hsp90 involvement with telomerase and implicate different functions of the N- and C-terminal Hsp90 domains in the telomerase reaction cycle. Hsp90 appears to be directly involved in primer binding and stabilizing the active telomerase complex as shown in the model presented in Figure 5.9. In this model, the N-terminal ATP binding site functions to maintain telomerase in a primer-binding competent state. The role of the putative C-terminal ATP binding site is to confer Hsp90-dependent

stability to hTERT. This proposal is consistent with the apparent instability of hTERT in the presence of NB and the rescue of GA inhibition by primer. Furthermore, though GA and NB target different ATP-binding sites on Hsp90, they both displace p23 from Hsp90 (Grenert et al., 1997; Prodromou et al., 1997; Marcu et al., 2000). This suggests that p23 displacement



**Figure 5.9 Conceptual model for Hsp90-mediated human telomerase maturation and the effects of GA and NB.** hTR and hTERT assemble after translation in the presence of Hsp90/p23 into an active complex that is competent in primer binding. The addition of geldanamycin (GA) or novobiocin (NB) to the assembled complex leads to the formation of an inactive complex, in part by preventing primer binding and in part by destabilizing the hTERT polypeptide. The addition of primer overcomes inhibition by GA, but not NB. The CR4-CR5 and pseudoknot domains are indicated in their respective dotted boxes. The N-terminus of Hsp90 is shown as the p23- and GA-binding sites. The Hsp90 C-terminus is shown as the NB-binding site. Note that the sizes of individual proteins are not drawn to scale.

is not related to primer binding. It therefore remains to be seen whether or not the addition of GA to an active primer-bound telomerase complex will result in the release of p23 (Figure 5.9).

Unlike the transient nature of some chaperones and client proteins, it is clear from our studies and those of the Holt lab that Hsp90 is stably associated with the telomerase holoenzyme both after hTERT/hTR assembly (Forsythe et al., 2001) and after primer binding (Figure 5.5). To explain this, we propose that Hsp90 remains bound to hTERT in order to maintain the holoenzyme in a conformation capable of primer binding and catalysis (Figure 5.9). By comparison to the DHBV holoenzyme (Wang et al., 2003), perhaps the role of Hsp90 in telomerase holoenzyme maturation may include the manipulation and offsetting of an autoinhibitory domain to allow primer binding. It is not clear if hTERT contains an autoinhibitory domain, but it is clear that the presence of functional Hsp90 does allow for efficient primer binding and thus increased telomerase activity. Further studies using truncated hTERT mutants will address the existence of an Hsp90-antagonized autoinhibitory domain on hTERT.

The mechanism of Hsp90-targeted inhibition of telomerase in cells appears more complex. Antisense oligonucleotides targeting cellular Hsp90 have been shown to directly reduce Hsp90 mRNA expression and decrease telomerase activity (Chang et al., 2002). However, Hsp90 plays a significant role in regulating many cellular signaling pathways including that for the serine/threonine kinase Akt. Akt has been found to increase human telomerase activity via phosphorylation of hTERT (Kang et al., 1999). The association with Hsp90 is required to maintain Akt in an active state (Sato et al., 2000) and GA- and NB-mediated Hsp90 inhibition has been found to inhibit the Akt cascade (Haendeler et al., 2003; Machida et al., 2003), thus leading to decreased telomerase activity. Furthermore, GA-induced Hsp90 inhibition in H1299 cells leads to the ubiquitination and proteasome-mediated degradation of hTERT (Kim et al., 2005). These indirect affects on telomerase

activity must therefore be taken into account when elucidating the cellular mechanisms of GA- and NB-induced telomerase inhibition. Additionally, these results suggest that Hsp90 inhibition will not lead to the formation of a dominant negative complex within cells. Therefore, though Hsp90 inhibitors do influence telomerase holoenzyme stability and primer-binding competence, they do not fit into our defined class of telomerase assemblage inhibitors.

In conclusion, we have found that the role of Hsp90 in the telomerase reaction cycle is more complex than expected. Hsp90 is not involved in allowing hTR to bind hTERT, though it may be involved in fine-tuning and stabilizing the structure of the complex. Active Hsp90 seems to be required both during translation of the nascent hTERT transcript and for the assembly of active telomerase. Importantly, Hsp90 appears to be involved in maintaining telomerase in a conformation that is competent to bind the telomere. While it remains to be seen if this is true *in vivo*, it is an attractive model for allowing the Hsp90-dependent regulation of telomerase, and is consistent with the role of Hsp90 in facilitating ligand binding to other Hsp90 clients.

#### D. Materials and methods

See Chapter II, section D for information on pET-28c-hTERT and phTR+HH expression plasmids and methods for synthesis of hTERT, reconstitution of human telomerase, direct telomerase assays and assemblage assays. p23 protein used in the direct telomerase assay was generously donated by Dr. David O. Toft.

### 1. Antibodies and chemical reagents

The anti-Hsp90 mouse monoclonal antibody (H90-10) was a generous gift from Dr. David O. Toft (Mayo Clinic, Rochester, MN) (Barent et al., 1998). GA was purchased from Alexis Biochemicals and NB was purchased from MP Biomedicals, Inc. Before use, GA and NB were dissolved in sterile DMSO and H<sub>2</sub>O, respectively.

### 2. In vitro transcription and purification of hTR

Wild-type hTR was in vitro transcribed as described in Chapter II with minor modifications. Briefly, 15 µg of phTR+HH was linearized with the restriction enzyme FokI and added to a 100 µL transcription reaction containing 1× Ampliscribe T7 Reaction Buffer (Epicentre Technologies), 7.5 mM each NTP, 10 mM DTT and 10 µL Ampliscribe T7 Enzyme Solution (containing an RNase inhibitor) (Epicentre Technologies). The reaction was incubated at 37 °C for 4 h. Hammerhead ribozyme cleavage was initiated by the addition of MgCl<sub>2</sub> to a final concentration of 12 mM followed by incubation at 45 °C for 1 h. The reaction was then treated with 5 µL RNase-Free DNase 1 (Epicentre, 1 MBU/µL), incubated at 37 °C for 20 min, extracted with phenol/chloroform/isoamyl alcohol and ethanol precipitated in the presence of 0.3 M NaOAc. The RNA was then resuspended in denaturing loading buffer (7 M urea/10% glycerol/1× TBE) and purified by denaturing PAGE. The resulting purified RNA was resuspended in a suitable volume of 1× TE (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) and quantified by UV absorbance.

### 3. Synthesis of [<sup>32</sup>P]-labeled pseudoknot and CR4-CR5 RNA fragments and full-length hTR

RNA fragments were generated using a modification of the protocol described in Chapter II (Chen and Greider, 2003; Keppler and Jarstfer, 2004). Briefly, the DNA template

required to transcribe the pseudoknot RNA fragment (nucleotides 46-209) and the CR4-CR5 RNA fragment (nucleotides 243-328) were generated by PCR. The resulting PCR products were purified using the Wizard PCR Preps DNA Purification system (Promega). A 100  $\mu$ L Ampliscribe T7 Transcription reaction contained 1 $\times$  Ampliscribe T7 Reaction Buffer, 7.5 mM CTP, 7.5 mM GTP, 7.5 mM UTP, 5.8 mM ATP,  $\sim$ 0.7  $\mu$ M [ $\alpha$ - $^{32}$ P]-ATP (3000 Ci/mmol, 10  $\mu$ Ci/ $\mu$ L; Perkin-Elmer), 10 mM DTT, 10  $\mu$ L Ampliscribe T7 Enzyme Solution and the required linear template. Reactions were incubated at 37  $^{\circ}$ C for 4 h. For full length hTR, hammerhead ribozyme cleavage was initiated as described. Reactions were treated with 5  $\mu$ L RNase-Free DNase 1 (Epicentre, 1 MBU/ $\mu$ L), incubated at 37  $^{\circ}$ C for 20 min, extracted with phenol/chloroform/isoamyl alcohol and ethanol precipitated in the presence of 0.3 M NaOAc. The RNA was resuspended in 1 $\times$  TE and passed through a Microspin G-25 column (Amersham) to remove any remaining unincorporated [ $\alpha$ - $^{32}$ P]-ATP.

#### 4. Synthesis of biotinylated pseudoknot or CR4-CR5 RNA fragment

A 100  $\mu$ L Ampliscribe T7 Transcription reaction contained 1 $\times$  Ampliscribe T7 Reaction Buffer, 7.5 mM GTP, 7.5 mM UTP, 7.5 mM ATP, 6.25 mM CTP, 1.25 mM bio-11-CTP (ENZO Life Sciences), 10 mM DTT, 10  $\mu$ L Ampliscribe T7 Enzyme Solution and the appropriate DNA template. Reactions were incubated for 4 h at 37  $^{\circ}$ C, treated with DNase, extracted with phenol/chloroform/isoamyl alcohol, ethanol precipitated and purified by PAGE as described.

#### 5. Inhibition studies

Telomerase activity assays, using both pre-assembled telomerase and telomerase assembled in the presence of an Hsp90 inhibitor were performed using varying

concentrations of inhibitor. Seven data points were used with GA or NB concentrations ranging from 0 to 5 mM. IC<sub>50</sub> values were calculated and inhibition data were plotted as described in Chapter II.

#### 6. Association of hTR and hTERT in the presence of GA and NB

The ability of hTERT to associate with hTR in the presence of Hsp90 inhibitors was determined using a co-immunoprecipitation assay as described in Chapter II with minor modifications. Briefly, a 75  $\mu$ L reaction contained 50  $\mu$ L (~250 fmol) of T7-tagged [<sup>35</sup>S]-hTERT from a reticulocyte lysate reaction and ~80-800 pmol [<sup>32</sup>P]-labeled pseudoknot or CR4-CR5 RNA fragment or full-length hTR (2.7-3.1 x 10<sup>6</sup> cpm per reaction). Inhibition studies also included GA (300  $\mu$ M) or NB (1 mM). Reactions were incubated at 30 °C for 90 min. Each reaction was immunoprecipitated using 25  $\mu$ L of pre-washed and pre-blocked anti-T7 antibody agarose beads (Novagen). Following incubation on a rotary platform for 2 h at 4 °C, the beads were washed three times, resuspended in SDS gel loading buffer containing 10 mM DTT and resolved on a 4-12% Bis-Tris SDS gel (Invitrogen). After phosphorimaging, the RNA band intensities were quantified, normalized to the [<sup>35</sup>S]-hTERT protein bands and compared to the positive control.

#### 7. Association of hTR and hTERT when GA or NB was present during translation of hTERT

The association of hTERT with hTR when Hsp90 was inhibited during translation of hTERT was assayed by affinity purification of full-length hTR using a modification of a previously described protocol for purification of human telomerase (Schnapp et al., 1998). [<sup>35</sup>S]-hTERT was synthesized in 50  $\mu$ L reactions as described in Chapter II in the presence of 5 pmol hTR and 100  $\mu$ M GA or 1 mM NB. Following a 90 min incubation at 30 °C the

reactions were combined with 50  $\mu$ L of UltraLink<sup>®</sup> Immobilized NeutrAvidin<sup>™</sup> Plus beads (Pierce) and 50  $\mu$ L buffer A (20 mM Hepes-KOH, pH 7.9, 1 mM EDTA, 300 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol) supplemented with 0.5% Triton X-100, 5  $\mu$ g yeast RNA (50  $\mu$ g/mL final concentration) and 100 pmol hTRbait, an affinity oligonucleotide complimentary to the template of hTR (5'-/biotin/-CTAGACCTGTCATCAmGmUmUmAmGmGmUmUmAmG-3' where m = 2' O-methyl ribose). Before use, the beads were washed once with 300  $\mu$ L buffer A, blocked twice with 250  $\mu$ L blocking buffer (buffer A supplemented with 0.5 mg/mL BSA) for 15 min at 4°C and then washed with 300  $\mu$ L buffer A. The beads were precipitated by centrifugation at 2500g for 1.5 min. The reactions were then incubated for 10 min at room temperature followed by 2 h at 4°C on a rotary platform. The resulting bead complexes were washed three times with 300  $\mu$ L buffer A supplemented with 0.5% Triton X-100, once with 300  $\mu$ L buffer A supplemented with 300 mM KCl and twice with buffer A alone. The precipitated bead samples were then resuspended in 1 $\times$  SDS gel loading buffer with 10 mM DTT, heated for 5 min at 95 °C and loaded onto a tris-glycine SDS gel (6% resolving, 5% stacking). Dried gels were exposed to a phosphorimager screen overnight and the [<sup>35</sup>S]-hTERT bands were quantified, normalized to inputs and compared to the positive control. Controls containing DMSO and H<sub>2</sub>O were conducted for comparison to GA and NB, respectively.

Association of the CR4-CR5 and pseudoknot domains of hTR with hTERT was determined using biotin-labeled RNA. [<sup>35</sup>S]-hTERT was synthesized in 50  $\mu$ L reactions in the presence of ~60 pmol biotinylated pseudoknot RNA fragment or biotinylated CR4-CR5 RNA fragment and 100  $\mu$ M GA or DMSO carrier. Each reaction was then affinity purified with 45  $\mu$ L of pre-blocked MPG<sup>®</sup> Streptavidin beads (Pure Biotech, LLC). Beads were



washed four times with 400  $\mu$ L wash buffer 1 and blocked twice with 250  $\mu$ L blocking buffer for 15 min at 4 °C before use as described in Chapter II. The beads were precipitated by centrifugation at 2500g for 2 min between each step. Each 50  $\mu$ L reaction was then mixed with 50  $\mu$ L blocking buffer and centrifuged at 17000g for 10 min at 4 °C in order to remove any precipitates. The supernatant was added to the blocked beads and the samples were incubated for 45 min at room temperature on a rotary platform. After washing the beads three times with 350  $\mu$ L wash buffer 2 and once with 350  $\mu$ L TMG, the samples were analyzed by SDS PAGE as described in Chapter II.

#### 8. Immunoprecipitation of telomerase using an Hsp90 antibody

Pre-assembled telomerase complexes were immunoprecipitated using a previously described protocol (Holt et al., 1999). Briefly, 75  $\mu$ L telomerase pre-assembled with [ $^{35}$ S]-hTERT was incubated at 30 °C in the presence of 1  $\mu$ g human telomeric primer, no telomeric primer or 1  $\mu$ g of non-telomeric primer. After 90 min, anti-Hsp90 mouse monoclonal antibody (H90-10), generously donated by Dr. David O. Toft, was added to a final concentration of  $\sim$ 0.5  $\mu$ g/mL and the samples were placed on ice for 1 h. A negative control with no primer and no antibody added was performed. Each reaction was immunoprecipitated by the addition of 22.5  $\mu$ L Protein-G Agarose Beads (Roche). Before use, the beads were washed three times with 400  $\mu$ L PBS. The reactions were incubated at 4 °C for 1 h with constant rotation. The beads were then washed three times with 400  $\mu$ L Wash Buffer (20 mM Hepes, pH 7.6, 20% glycerol, 100 mM NaCl, 0.2 mM EGTA, 1 mM MgCl<sub>2</sub>, 0.1% NP-40, 0.1% BSA). The precipitated bead samples were analyzed by SDS PAGE as described.

A similar immunoprecipitation was performed utilizing the H90-10 antibody in which pre-assembled,  $^{35}\text{S}$ -labeled telomerase was incubated in the presence of 100  $\mu\text{M}$  GA or 500  $\mu\text{M}$  NB. Positive controls contained DMSO or  $\text{H}_2\text{O}$ . The remainder of this pull-down was performed as above.

#### 9. Lys-C proteolysis of hTERT

The stability of hTERT was determined by Lys-C proteolysis using a modification of a previously described protocol (Bryan et al., 2000).  $\sim 700$  fmol T7-tagged [ $^{35}\text{S}$ ]-hTERT and 3  $\mu\text{g}$  hTR were allowed to assemble either in the presence or absence of 100  $\mu\text{M}$  geldanamycin or 1 mM novobiocin at 30  $^{\circ}\text{C}$  for 90 min. Each reaction was then immunoprecipitated using 30  $\mu\text{L}$  of pre-washed and pre-blocked anti-T7 antibody agarose beads (Novagen), as described in Chapter II. Following immunoprecipitation, the beads were washed with and resuspended in 80  $\mu\text{L}$  Digestion Buffer (25 mM Tris-HCl, pH 8.5, 1 mM EDTA). Each sample was then treated with  $\sim 90$  ng endoproteinase Lys-C (Roche, sequencing grade) and incubated at 30  $^{\circ}\text{C}$ . Aliquots were taken out of each reaction at various time points and quenched with an equal volume of Laemmli's sample buffer (125 mM Tris-Cl, pH 6.8, 4% SDS, 0.005% bromophenol blue, 20% glycerol, 0.72 M  $\beta$ -mercaptoethanol). The samples were then heated at 95  $^{\circ}\text{C}$  for 5 min and resolved on a 4-12% Bis-Tris SDS gel (Invitrogen). Following phosphorimaging, the [ $^{35}\text{S}$ ]-hTERT and hTERT fragment bands were quantified.

#### 10. Pulse-chase

325  $\mu\text{L}$  ( $\sim 1.625$  pmol) T7-tagged [ $^{35}\text{S}$ ]-hTERT was incubated with 16.25 pmol [ $^{32}\text{P}$ ]-CR4-CR5 RNA ( $\sim 32 \times 10^6$  cpm), 16.25 pmol [ $^{32}\text{P}$ ]-PD RNA ( $\sim 24 \times 10^6$  cpm) and either 100

$\mu$ M GA or 1 mM NB. Positive controls contained DMSO or H<sub>2</sub>O. Following a 90 min incubation at 30 °C, 70-fold excess (~1137 pmol) CR4-CR5 and PD RNA were added to the reaction. A 50  $\mu$ L aliquot was taken immediately and placed on ice (Time = 0). The reaction was incubated at 30 °C for 4 h and additional 50  $\mu$ L aliquots were taken at 30, 60, 120 and 240 min and placed on ice. Each 50  $\mu$ L sample was then immunopurified via a T7-tag on hTERT and resolved on 4-12% Bis-Tris SDS gels (Invitrogen) as described in Chapter II (Keppler and Jarstfer, 2004). Following phosphorimaging, the RNA band intensities were quantified, normalized to the [<sup>35</sup>S]-hTERT protein bands and compared to the positive control. Pulse-chase experiments were performed in partnership with Allen T. Grady.

## **Chapter VI. Development of a high-throughput screen capable of identifying novel inhibitors of a specific hTERT/hTR interaction**

Adapted from: Keppler, B. R. and Jarstfer, M. B. (2005) A High-Throughput Screen Capable of Identifying Novel Inhibitors of Human Telomerase Assemblage, accepted to Analytical Biochemistry.

### **A. Introduction**

Although active telomerase is naturally present in only select, non-somatic cells, the vast majority of cancer cell types also express active telomerase in order to maintain genetic stability throughout their infinite lifespan. The loss of telomerase activity in a telomerase-dependent cancer cell adversely affects telomeric integrity and results in senescence or cell death, thus making telomerase an appealing drug target. In fact, the telomerase inhibitor GRN163L has entered phase I/II clinical trials for the treatment of chronic lymphocytic leukemia (Djojosebroto et al., 2005). Past approaches towards manipulating telomerase activity include, but are not limited to, targeting hTERT, hTR, telomerase enzymatic activity or the telomere (White et al., 2001; Saretzki, 2003). The use of dominant negative mutants (Hahn et al., 1999; Zhang, X. et al., 1999b) or ribozymes (Yokoyama et al., 2000; Qu et al., 2002) are the most common methods of hTERT-targeted telomerase inhibition, and hTERT-targeted immunotherapy is a more recent approach that is showing great promise (Shay and Wright, 2002). Targeting hTR is carried out mainly through antisense interactions using various modified oligonucleotides including phosphorothioate (Pitts and Corey, 1998; Elayadi et al., 2001), 2'-*O*-methyl (Pitts and Corey, 1998), 2'-*O*-(2-methoxyethyl) (2'-MOE)

(Elayadi et al., 2001), N3'→P5' *thio*-phosphoramidate (NPS) (Asai et al., 2003) and peptide nucleic acids (PNA) (Norton et al., 1996). The most potent antisense oligonucleotide inhibitors found to date target the telomerase RNA template (Elayadi et al., 2001; Corey, 2002; Asai et al., 2003). Targeting the telomere as an anti-telomerase approach has been achieved using compounds that bind and stabilize G-quadruplexes at the end of the telomere. This class of telomerase inhibitors, which includes various derivatives of anthraquinones (Sun et al., 1997; Read, M. A. et al., 1999; Read, M. et al., 2001), acridines (Harrison et al., 1999), porphyrins (Han et al., 2001; Shi et al., 2001), perylenes (Fedoroff et al., 1998; Han et al., 1999; Rangan et al., 2001) and ethidium (Koeppel et al., 2001), inhibits telomerase activity by preventing its access to the telomere. Recently, our laboratory demonstrated that preventing proper telomerase holoenzyme assemblage is a viable approach to inhibiting the telomere-extension capabilities of telomerase (Keppler and Jarstfer, 2004). We have advanced this approach by confirming that small molecules such as known nucleic acid-binding compounds can also adversely affect telomerase assembly and thus its activity (Dominick et al., 2004). Because the identification of an ideal, efficacious telomerase inhibitor has the potential to be a universal anticancer drug, there lies a need to develop rapid methods to identify novel inhibitors of telomerase.

Several high-throughput methods to identify inhibitors of telomerase have been developed. Originally, telomerase activity was detected using a direct assay that required large amounts of reagents including partially purified telomerase from cell extracts and radioactive substrates. Issues related to the minute amounts of telomerase available from cultured cells were partially overcome by a PCR-based telomerase assay called the Telomeric Repeat Amplification Protocol (TRAP) (Kim et al., 1994). By amplifying the telomerase

extension products, the TRAP assay allows detection of very small amounts of telomerase activity, though without the proper controls the TRAP assay is liable to yield problems such as false positives due to *Taq* DNA polymerase inhibition (Wright et al., 1995). Both the direct telomerase assay and the TRAP assay, however, require gel analysis in order to visualize and quantify activity. In order to bypass the requirement for gel electrophoresis, Francis and Friedman used a biotinylated primer that was bound to streptavidin-coated 96-well plates after extension by telomerase with radioactive nucleotides (Francis and Friedman, 2002). Subsequent washing steps in the plate precluded the need for gel analysis. Francis and Friedman also developed a modification of this assay that utilized PCR and PicoGreen to detect the amplification of double-stranded DNA (Francis and Friedman, 2003). Their use of streptavidin-coated 96-well PCR plates allowed for rapid screening and inhibitor removal to prevent false positives resulting from inhibition of *Taq* polymerase during PCR (Francis and Friedman, 2003). Kha et al. developed a similar high-throughput screen using streptavidin-coated plates, however, this assay quantified activity using ELISA-based chemiluminescence and therefore did not require the use of radioactivity or electrophoresis (Kha et al., 2004). Atha et al. developed a high-throughput method to detect telomerase activity using a fluorescent-labeled primer, PCR-amplified products and capillary electrophoresis (Atha et al., 2003). Savovsky et al. developed a screen utilizing both TRAP and scintillation proximity assay (SPA) technology (Bosworth and Towers, 1989; Savovsky et al., 1996). A biotinylated primer was used to amplify telomerase extension products with [<sup>3</sup>H]-thymidine as one of the dNTPs. Following extension and amplification, the products were captured on fluorophore-containing streptavidin-coated beads (Savovsky et al., 1996). Notably, each of these screens reports directly on the enzymatic activity of telomerase.

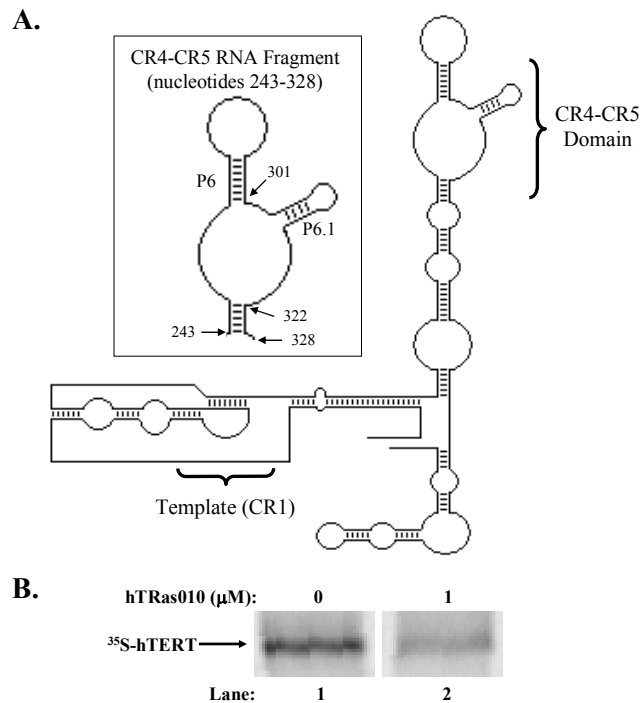
We previously showed that hTRas010, a 2'-*O*-methyl modified oligonucleotide that targets nucleotides 301-322 of hTR and envelops the entire P6.1 stem-loop (targets nt's 301-322 of hTR), inhibits telomerase activity when added prior to hTR/hTERT assembly (Keppler and Jarstfer, 2004). By using a co-immunoprecipitation assay, we determined that this inhibition was the result of blocking the association between hTERT and the CR4-CR5 portion of hTR (Keppler and Jarstfer, 2004). With this in mind, we developed a high-throughput screen proficient in identifying telomerase assemblage modulators that specifically affected the interaction of hTERT with the CR4-CR5 domain of hTR. Using SPA technology, we were able to develop a robust assay using a biotin-labeled derivative of the CR4-CR5 domain of hTR to capture [<sup>35</sup>S]-hTERT.

## B. Results

### 1. Biotin-labeled CR4-CR5 domain binds hTERT

Previously, we identified oligonucleotide hTRas010 as a nM telomerase inhibitor that functions by blocking the interaction between the CR4-CR5 domain of hTR and hTERT and therefore preventing assemblage of active telomerase (Keppler and Jarstfer, 2004). This highly conserved RNA domain contains a short stem-loop, termed P6.1, that was identified by Chen and Greider (Chen et al., 2002) and subsequently characterized by NMR (Leeper and Varani, 2005) (Figure 6.1A, inset). To facilitate the identification of small molecules that block this critical interaction we developed a high-throughput screen. Due to the established use and commercial support of the streptavidin-biotin interaction for monitoring biomolecular interactions, we chose to use biotin-labeled CR4-CR5 domain RNA to capture hTERT on streptavidin beads. Before developing the high-throughput screen, we first

confirmed that biotin labeled CR4-CR5 domain RNA could associate with hTERT. The ability to capture [<sup>35</sup>S]-labeled hTERT with biotin-labeled CR4-CR5 RNA was determined biochemically using MPG streptavidin-coated beads (~20% efficiency). We confirmed that biotin-labeled CR4-CR5 domain RNA could associate with hTERT and demonstrated that the addition of the known telomerase inhibitor hTRas010 inhibited the interaction when added prior to RNA/protein association (Figure 6.1B). Importantly, negative controls with unbiotinylated RNA were unable to efficiently capture hTERT on the streptavidin beads (< 5%).

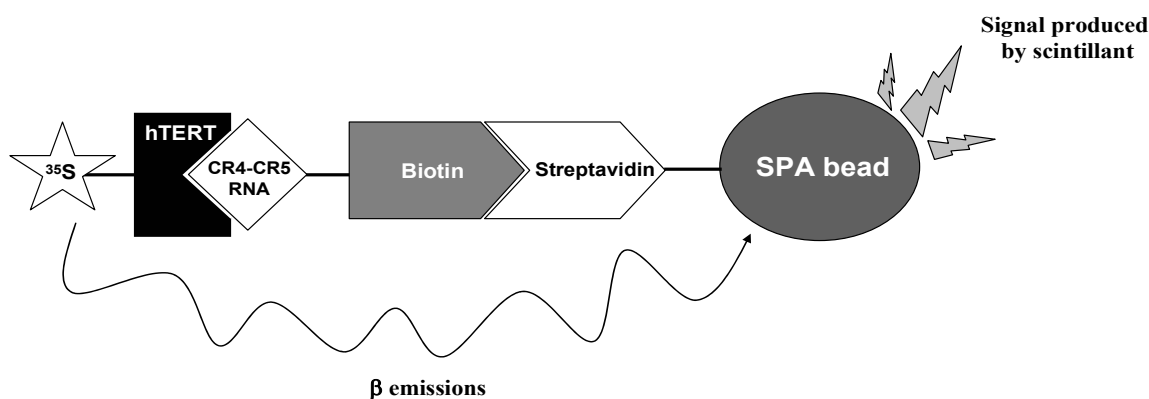


**Figure 6.1 Structure and hTERT-binding inhibition of the CR4-CR5 domain of hTR.** (A) Proposed secondary structure of hTR. The template and CR4-CR5 domain are indicated. Inset: the P6.1 stem-loop-containing CR4-CR5 RNA fragment (nucleotides 243-328) used in the SPA. Structure modified from Chen et al., 2002. (B) Effect of 1 μM hTRas010 on the affinity purification of [<sup>35</sup>S]-hTERT using biotinylated CR4-CR5 RNA when added prior to protein/RNA association.



## 2. A Scintillation Proximity Assay for hTERT-hTR interactions

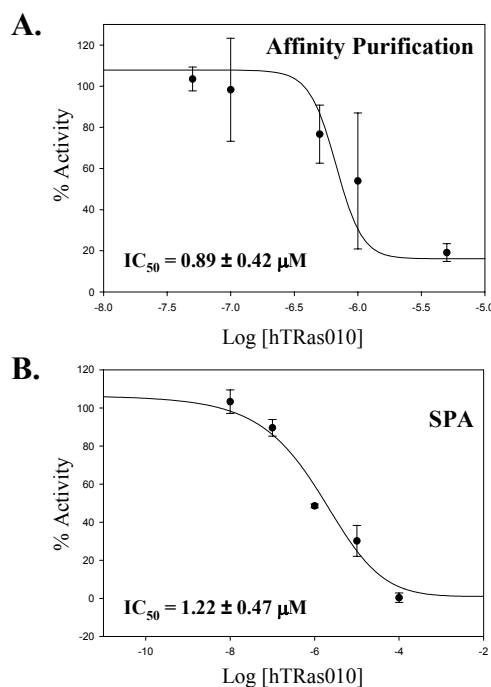
We examined several analytical techniques that are compatible with the demands of a high-throughput screen. Filter binding assays were limited by the high background and fluorescence-based assays were incompatible with our current source of recombinant hTERT. We therefore settled on and developed a SPA. For the SPA, the same affinity purification principle as described above was used, except the readout was by scintillation counting as opposed to phosphorimaging. In the absence of inhibitor, [ $^{35}\text{S}$ ]-hTERT can bind biotinylated RNA. Upon the addition of streptavidin-coated SPA beads (Amersham Biosciences), the RNA can bring the [ $^{35}\text{S}$ ]-hTERT into close proximity with the fluorophore contained in the bead, thus eliciting a signal (Figure 6.2).



**Figure 6.2** Assay schematics for scintillation proximity assay. Biotinylated CR4-CR5 RNA and radio-labeled hTERT associate. The addition of streptavidin-SPA beads brings the [ $^{35}\text{S}$ ]-hTERT into close proximity to the beads and produces a scintillant signal.

### 3. Validation of SPA

Initially, prior to assay optimization, the SPA was run in concert with a low-throughput affinity purification using a range of concentrations of hTRas010 to determine if the two assays reported similar results. We found that the addition of hTRas010 to both the affinity purification and the SPA impeded the RNA/protein interaction in a concentration dependent manner yielding  $IC_{50}$  values of  $0.89 \mu M$  and  $1.22 \mu M$ , respectively (Figure 6.3). Notably, high concentration of hTRas010 completely impeded RNA/protein binding in the SPA. The close comparison to the biochemical assay validated the SPA approach.

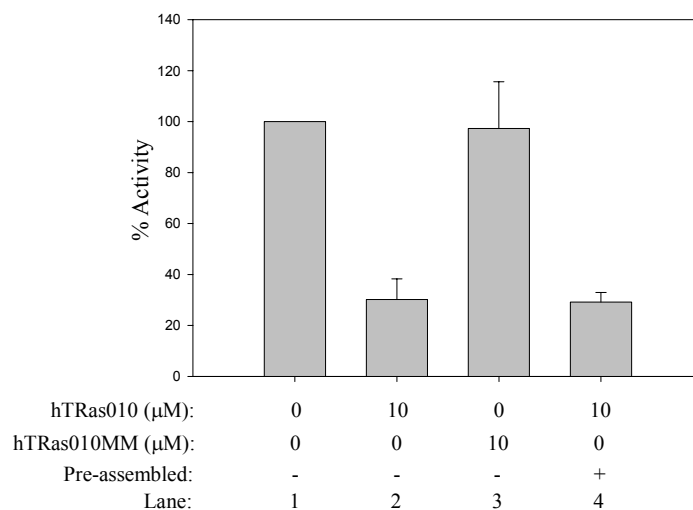


**Figure 6.3 Concentration dependence of hTRas010 in an affinity purification and a scintillation proximity assay.** Each concentration of hTRas010 was assayed in duplicate (A) or triplicate (B). “% Activity” indicates the amount of residual activity as compared to the positive control.  $IC_{50}$  curves and values were obtained as described in the Materials and methods. Error bars indicate standard deviation and  $IC_{50}$  error indicates standard error.

#### 4. Optimization of SPA

Several aspects of the CR4-CR5-hTERT SPA were optimized to ensure its efficient and accurate application for high-throughput data collection. Various assay conditions were compared by calculating the Z-factor for each. The Z-factor, first defined by Zhang et al., is a statistical parameter used to evaluate the robustness and usefulness of an assay (Zhang, J. H. et al., 1999a). Generally, a Z-factor value between 0.5 and 1 is considered an excellent assay, whereas one between zero and 0.5 is marginal (Zhang, J. H. et al., 1999a). Optimizations included varying amount of biotin-labeled RNA, amounts of protein, bead concentration, blocking buffer components and concentrations, incubation time and order of addition. Components were titrated and the amounts yielding the best Z-factor were implemented. The optimum conditions are those listed in the Materials and methods section. Notably, the use and optimization of blocking buffer was a key step in the development of this assay as the absence of a blocking step resulted in both increased background signal and decreased RNA-bead interaction. Furthermore, using a blocking buffer that contained herring sperm DNA and BSA yielded an assay with a better Z-factor when compared to assays containing yeast RNA and Casein as the blocking reagents. Cross-talk between adjacent wells was also determined and found to be negligible.

To demonstrate assay specificity, hTRas010MM, a modified mismatch oligonucleotide that is complimentary to the CR4-CR5 domain at 15 of its 22 nucleotides, was added to the SPA in place of hTRas010. At 10  $\mu$ M, the concentration at which hTRas010 inhibited the SPA by ~70%, hTRas010MM had a negligible effect (Figure 6.4,

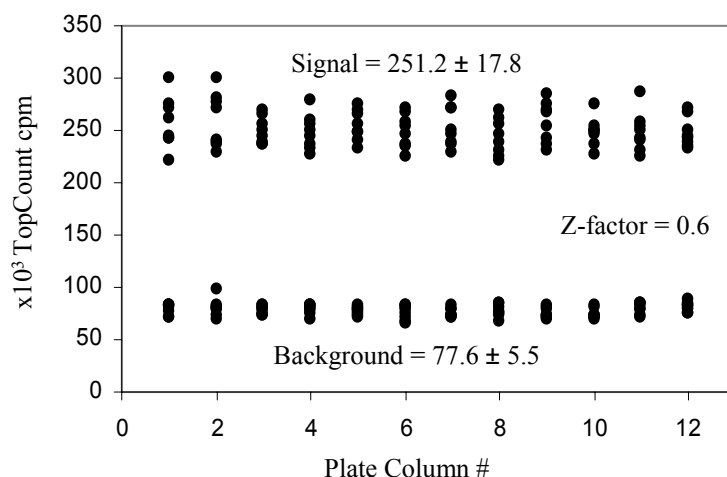


**Figure 6.4 Effects of hTRas010 and hTRas010MM on the interaction between hTERT and the CR4-CR5 fragment of hTR.** “Pre-assembled” refers to hTERT and CR4-CR5 RNA pre-assembled prior to oligonucleotide addition. hTERT pre-assembled with RNA was prepared by adding 855 ng of in vitro transcribed biotinylated CR4-CR5 RNA to a 100 μL reticulocyte lysate reaction during translation of hTERT. “% Activity” indicates the amount of residual activity as compared to the positive control. Error bars indicate standard deviation

lane 3). Negative controls using unbiotinylated RNA, no RNA or an empty vector control were performed with no significant increase in signal. We also performed a control in which hTERT and the CR4-CR5 RNA were pre-assembled prior to the addition of hTRas010. Surprisingly, we found that the oligonucleotide was equally effective at preventing the RNA/protein interaction in this instance as it is when added prior to assembly (Figure 6.4, compare lane 4 to lane 2). This suggests that other hTR/hTERT contacts, such as those identified in the pseudoknot domain (Tesmer et al., 1999; Mitchell and Collins, 2000; Keppler and Jarstfer, 2004), affect the affinity or dissociation rate of the CR4-CR5 domain.

Over a span of 11 days, the SPA was tested for inter-day and intra-column variability. We obtained a positive signal of  $251.2 \times 10^3$  cpm and a background signal of  $77.6 \times 10^3$  cpm, with a Z-factor of 0.6 (Figure 6.5). Due to  $^{35}\text{S}$  nuclide decay and the use of different [ $^{35}\text{S}$ ]-

Met batches to translate hTERT, the scintillant signals from different assays were normalized to the background signals.



**Figure 6.5 Precision of scintillation proximity assay for assembly of hTERT with CR4-CR5 RNA under optimal conditions.** Inter-day and intra-column assay variation is represented.

### C. Discussion

There are many approaches used to detect telomerase activity. Direct assays, PCR-based TRAP assays and bead-based assays have all been adapted to high-throughput protocols with the goal of identifying novel, efficacious telomerase inhibitors (Bosworth and Towers, 1989; Savoyesky et al., 1996; Francis and Friedman, 2002; Francis and Friedman, 2003). To date, however, there is presently no gold-standard FDA-approved anti-telomerase drug. The identification of such a compound would have dramatic implications on cancer therapy as telomerase is found almost universally in cancer cells and its activity is required for cancer cell proliferation. Therefore, there lies a need for the identification of a specific, high affinity telomerase inhibitor. To facilitate the discovery of such compounds, we

considered blocking telomerase-specific protein-RNA interactions. Towards this end, the SPA described here was developed.

Rather than screening for compounds which directly affect the enzymatic activity of telomerase, i.e. telomere extension, we set out to develop a high-throughput method to screen for compounds that inhibit proper telomerase assemblage. We choose to target the CR4-CR5 domain of hTR (Figure 6.1A) because it is a well-defined and structurally conserved region that has received much attention (Chen et al., 2000; Chen et al., 2002; Chen and Greider, 2003). Screening with full-length hTR was not performed because there are multiple hTR/hTERT contact sites that preclude the use of simplistic analytic methods required for high-throughput screening (Tesmer et al., 1999; Mitchell and Collins, 2000; Chen et al., 2002; Chen and Greider, 2003; Keppler and Jarstfer, 2004). The identification of a compound that perturbs all of these required contacts is unlikely, and the disruption of the CR4-CR5 domain binding to hTERT has been shown to be all that is needed to disrupt reconstituted telomerase activity (Chen et al., 2002; Keppler and Jarstfer, 2004). Furthermore, the NMR structure of the CR4-CR5 domain has been solved by Leeper and Varani, foreshadowing structural studies on CR4-CR5 domain-ligand complexes (Leeper and Varani, 2005). Here we describe a method to rapidly identify novel inhibitors of telomerase assemblage in high-throughput format using scintillation proximity assay technology.

Prior to SPA development, we confirmed that [ $^{35}\text{S}$ ]-hTERT could be affinity purified using biotinylated CR4-CR5 RNA and streptavidin beads (Figure 6.1B). The same methodology was implemented for the SPA (Figure 6.2). In the absence of inhibitor, the RNA/protein complex forms and binds to the streptavidin-coated SPA beads. The close

proximity of the radio-labeled hTERT to the bead generates a positive signal when read on a microplate scintillation counter.

The SPA was validated using a modified oligonucleotide previously shown to completely inhibit the association of hTERT with the CR4-CR5 portion of hTR (Keppler and Jarstfer, 2004). Biochemical affinity purifications and scintillation proximity assays were performed concurrently to determine the concentration dependence of hTRas010 using these different methods. Both techniques generated  $IC_{50}$ 's in the 1  $\mu$ M range thus validating the SPA (Figure 6.3). The proven ability to completely eradicate binding in the SPA at high concentration of hTRas010 is important for the usefulness and quality of the assay. Noticeably there is a much greater error associated with the biochemical affinity purification than the SPA, particularly with the intermediate concentrations of hTRas010 (Figure 6.3A). This deviation is most likely due to human error during the many handling steps required for this procedure. The error in the SPA data (Figure 6.3B), however, is minimal as the beads are handled only once and the filter plate washing steps are not prone to loss of beads, as is common in centrifugation-mediated affinity purifications. Thus, the SPA could also find applications in biochemical-binding studies of the telomerase complex.

SPA optimization was performed in order to increase the speed and sensitivity of the assay while decreasing nonspecific binding and cost. The amounts of RNA, protein and beads were minimized as much as possible without greatly affecting signal strength. Various common blocking buffer components were used in different combinations and concentrations in order to decrease the background signal and increase the Z-factor of the screen. The order of addition was also optimized. Ultimately, the delayed addition of the beads to the samples allowed for optimum RNA/protein association. Importantly, this screen generated a Z-factor

value of 0.6, which is within the defined range of an “excellent assay,” as described by Zhang et al. (Zhang, J. H. et al., 1999a). To determine assay specificity, we used a mismatch oligonucleotide. hTRas010MM was found to have little effect on the SPA (Figure 6.4, lane 3), indicating that the decrease in signal in the presence of hTRas010 is due to specific targeting and not a nonspecific effect of 2'-*O*-methyl RNA.

The SPA described here has several advantages over other high-throughput screens in that there is no false positive-laden PCR step, a lower energy radionuclide ( $^{35}\text{S}$ ) is used when compared to  $^{32}\text{P}$  and the use of scintillation proximity beads negates the need for a scintillation cocktail. Furthermore, by detecting changes in telomerase assemblage rather than changes in activity, this screen will identify telomerase inhibitors that would be missed by activity screens that use full-length hTR pre-assembled with hTERT. For example, previous reports have shown that the addition of hTRas010 to a direct telomerase assay with pre-assembled telomerase has little effect on activity, whereas telomerase assembled in the presence of hTRas010 is inactive (Keppler and Jarstfer, 2004). This is because hTRas010, when targeting the association between hTERT and full-length hTR, is only effective at inhibiting telomerase activity when added prior to hTERT interaction with the CR4-CR5 domain of hTR. The inhibitory potential of hTRas010, when targeting the association between hTERT and the CR4-CR5 domain only, however, is effective both before and after protein/RNA assembly (Figure 6.4, lanes 2 and 4). This is presumably because when using full-length hTR, other hTR/hTERT contact points, such as those identified in the pseudoknot domain (Tesmer et al., 1999; Mitchell and Collins, 2000; Keppler and Jarstfer, 2004), hold the CR4-CR5 domain in place, thus preventing hTRas010 from inhibiting. Because of the rapid rate at which hTERT-positive cancer cells divide, the high transcription rate of hTR (Yi



et al., 1999) and thus the likely high turnover rate of telomerase complexes, the identification of compounds that can only perturb hTR/hTERT interactions prior to telomerase assembly is therapeutically relevant. Additionally, we have shown that hTRas010 inhibits telomerase in cultured cells (unpublished data), which is not surprising as a PNA that binds to a similar region of hTR has been reported to inhibit cellular telomerase (Hamilton et al., 1999). Therefore, our assay will identify highly specific compounds that inhibit telomerase assemblage and therefore its enzymatic activity. By conducting the assay with hTERT translated in rabbit reticulocyte lysates, the assay will identify compounds with greater selectivity than compounds identified in screens using purified materials.

In comparison to performing direct activity assays or affinity purifications, our method greatly reduces both assay time and cost. One 96-well SPA plate can be assayed in 4 h, whereas it takes 10 h plus an overnight gel exposure for up to 25 samples in a direct assay or 6 h plus an overnight gel exposure for up to 25 samples when using affinity purification. Presently, our screen can be run at a cost of about \$1 per compound. Despite these advancements to past high-throughput assays, there obviously remains room for improvement particularly in decreasing the cost.

Unlike the typical SPA protocol, our procedure does involve the removal of unbound ligand. This is not due to non-proximity effects from the [<sup>35</sup>S]-hTERT, but because the protein is translated in rabbit reticulocyte lysates and the scintillant signal could not be detected through such a medium. Though this necessitates an extra step, the use of 96-well filter plates to remove the surrounding medium allows for rapid simultaneous analysis of a large number of samples and greatly reduces assay time as compared to individual affinity purification washes. However, it would be beneficial to express hTERT in a different

heterologous system other than reticulocyte lysates to remove this step and improve the cost effectiveness. Unfortunately, hTERT expression in baculovirus is not trivial and bacterial expression systems are unable to translate hTERT, owing to its size (~127 kDa), poor solubility and other issues related to the complexity of this polypeptide. Elucidation of RNA binding sites on hTERT could allow bacterial expression of smaller hTERT fragments that bind the CR4-CR5 domain of hTR. In fact, this SPA is adaptable to the high-throughput characterization of hTERT point and truncation mutants that are capable of binding hTR, thus potentially revealing as yet undiscovered RNA-binding domains in the protein.

#### D. Materials and methods

See Chapter II, section D for information on the pET-28c-hTERT expression plasmid and synthesis of hTERT. See Chapter V, section D for a protocol for the synthesis of biotinylated CR4-CR5 RNA fragment. We thank Dr. Gary M. Pollack for use of the TopCount™ NXT scintillation counter.

##### 1. Oligonucleotides.

Modified oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, Iowa). Concentrations were determined by UV absorbance at 260 nm using the molar extinction coefficient supplied by the manufacturer. The sequence of hTRas010 is CGGCUGACAGAGCCCAACUCUU and the sequence of hTRas010MM is CGGCUCAGACACGGGAACUCUU. All bases are 2'-O-methyl modified and underlined bases indicate phosphorothioate linkages.

## 2. Biochemical assay for the interaction between hTERT and the CR4-CR5 domain of hTR.

Biotinylated RNA was used to capture [ $^{35}\text{S}$ ]-hTERT. 75  $\mu\text{L}$  reactions contained 50  $\mu\text{L}$  (250 fmol) of  $^{35}\text{S}$ -labeled hTERT from a reticulocyte lysate reaction and 29 pmol of biotinylated CR4-CR5 RNA. Inhibition studies also included varying amounts of hTRas010. Reactions were incubated at 30 °C for 90 min to allow assembly between the RNA and the protein. Each reaction was affinity purified using 50  $\mu\text{L}$  of MPG<sup>®</sup> Streptavidin beads (Pure Biotech, LLC). Prior to use, the beads were washed and blocked as described in Chapters II and V. The beads were precipitated by centrifugation at 1500g for 30 sec. The 75  $\mu\text{L}$  assembly reaction was combined with 75  $\mu\text{L}$  blocking buffer and centrifuged at 17000g for 10 min at 4 °C to remove any precipitates. The assembly reaction was then added to the blocked beads and the samples were mixed on a rotary platform for 30 min at room temperature. The beads were then washed and processed as described in Chapters II and V.

## 3. Scintillation Proximity Assay

Each 15  $\mu\text{L}$  assembly reaction contained 10  $\mu\text{L}$  (50 fmol) [ $^{35}\text{S}$ ]-hTERT from an in vitro translation reaction and 2.9 pmol biotinylated CR4-CR5 RNA. Negative controls contained either unbiotinylated RNA, no RNA or 10  $\mu\text{L}$  of a translation reaction using an empty vector (pET-28c) in place of pET-28c-hTERT. Inhibition-control reactions also included varying concentrations of hTRas010. Prior to use, all reticulocyte lysate reactions were centrifuged at 17000g for 10 min at 4 °C to remove any precipitates. Protein/RNA assembly reactions were incubated at 30 °C for 90 min in 96-well polystyrene microplates (OptiPlate-96, Packard). For each SPA reaction, 0.4 mg Streptavidin PVT-SPA beads (Amersham Biosciences, Cat. # RPNQ0006) were used. Prior to use, beads were washed

four times with 200  $\mu$ L SPA buffer 1 (10 mM Tris-acetate, pH 7.5, 5% glycerol, 0.5 mM EDTA, 2.5 mM  $MgCl_2$ , 50 mM potassium glutamate, 0.05% IGEPAL, 0.5 mM DTT) and blocked twice for 15 min at 4  $^{\circ}C$  with 200  $\mu$ L of SPA blocking buffer (20 mM Tris-acetate, pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM  $MgCl_2$ , 100 mM potassium glutamate, 0.1% IGEPAL, 1 mM DTT, 0.05 mg/mL lysozyme, 0.5 mg/mL BSA, 0.05 mg/mL glycogen, 0.1 mg/mL herring sperm DNA (Promega)). The beads were precipitated by centrifugation at 4000g for 2 min. Precipitating the beads from the SPA blocking buffer first required the addition of an equal volume of  $H_2O$  in order to prevent bead buoyancy due to high glycerol concentrations. Large batch preparations of beads were possible by scaling up the above procedure. To initiate the SPA, 15  $\mu$ L assembly reactions were combined with 15  $\mu$ L blocking buffer and 0.4 mg blocked beads and incubated for 30 min at room temperature. Reactions were transferred to a 96-well MultiScreen<sup>®</sup> HTS filter plate (Millipore, Cat. #MSDNV6B) that had been pre-wet with 200  $\mu$ L SPA buffer 2 (10 mM Tris-acetate, pH 7.5, 5% glycerol, 0.5 mM EDTA, 2.5 mM  $MgCl_2$ , 150 mM potassium glutamate, 0.05% IGEPAL, 0.5 mM DTT). Samples were filtered using a MultiScreen<sup>®</sup> Resist Vacuum Manifold (Millipore, Cat. #MAVM0960R). The trapped beads were washed four times with 200  $\mu$ L of SPA buffer 2, ceasing the vacuum pressure in between each wash. Once completed, the underdrain was removed from the filter plate and the top and bottom of the plate were covered with clear and opaque tape, respectively, per the manufacturer's instructions. The plate was then read on a TopCount<sup>™</sup> NXT scintillation counter (Packard, version 1.06). TopCount<sup>™</sup> instrument settings used can be found at [www.amershambiosciences.com/spa](http://www.amershambiosciences.com/spa). After subtracting the background signal (negative control with no RNA), the SPA signals for the experimental samples were compared to that

for the positive control. Unused beads were stored in SPA buffer 1 (minus the DTT) at 4 °C and were used within 10 days. All liquid transfer steps could be conducted with multi-channel pipettors or robotic liquid handling devices.

#### 4. Inhibition studies

Affinity purifications and SPA assays were conducted as described above with varying concentrations of hTRas010. Five data points were used with oligonucleotide concentrations ranging from 10 nM to 100 µM. IC<sub>50</sub> values were calculated and inhibition data were plotted as described in Chapter II.

#### 5. Z-factor determination

The Z-factor (Z'), first described by Zhang et al. (Zhang, J. H. et al., 1999a), was calculated using the following equation

$$Z' = 1 - \frac{(3\sigma_{\max} + 3\sigma_{\min})}{(|SPA_{\max} - SPA_{\min}|)}$$

where  $\sigma_{\max}$  is the standard deviation of the maximum signal (positive control),  $\sigma_{\min}$  is the standard deviation of the minimum signal (negative control),  $SPA_{\max}$  is the mean of the maximum signal and  $SPA_{\min}$  is the mean of the minimum signal.

## **Chapter VII. Conclusions and future directions of this research**

This research describes the design, development and validation of a new mode of human telomerase inhibition. Through the use of oligonucleotides and known nucleic-binding compounds, it was shown that this methodology of perturbing proper telomerase assemblage represents an innovative modality for inhibiting telomerase. The screening of a small library of compounds and identification of a structurally related group of natural products and derivatives as telomerase inhibitors further solidified this approach. This work has also further elucidated the roles of hsp90 in maintaining an active telomerase complex for the purpose of understanding telomerase holoenzyme assemblage requirements. It was shown that hsp90 is involved in both telomerase primer-binding competence and holoenzyme stability. In order to adapt this method of telomerase inhibition to a high-throughput approach a scintillation proximity assay screen was initiated. The development of this screen is the first step toward identifying a new class of telomerase inhibitor.

Recently, other functions of telomerase besides DNA synthesis have been suggested to contribute to the survival of cancer cells (Stewart et al., 2002). Notably, telomerase appears capable of inhibiting apoptosis by a mechanism that is independent of telomere maintenance (Cao et al., 2002; Rahman et al., 2004). Some believe that this is achieved via telomerase's potential involvement in capping and protecting the telomere (Blackburn, E., 1999; Blackburn, E. H., 2000; Blackburn, E. H., 2001; Masutomi et al., 2003). This suggests that simply targeting its enzymatic activity may not achieve the maximum potential of

telomerase-targeted therapeutics. It is believed that the method of telomerase inhibition described in this thesis will be a more effective approach in the abrogation of cancer cells as telomerase assemblage inhibitors may have the potential to not only perturb telomerase's ability to maintain telomere length, but also its anti-apoptotic role in tumorigenesis. Preventing the proper assemblage of the holoenzyme could seize telomerase in an inactive state by preventing the dissociation of hTR and hTERT, thus generating an in situ dominant negative complex that is incapable of performing its telomere capping function or other functions unrelated to DNA synthesis.

The identification of potent telomerase assemblage inhibitors will likely have a profound impact on the relentless fight against cancer. Future clinically approved telomerase inhibitors will almost certainly be used as an adjuvant to increase the efficacy of additional chemotherapeutics as a two-pronged approach toward cancer cell attack.

Future studies in the field of telomerase assembly inhibition will focus on the characterization of hTR binding sites on hTERT and the determination of compound target sites on telomerase, as well as cellular in vivo experiments. Presently, not much is known about the structure of hTERT due to the inability to completely purify it to homogeneity. Additionally, large scale translation of recombinant wild-type hTERT with commonly used viral and bacterial expression systems has proven to be an arduous task. Recently, however, the Cech laboratory has used high-throughput methods to characterize portions of the telomerase catalytic protein from *Tetrahymena* by identifying and over-expressing soluble truncated tTERT fragments in *E. coli* (Jacobs et al., 2005). Analysis of these fragments, which can be purified to homogeneity, revealed the presence of an independently folded domain at the N-terminus of tTERT. This approach is also adaptable to hTERT structural

identification, which, along with other studies such as limited proteolytic digestion, NMR spectroscopy and hTR binding studies, will ultimately reveal the specific hTERT amino acids involved in hTR/hTERT assembly. Furthermore, once soluble hTERT fragments are expressed, the scintillation proximity assay screen described in Chapter VI can also be adapted identify the region(s) of hTERT that interact with the CR4-CR5 domain of hTR. Subsequently, additional RNA-binding domains of hTERT can then be identified by replacing the biotinylated CR4-CR5 RNA fragment in the SPA with other biotinylated hTR domains known to independently bind hTERT.

Surface Plasmon Resonance (SPR) can be utilized to determine if a compound binds to either hTR or hTERT. SPR involves the immobilization of a target on a sensor chip and the measurement of the change in the angle of polarized reflected light off of the chip as the compound is passed over the target (Biacore website: [http://www.biacore.com/technology/spr\\_technology.lasso#](http://www.biacore.com/technology/spr_technology.lasso#)). This technique yields real-time measurements and allows for the determination of stoichiometry and on/off rates for the binding between target and compound. Determination of compound binding to hTERT, however, will have to await the development of a high-yield hTERT expression protocol as the use of a protein as the target in SPR requires more purified material than is presently reasonably able to be expressed. Alternatively, verification of compound/target interaction can be accomplished using amine-reactive fluorescent dyes such as 5-carboxytetramethylrhodamine, succinimidyl ester (5-TAMRA, SE) or 5-carboxyfluorescein, succinimidyl ester (5-FAM, SE) (Molecular Probes). After allowing fluorescent compound/target association, detection of the relative fluorescence on various independent domains of hTR for example should shed light on the compound's specific target site(s). The



protocols for conjugation and measuring the change in fluorescent absorbance are relatively simple, however, this method can only be used to test amine-containing compounds.

Following the identification and binding characterization of telomerase assemblage inhibitors *in vitro*, cellular studies should be performed to confirm that the compound not only inhibits enzymatic activity, but also decreases telomere length, limits proliferation and induces apoptosis. Compound-induced inhibition of activity will typically be detected by the PCR-based TRAP assay since the low levels of telomerase expressed in cancer cells are difficult to detect using a direct assay. Changes in telomere length upon inhibitor treatment should be performed by restriction digest of genomic DNA followed by Southern blot analysis using a telomeric probe, for example. Proliferation rates can be determined using basic cell culture and counting methods while the induction of apoptosis can be monitored by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL). Furthermore, in terms of compound specificity for telomerase, the time it takes a cell to senesce after being treated with a telomerase assemblage inhibitor should be proportional to the initial telomere length of the cell (White et al., 2001).

Cellular studies to define the mechanism(s) of compound-induced telomerase inhibition should also be performed. Even if a compound is shown to bind to hTR or hTERT *in vivo*, it may also interact with other cellular entities (enzymes, nucleic acids, etc.) resulting in decreased proliferation and/or cell death if it is promiscuous and has low specificity for telomerase. Prior to cell studies, therefore, compounds should be screened *in vitro* for their specificity toward telomerase versus other polymerases, for example (see Figure 4.4). Compound-induced cytotoxicity should also be assayed in multiple cell lines, including

those that are hTERT-negative, in order to determine if the toxicity is telomerase-dependent (see Figure 4.5A).

Additionally, cellular studies to test the hypothesis that telomerase assemblage inhibitors induce the formation of an in vivo dominant negative complex will also further define and advance the field of telomerase assemblage inhibition. Most screened compounds identified as telomerase assemblage inhibitors are expected to affect only a single hTR-hTERT contact point, which will continue to allow the two subunits to associate, however in an inactive and misshapen form that is unable to bind and maintain telomere length or telomere structure. Therefore, this could possibly be achieved by first treating a cancer cell line with a defined telomerase assemblage inhibitor and affinity purifying hTR from cells to detect the presence of hTERT via Western blot. The continued association of hTERT with hTR would suggest that the inhibitor paralyzes the complex, thus perturbing activity and preventing hTR/hTERT dissociation. This, however, would not decidedly reveal if the inactive telomerase complex was incapable of binding the telomere. This feat could be approached with electron microscopy, though large amounts of the enzyme purified to homogeneity would most likely be required. Alternatively, affecting this secondary role of telomerase in tumorigenesis has been shown to lead to apoptosis without affecting telomere length or inducing senescence (Hao et al., 2005). Telomerase assemblage inhibitors eliciting this same behavior would thus be evidence that they also have adverse effects on the anti-apoptotic role of telomerase.

## **Representative works**

### *Refereed Publications:*

Keppler, B. R. and Jarstfer, M. B. (2004) Inhibition of Telomerase Activity by Preventing Proper Assemblage, *Biochemistry* 43, 334-43.

Dominick, P. K., Keppler, B. R., Legassie, J. D., Moon, I. K. and Jarstfer, M. B. (2004) Nucleic-Acid Binding Ligands Identify New Mechanisms to Inhibit Telomerase, *Bioorganic and Medicinal Chemistry Letters* 14,3467-3471.

Keppler, B. R. and Jarstfer, M. B. (2005) A High-Throughput Screen Capable of Identifying Novel Inhibitors of Human Telomerase Assemblage, accepted to *Analytical Biochemistry*.

Fouché, N., Moon, I. K., Keppler, B. R., Griffith, J. and Jarstfer, M. B. (2005) Electron Microscopic Visualization of Telomerase from *Euplotes aediculatus* Bound to a Model Telomere DNA, submitted to *Journal of Biological Chemistry*.

Keppler, B. R., Grady, A. and Jarstfer, M. B. (2005) The Biochemical Role of the Heat Shock Protein 90 Chaperone Complex in Human Telomerase Ribonucleoprotein Activity, submitted to *Journal of Biological Chemistry*.

Keppler, B.K., Soares, J., Wang, X., Lee, K.-H., and Jarstfer, M.B. (2005) Tanshinones Inhibit Telomerase Activity by Inactivating hTERT, manuscript in preparation.

### *Conference Presentations:*

Keppler, B. R. and Jarstfer, M. B. "A Role for Hsp90 in Primer Loading of Human Telomerase" (oral). Telomeres and Telomerase Conference - Cold Spring Harbor, New York, May 4-8, 2005.

Invited Speaker, "Cancer and the 21<sup>st</sup> Century" (oral), Graduate Education Advancement Board (GEAB) Meeting, University of North Carolina, Chapel Hill, November 5, 2004.

Keppler, B. R. and Jarstfer, M. B. "Targeting Telomerase Assemblage: A Novel Anticancer Approach" (poster). Symposium on RNA Biology V: RNA, Tool and Target, North Carolina Biotechnology Center, October 17-18, 2003.

Keppler, B. R. and Jarstfer, M. B. "A New Platform for Telomerase Inhibition" (oral). American Chemical Society Sectional Conference – North Carolina, April 26, 2003.

Keppler, B. R. and Jarstfer, M. B. "Targeting Telomerase Assemblage: A Novel Anticancer Approach" (poster). Pharmaceutical Sciences Research Conference, UNC School of Pharmacy, April 25-26, 2003.

## Literature cited

- Agarwal, R., S. K. Katiyar, S. I. Zaidi and H. Mukhtar (1992). Inhibition of skin tumor promoter-caused induction of epidermal ornithine decarboxylase in SENCAR mice by polyphenolic fraction isolated from green tea and its individual epicatechin derivatives. *Cancer Res.* **52**(13): 3582-8.
- Akalin, A., L. W. Elmore, H. L. Forsythe, B. A. Amaker, E. D. McCollum, P. S. Nelson, J. L. Ware and S. E. Holt (2001). A novel mechanism for chaperone-mediated telomerase regulation during prostate cancer progression. *Cancer Res.* **61**(12): 4791-6.
- Aldous, W. K., A. J. Marean, M. J. DeHart, L. A. Matej and K. H. Moore (1999). Effects of tamoxifen on telomerase activity in breast carcinoma cell lines. *Cancer* **85**(7): 1523-9.
- Ancelin, K., M. Brunori, S. Bauwens, C. E. Koering, C. Brun, M. Ricoul, J. P. Pommier, L. Sabatier and E. Gilson (2002). Targeting assay to study the cis functions of human telomeric proteins: evidence for inhibition of telomerase by TRF1 and for activation of telomere degradation by TRF2. *Mol. Cell. Biol.* **22**(10): 3474-87.
- Antal, M., E. Boros, F. Solymosy and T. Kiss (2002). Analysis of the structure of human telomerase RNA in vivo. *Nucleic Acids Res.* **30**(4): 912-20.
- Asai, A., Y. Oshima, Y. Yamamoto, T. A. Uochi, H. Kusaka, S. Akinaga, Y. Yamashita, K. Pongracz, R. Pruzan, E. Wunder, M. Piatyszek, S. Li, A. C. Chin, C. B. Harley and S. Gryaznov (2003). A novel telomerase template antagonist (GRN163) as a potential anticancer agent. *Cancer Res.* **63**(14): 3931-9.
- Asker, C., K. G. Wiman and G. Selivanova (1999). p53-induced apoptosis as a safeguard against cancer. *Biochem. Biophys. Res. Commun.* **265**(1): 1-6.
- Atha, D. H., K. Miller, A. D. Sanow, J. Xu, J. L. Hess, O. C. Wu, W. Wang, S. Srivastava and W. E. Highsmith (2003). High-throughput analysis of telomerase by capillary electrophoresis. *Electrophoresis* **24**(1-2): 109-14.
- Avantaggiati, M. L., V. Ogryzko, K. Gardner, A. Giordano, A. S. Levine and K. Kelly (1997). Recruitment of p300/CBP in p53-dependent signal pathways. *Cell* **89**(7): 1175-84.
- Avilion, A. A., M. A. Piatyszek, J. Gupta, J. W. Shay, S. Bacchetti and C. W. Greider (1996). Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. *Cancer Res.* **56**(3): 645-50.

- Bachand, F. and C. Autexier (2001). Functional regions of human telomerase reverse transcriptase and human telomerase RNA required for telomerase activity and RNA-protein interactions. *Mol. Cell. Biol.* **21**(5): 1888-97.
- Bagatell, R. and L. Whitesell (2004). Altered Hsp90 function in cancer: a unique therapeutic opportunity. *Mol. Cancer Ther.* **3**(8): 1021-30.
- Baillie, A. C. and R. H. Thomson (1968). Naturally occurring quinones. Part XI. The tanshinones. *J. Chem. Soc. (C)*: 48-52.
- Barent, R. L., S. C. Nair, D. C. Carr, Y. Ruan, R. A. Rimerman, J. Fulton, Y. Zhang and D. F. Smith (1998). Analysis of FKBP51/FKBP52 chimeras and mutants for Hsp90 binding and association with progesterone receptor complexes. *Mol. Endocrinol.* **12**(3): 342-54.
- Basso, A. D., D. B. Solit, G. Chiosis, B. Giri, P. Tschlis and N. Rosen (2002). Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. *J. Biol. Chem.* **277**(42): 39858-66.
- Baumann, P. and T. R. Cech (2001). Pot1, the putative telomere end-binding protein in fission yeast and humans. *Science* **292**(5519): 1171-5.
- Beattie, T. L., W. Zhou, M. O. Robinson and L. Harrington (1997). Reconstitution of human telomerase activity *in vitro*. *Current Biology* **8**: 177-180.
- Beattie, T. L., W. Zhou, M. O. Robinson and L. Harrington (2000). Polymerization defects within human telomerase are distinct from telomerase RNA and TEP1 binding. *Mol. Biol. Cell* **11**(10): 3329-40.
- Beliakoff, J. and L. Whitesell (2004). Hsp90: an emerging target for breast cancer therapy. *Anticancer Drugs* **15**(7): 651-62.
- Beltz, L. A. (2002). The 'other' telomerase inhibitors: non-G-quadruplex interactive agent, non-antisense, non-reverse transcriptase telomerase inhibitors. *Curr. Med. Chem. Anti-Canc. Agents* **2**(5): 589-603.
- Blackburn, E. (1999). The telomere and telomerase: how Do they interact? *Mt. Sinai J. Med.* **66**(5-6): 292-300.
- Blackburn, E. H. (2000). Telomere states and cell fates. *Nature* **408**(6808): 53-6.
- Blackburn, E. H. (2001). Switching and signaling at the telomere. *Cell* **106**(6): 661-73.
- Blank, M., M. Mandel, Y. Keisari, D. Meruelo and G. Lavie (2003). Enhanced ubiquitinylation of heat shock protein 90 as a potential mechanism for mitotic cell death in cancer cells induced with hypericin. *Cancer Res.* **63**(23): 8241-7.

- Blasco, M. A., W. Funk, B. Villeponteau and C. W. Greider (1995). Functional characterization and developmental regulation of mouse telomerase RNA. *Science* **269**(5228): 1267-70.
- Bledsoe, R. K., V. G. Montana, T. B. Stanley, C. J. Delves, C. J. Apolito, D. D. McKee, T. G. Consler, D. J. Parks, E. L. Stewart, T. M. Willson, M. H. Lambert, J. T. Moore, K. H. Pearce and H. E. Xu (2002). Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. *Cell* **110**(1): 93-105.
- Bodnar, A. G., N. W. Kim, R. B. Effros and C. P. Chiu (1996). Mechanism of telomerase induction during T cell activation. *Exp. Cell Res.* **228**(1): 58-64.
- Bohen, S. P. (1998). Genetic and biochemical analysis of p23 and ansamycin antibiotics in the function of Hsp90-dependent signaling proteins. *Mol. Cell. Biol.* **18**(6): 3330-9.
- Borras, C., J. M. Esteve, J. R. Vina, J. Sastre, J. Vina and F. V. Pallardo (2004). Glutathione regulates telomerase activity in 3T3 fibroblasts. *J. Biol. Chem.* **279**(33): 34332-5.
- Bosoy, D., Y. Peng, I. S. Mian and N. F. Lue (2003). Conserved N-terminal motifs of telomerase reverse transcriptase required for ribonucleoprotein assembly in vivo. *J. Biol. Chem.* **278**(6): 3882-90.
- Bosworth, N. and P. Towers (1989). Scintillation proximity assay. *Nature* **341**(6238): 167-8.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-54.
- Bryan, T. M., K. J. Goodrich and T. R. Cech (2000a). Telomerase RNA bound by protein motifs specific to telomerase reverse transcriptase. *Mol. Cell* **6**(2): 493-9.
- Bryan, T. M., K. J. Goodrich and T. R. Cech (2000b). A mutant of *Tetrahymena* telomerase reverse transcriptase with increased processivity. *J. Biol. Chem.* **275**: 24199-24207.
- Burger, A. M., F. Dai, C. M. Schultes, A. P. Reszka, M. J. Moore, J. A. Double and S. Neidle (2005). The G-quadruplex-interactive molecule BRACO-19 inhibits tumor growth, consistent with telomere targeting and interference with telomerase function. *Cancer Res.* **65**(4): 1489-96.
- Cao, Y., H. Li, S. Deb and J. P. Liu (2002). TERT regulates cell survival independent of telomerase enzymatic activity. *Oncogene* **21**(20): 3130-8.

- Carrello, A., E. Ingley, R. F. Minchin, S. Tsai and T. Ratajczak (1999). The common tetratricopeptide repeat acceptor site for steroid receptor-associated immunophilins and hop is located in the dimerization domain of Hsp90. *J. Biol. Chem.* **274**(5): 2682-9.
- Chadli, A., I. Bouhouche, W. Sullivan, B. Stensgard, N. McMahon, M. G. Catelli and D. O. Toft (2000). Dimerization and N-terminal domain proximity underlie the function of the molecular chaperone heat shock protein 90. *Proc. Natl. Acad. Sci. U S A* **97**(23): 12524-9.
- Chang, J. T., Y. L. Chen, H. T. Yang, C. Y. Chen and A. J. Cheng (2002). Differential regulation of telomerase activity by six telomerase subunits. *Eur. J. Biochem.* **269**(14): 3442-50.
- Chen, G., P. Cao and D. V. Goeddel (2002). TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. *Mol. Cell* **9**(2): 401-10.
- Chen, J. L., M. A. Blasco and C. W. Greider (2000). Secondary structure of vertebrate telomerase RNA. *Cell* **100**(5): 503-14.
- Chen, J. L., K. K. Opperman and C. W. Greider (2002). A critical stem-loop structure in the CR4-CR5 domain of mammalian telomerase RNA. *Nucleic Acids Res.* **30**(2): 592-7.
- Chen, J. L. and C. W. Greider (2003). Determinants in mammalian telomerase RNA that mediate enzyme processivity and cross-species incompatibility. *Embo J.* **22**(2): 304-14.
- Cheung, J. and D. F. Smith (2000). Molecular chaperone interactions with steroid receptors: an update. *Mol. Endocrinol.* **14**(7): 939-46.
- Chiosis, G., M. Vilenchik, J. Kim and D. Solit (2004). Hsp90: the vulnerable chaperone. *Drug Discov. Today* **9**(20): 881-8.
- Cho, J. and R. R. Rando (2000). Specific binding of Hoechst 33258 to site 1 thymidylate synthase mRNA. *Nucleic Acids Res.* **28**(10): 2158-63.
- Ciocca, D. R., G. M. Clark, A. K. Tandon, S. A. Fuqua, W. J. Welch and W. L. McGuire (1993). Heat shock protein hsp70 in patients with axillary lymph node-negative breast cancer: prognostic implications. *J. Natl. Cancer Inst.* **85**(7): 570-4.
- Colgin, L. M., C. Wilkinson, A. Englezou, A. Kilian, M. O. Robinson and R. R. Reddel (2000). The hTERT $\alpha$  splice variant is a dominant negative inhibitor of telomerase activity. *Neoplasia* **2**: 426-432.

- Colgin, L. M., K. Baran, P. Baumann, T. R. Cech and R. R. Reddel (2003). Human POT1 facilitates telomere elongation by telomerase. *Curr. Biol.* **13**(11): 942-6.
- Collins, K. and J. R. Mitchell (2002). Telomerase in the human organism. *Oncogene* **21**(4): 564-79.
- Cong, Y. S., J. Wen and S. Bacchetti (1999). The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter. *Hum. Mol. Genet.* **8**(1): 137-42.
- Cong, Y. S. and S. Bacchetti (2000). Histone deacetylation is involved in the transcriptional repression of hTERT in normal human cells. *J. Biol. Chem.* **275**(46): 35665-8.
- Cong, Y. S., W. E. Wright and J. W. Shay (2002). Human telomerase and its regulation. *Microbiol. Mol. Biol. Rev.* **66**(3): 407-25.
- Conroy, S. E., P. D. Sasieni, I. Fentiman and D. S. Latchman (1998). Autoantibodies to the 90kDa heat shock protein and poor survival in breast cancer patients. *Eur. J. Cancer* **34**(6): 942-3.
- Corey, D. R. (2002). Telomerase inhibition, oligonucleotides, and clinical trials. *Oncogene* **21**(4): 631-7.
- Crowe, D. L. and D. C. Nguyen (2001). Rb and E2F-1 regulate telomerase activity in human cancer cells. *Biochim. Biophys. Acta* **1518**(1-2): 1-6.
- Csermely, P. and C. R. Kahn (1991). The 90-kDa heat shock protein (hsp-90) possesses an ATP binding site and autophosphorylating activity. *J. Biol. Chem.* **266**(8): 4943-50.
- Cuesta, J., M. A. Read and S. Neidle (2003). The design of G-quadruplex ligands as telomerase inhibitors. *Mini Rev. Med. Chem.* **3**(1): 11-21.
- Damm, K., U. Hemmann, P. Garin-Chesa, N. Hael, I. Kauffmann, H. Priepke, C. Niestroj, C. Daiber, B. Enenkel, B. Guilliard, I. Lauritsch, E. Muller, E. Pascolo, G. Sauter, M. Pantic, U. M. Martens, C. Wenz, J. Lingner, N. Kraut, W. J. Rettig and A. Schnapp (2001). A highly selective telomerase inhibitor limiting human cancer cell proliferation. *Embo J.* **20**(24): 6958-68.
- Darimont, C., O. Avanti, Y. Tromvoukis, P. Vautravers-Leone, N. Kurihara, G. D. Roodman, L. M. Colgin, H. Tullberg-Reinert, A. M. Pfeifer, E. A. Offord and K. Mace (2002). SV40 T antigen and telomerase are required to obtain immortalized human adult bone cells without loss of the differentiated phenotype. *Cell Growth Differ.* **13**(2): 59-67.



- Dassonneville, L., F. Hamy, P. Colson, C. Houssier and C. Bailly (1997). Binding of Hoechst 33258 to the TAR RNA of HIV-1. Recognition of a pyrimidine bulge-dependent structure. *Nucleic Acids Res.* **25**(22): 4487-92.
- de Lange, T. (2002). Protection of mammalian telomeres. *Oncogene* **21**(4): 532-40.
- DePinho, R. A., N. Schreiber-Agus and F. W. Alt (1991). myc family oncogenes in the development of normal and neoplastic cells. *Adv. Cancer Res.* **57**: 1-46.
- Djojosebroto, M. W., A. C. Chin, N. Go, S. Schaetzlein, M. P. Manns, S. Gryaznov, C. B. Harley and K. L. Rudolph (2005). Telomerase antagonists GRN163 and GRN163L inhibit tumor growth and increase chemosensitivity of human hepatoma. *Hepatology*.
- Dominick, P. K., B. R. Keppler, J. D. Legassie, I. K. Moon and M. B. Jarstfer (2004). Nucleic acid-binding ligands identify new mechanisms to inhibit telomerase. *Bioorg. Med. Chem. Lett.* **14**(13): 3467-71.
- Duan, W., A. Rangan, H. Vankayalapati, M. Y. Kim, Q. Zeng, D. Sun, H. Han, O. Y. Fedoroff, D. Nishioka, S. Y. Rha, E. Izbicka, D. D. Von Hoff and L. H. Hurley (2001). Design and synthesis of fluoroquinophenoxazines that interact with human telomeric G-quadruplexes and their biological effects. *Mol. Cancer Ther.* **1**(2): 103-20.
- Dutta, R. and M. Inouye (2000). GHKL, an emergent ATPase/kinase superfamily. *Trends Biochem. Sci.* **25**(1): 24-8.
- Eckstein, F. (2000). Phosphorothioate oligodeoxynucleotides: what is their origin and what is unique about them? *Antisense Nucleic Acid Drug Dev.* **10**(2): 117-21.
- Eickbush, T. H. (1997). Telomerase and retrotransposons: which came first? *Science* **277**(5328): 911-2.
- Elayadi, A. N., A. Demieville, E. V. Wancewicz, B. P. Monia and D. R. Corey (2001). Inhibition of telomerase by 2'-O-(2-methoxyethyl) RNA oligomers: effect of length, phosphorothioate substitution and time inside cells. *Nucleic Acids Res.* **29**(8): 1683-9.
- Emili, A., J. Greenblatt and C. J. Ingles (1994). Species-specific interaction of the glutamine-rich activation domains of Sp1 with the TATA box-binding protein. *Mol. Cell. Biol.* **14**(3): 1582-93.
- Etheridge, K. T., S. S. Banik, B. N. Armbruster, Y. Zhu, R. M. Terns, M. P. Terns and C. M. Counter (2002). The nucleolar localization domain of the catalytic subunit of human telomerase. *J. Biol. Chem.* **277**(27): 24764-70.

- Evans, S. K. and V. Lundblad (1999). Est1 and Cdc13 as comediators of telomerase access. *Science* **286**(5437): 117-20.
- Evans, S. K. and V. Lundblad (2000). Positive and negative regulation of telomerase access to the telomere. *J. Cell Sci.* **113 Pt 19**: 3357-64.
- Fang, Y., A. E. Fliss, J. Rao and A. J. Caplan (1998). SBA1 encodes a yeast hsp90 cochaperone that is homologous to vertebrate p23 proteins. *Mol. Cell. Biol.* **18**(7): 3727-34.
- Fedoroff, O. Y., M. Salazar, H. Han, V. V. Chemeris, S. M. Kerwin and L. H. Hurley (1998). NMR-Based model of a telomerase-inhibiting compound bound to G-quadruplex DNA. *Biochemistry* **37**(36): 12367-74.
- Feng, J., W. D. Funk, S. S. Wang, S. L. Weinrich, A. A. Avilion, C. P. Chiu, R. R. Adams, E. Chang, R. C. Allsopp, J. Yu and et al. (1995). The RNA component of human telomerase. *Science* **269**(5228): 1236-41.
- Filipowsky, M. E., M. L. Kopka, M. Brazil-Zison, J. W. Lown and R. E. Dickerson (1996). Linked lexitropsins and the in vitro inhibition of HIV-1 reverse transcriptase RNA-directed DNA polymerization: a novel induced-fit of 3,5 m-pyridyl bisdistamycin to enzyme-associated template-primer. *Biochemistry* **35**(48): 15397-410.
- Forsythe, H. L., J. L. Jarvis, J. W. Turner, L. W. Elmore and S. E. Holt (2001). Stable association of hsp90 and p23, but Not hsp70, with active human telomerase. *J. Biol. Chem.* **276**(19): 15571-4.
- Fortugno, P., E. Beltrami, J. Plescia, J. Fontana, D. Pradhan, P. C. Marchisio, W. C. Sessa and D. C. Altieri (2003). Regulation of survivin function by Hsp90. *Proc. Natl. Acad. Sci. U S A* **100**(24): 13791-6.
- Francis, R. and S. H. Friedman (2002). A rapid direct telomerase assay method using 96-well streptavidin plates. *Biotechniques* **32**(5): 1154-6, 1158, 1160.
- Francis, R. and S. H. Friedman (2003). An interference-free fluorescent assay of telomerase for the high-throughput analysis of inhibitors. *Anal. Biochem.* **323**(1): 65-73.
- Freitag, D. G., P. M. Ouimet, T. L. Girvitz and M. Kapoor (1997). Heat shock protein 80 of *Neurospora crassa*, a cytosolic molecular chaperone of the eukaryotic stress 90 family, interacts directly with heat shock protein 70. *Biochemistry* **36**(33): 10221-9.
- Fu, D. and K. Collins (2003). Distinct biogenesis pathways for human telomerase RNA and H/ACA small nucleolar RNAs. *Mol. Cell* **11**(5): 1361-72.

- Garnier, C., D. Lafitte, P. O. Tsvetkov, P. Barbier, J. Leclerc-Devin, J. M. Millot, C. Briand, A. A. Makarov, M. G. Catelli and V. Peyrot (2002). Binding of ATP to heat shock protein 90: evidence for an ATP-binding site in the C-terminal domain. *J. Biol. Chem.* **277**(14): 12208-14.
- Gewin, L. and D. A. Galloway (2001). E box-dependent activation of telomerase by human papillomavirus type 16 E6 does not require induction of c-myc. *J. Virol.* **75**(15): 7198-201.
- Glukhov, A. I., O. V. Zimnik, S. A. Gordeev and S. E. Severin (1998). Inhibition of telomerase activity of melanoma cells in vitro by antisense oligonucleotides. *Biochem. Biophys. Res. Commun.* **248**(2): 368-71.
- Goetz, M. P., D. O. Toft, M. M. Ames and C. Erlichman (2003). The Hsp90 chaperone complex as a novel target for cancer therapy. *Ann. Oncol.* **14**(8): 1169-76.
- Gomez, D. E., A. M. Tejera and O. A. Olivero (1998). Irreversible telomere shortening by 3'-azido-2',3'-dideoxythymidine (AZT) treatment. *Biochem. Biophys. Res. Commun.* **246**(1): 107-10.
- Gowan, S. M., R. Heald, M. F. Stevens and L. R. Kelland (2001). Potent inhibition of telomerase by small-molecule pentacyclic acridines capable of interacting with G-quadruplexes. *Mol. Pharmacol.* **60**(5): 981-8.
- Gowan, S. M., J. R. Harrison, L. Patterson, M. Valenti, M. A. Read, S. Neidle and L. R. Kelland (2002). A G-quadruplex-interactive potent small-molecule inhibitor of telomerase exhibiting in vitro and in vivo antitumor activity. *Mol. Pharmacol.* **61**(5): 1154-62.
- Grandori, C., S. M. Cowley, L. P. James and R. N. Eisenman (2000). The Myc/Max/Mad network and the transcriptional control of cell behavior. *Annu. Rev. Cell Dev. Biol.* **16**: 653-99.
- Granger, M. P., W. E. Wright and J. W. Shay (2002). Telomerase in cancer and aging. *Crit. Rev. Oncol. Hematol.* **41**(1): 29-40.
- Greenberg, R. A., R. C. O'Hagan, H. Deng, Q. Xiao, S. R. Hann, R. R. Adams, S. Lichtsteiner, L. Chin, G. B. Morin and R. A. DePinho (1999). Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation. *Oncogene* **18**(5): 1219-26.
- Grenert, J. P., W. P. Sullivan, P. Fadden, T. A. Haystead, J. Clark, E. Mimnaugh, H. Krutzsch, H. J. Ochel, T. W. Schulte, E. Sausville, L. M. Neckers and D. O. Toft (1997). The amino-terminal domain of heat shock protein 90 (hsp90) that binds

- geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. *J. Biol. Chem.* **272**(38): 23843-50.
- Grenert, J. P., B. D. Johnson and D. O. Toft (1999). The importance of ATP binding and hydrolysis by hsp90 in formation and function of protein heterocomplexes. *J. Biol. Chem.* **274**(25): 17525-33.
- Griffith, J., A. Bianchi and T. de Lange (1998). TRF1 promotes parallel pairing of telomeric tracts in vitro. *J. Mol. Biol.* **278**(1): 79-88.
- Griffith, J. D., L. Comeau, S. Rosenfield, R. M. Stansel, A. Bianchi, H. Moss and T. de Lange (1999). Mammalian telomeres end in a large duplex loop. *Cell* **97**(4): 503-14.
- Gunes, C., S. Lichtsteiner, A. P. Vasserot and C. Englert (2000). Expression of the hTERT gene is regulated at the level of transcriptional initiation and repressed by Mad1. *Cancer Res.* **60**(8): 2116-21.
- Guo, C., B. N. Armbruster, D. T. Price and C. M. Counter (2003). In vivo regulation of hTERT expression and telomerase activity by androgen. *J. Urol.* **170**(2 Pt 1): 615-8.
- Haendeler, J., J. Hoffmann, S. Rahman, A. M. Zeiher and S. Dimmeler (2003). Regulation of telomerase activity and anti-apoptotic function by protein-protein interaction and phosphorylation. *FEBS Lett.* **536**(1-3): 180-6.
- Hahn, W. C., S. A. Stewart, M. W. Brooks, S. G. York, E. Eaton, A. Kurachi, R. L. Beijersbergen, J. H. Knoll, M. Meyerson and R. A. Weinberg (1999). Inhibition of telomerase limits the growth of human cancer cells. *Nat. Med.* **5**(10): 1164-70.
- Hamilton, S. E., C. G. Simmons, I. S. Kathiriya and D. R. Corey (1999). Cellular delivery of peptide nucleic acids and inhibition of human telomerase. *Chem. Biol.* **6**(6): 343-51.
- Han, H., C. L. Cliff and L. H. Hurley (1999). Accelerated assembly of G-quadruplex structures by a small molecule. *Biochemistry* **38**(22): 6981-6.
- Han, H., D. R. Langle, A. Rangan and L. H. Hurley (2001). Selective interactions of cationic porphyrins with G-quadruplex structures. *J. Am. Chem. Soc.* **123**(37): 8902-13.
- Hao, Z. M., J. Y. Luo, J. Cheng, L. Li, D. He, Q. Y. Wang and G. X. Yang (2005). Intensive Inhibition of hTERT Expression by a Ribozyme Induces Rapid Apoptosis of Cancer Cells through a Telomere Length-Independent Pathway. *Cancer Biol. Ther.* **4**(10).

- Harbour, J. W. and D. C. Dean (2000). Chromatin remodeling and Rb activity. *Curr. Opin. Cell Biol.* **12**(6): 685-9.
- Harrington, L., W. Zhou, T. McPhail, R. Oulton, D. S. Yeung, V. Mar, M. B. Bass and M. O. Robinson (1997). Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes Dev.* **11**(23): 3109-15.
- Harrington, L. (2003). Biochemical aspects of telomerase function. *Cancer Lett.* **194**(2): 139-54.
- Harrison, R. J., S. M. Gowan, L. R. Kelland and S. Neidle (1999). Human telomerase inhibition by substituted acridine derivatives. *Bioorg. Med. Chem. Lett.* **9**(17): 2463-8.
- Hasaniya, N., K. Youn, M. Xu, J. Hernaez and R. Dashwood (1997). Inhibitory activity of green and black tea in a free radical-generating system using 2-amino-3-methylimidazo[4,5-f]quinoline as substrate. *Jpn. J. Cancer Res.* **88**(6): 553-8.
- Hayakawa, N., K. Nozawa, A. Ogawa, N. Kato, K. Yoshida, K. Akamatsu, M. Tsuchiya, A. Nagasaka and S. Yoshida (1999). Isothiazolone derivatives selectively inhibit telomerase from human and rat cancer cells in vitro. *Biochemistry* **38**(35): 11501-7.
- Henderson, Y. C., R. L. Breau, T. J. Liu and G. L. Clayman (2000). Telomerase activity in head and neck tumors after introduction of wild-type p53, p21, p16, and E2F-1 genes by means of recombinant adenovirus. *Head Neck* **22**(4): 347-54.
- Hernandez, M. P., A. Chadli and D. O. Toft (2002). HSP40 binding is the first step in the HSP90 chaperoning pathway for the progesterone receptor. *J. Biol. Chem.* **277**(14): 11873-81.
- Hickey, E., S. E. Brandon, S. Sadis, G. Smale and L. A. Weber (1986). Molecular cloning of sequences encoding the human heat-shock proteins and their expression during hyperthermia. *Gene* **43**(1-2): 147-54.
- Hoey, T., R. O. Weinzierl, G. Gill, J. L. Chen, B. D. Dynlacht and R. Tjian (1993). Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. *Cell* **72**(2): 247-60.
- Hollstein, M., K. Rice, M. S. Greenblatt, T. Soussi, R. Fuchs, T. Sorlie, E. Hovig, B. Smith-Sorensen, R. Montesano and C. C. Harris (1994). Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic Acids Res.* **22**(17): 3551-5.
- Holt, S. E., D. L. Aisner, J. Baur, V. M. Tesmer, M. Dy, M. Ouellette, J. B. Trager, G. B. Morin, D. O. Toft, J. W. Shay, W. E. Wright and M. A. White (1999). Functional

- requirement of p23 and Hsp90 in telomerase complexes. *Genes Dev.* **13**(7): 817-26.
- Horikawa, I., P. L. Cable, C. Afshari and J. C. Barrett (1999). Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. *Cancer Res.* **59**(4): 826-30.
- Hostein, I., D. Robertson, F. DiStefano, P. Workman and P. A. Clarke (2001). Inhibition of signal transduction by the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin results in cytostasis and apoptosis. *Cancer Res.* **61**(10): 4003-9.
- Hou, M., X. Wang, N. Popov, A. Zhang, X. Zhao, R. Zhou, A. Zetterberg, M. Bjorkholm, M. Henriksson, A. Gruber and D. Xu (2002). The histone deacetylase inhibitor trichostatin A derepresses the telomerase reverse transcriptase (hTERT) gene in human cells. *Exp. Cell Res.* **274**(1): 25-34.
- Hu, J. and C. Seeger (1996). Hsp90 is required for the activity of a hepatitis B virus reverse transcriptase. *Proc. Natl. Acad. Sci. U S A* **93**(3): 1060-4.
- Hutchison, K. A., K. D. Dittmar and W. B. Pratt (1994). All of the factors required for assembly of the glucocorticoid receptor into a functional heterocomplex with heat shock protein 90 are preassociated in a self-sufficient protein folding structure, a "foldosome". *J. Biol. Chem.* **269**(45): 27894-9.
- Iannotti, A. M., D. A. Rabideau and J. J. Dougherty (1988). Characterization of purified avian 90,000-Da heat shock protein. *Arch. Biochem. Biophys.* **264**(1): 54-60.
- Ishii, Y., N. Tsuyama, S. Maeda, H. Tahara and T. Ide (1999). Telomerase activity in hybrids between telomerase-negative and telomerase-positive immortal human cells is repressed in the different complementation groups but not in the same complementation group of immortality. *Mech. Ageing Dev.* **110**(3): 175-93.
- Ito, H., S. Kyo, T. Kanaya, M. Takakura, M. Inoue and M. Namiki (1998). Expression of human telomerase subunits and correlation with telomerase activity in urothelial cancer. *Clin. Cancer Res.* **4**(7): 1603-8.
- Jacobs, S. A., E. R. Podell, D. S. Wuttke and T. R. Cech (2005). Soluble domains of telomerase reverse transcriptase identified by high-throughput screening. *Protein Sci.* **14**(8): 2051-8.
- Jaiswal, R. K., E. Weissinger, W. Kolch and G. E. Landreth (1996). Nerve growth factor-mediated activation of the mitogen-activated protein (MAP) kinase cascade involves a signaling complex containing B-Raf and HSP90. *J. Biol. Chem.* **271**(39): 23626-9.

- Jameel, A., R. A. Skilton, T. A. Campbell, S. K. Chander, R. C. Coombes and Y. A. Luqmani (1992). Clinical and biological significance of HSP89 alpha in human breast cancer. *Int. J. Cancer* **50**(3): 409-15.
- Jankun, J., S. H. Selman, R. Swiercz and E. Skrzypczak-Jankun (1997). Why drinking green tea could prevent cancer. *Nature* **387**(6633): 561.
- Jarstfer, M. B. and T. R. Cech (2002). Effects of nucleotide analogues on Euplotes aediculatus telomerase processivity: evidence for product-assisted translocation. *Biochemistry* **41**(1): 151-61.
- Johnson, J. L. and D. O. Toft (1994). A novel chaperone complex for steroid receptors involving heat shock proteins, immunophilins, and p23. *J. Biol. Chem.* **269**(40): 24989-93.
- Johnson, J. L. and D. O. Toft (1995). Binding of p23 and hsp90 during assembly with the progesterone receptor. *Mol. Endocrinol.* **9**(6): 670-8.
- Jolly, C. and R. I. Morimoto (2000). Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *J. Natl. Cancer Inst.* **92**(19): 1564-72.
- Kanaya, T., S. Kyo, M. Takakura, H. Ito, M. Namiki and M. Inoue (1998). hTERT is a critical determinant of telomerase activity in renal-cell carcinoma. *Int. J. Cancer* **78**(5): 539-43.
- Kanaya, T., S. Kyo, K. Hamada, M. Takakura, Y. Kitagawa, H. Harada and M. Inoue (2000). Adenoviral expression of p53 represses telomerase activity through down-regulation of human telomerase reverse transcriptase transcription. *Clin. Cancer Res.* **6**(4): 1239-47.
- Kanelakis, K. C., D. S. Shewach and W. B. Pratt (2002). Nucleotide binding states of hsp70 and hsp90 during sequential steps in the process of glucocorticoid receptor.hsp90 heterocomplex assembly. *J. Biol. Chem.* **277**(37): 33698-703.
- Kang, S. S., T. Kwon, D. Y. Kwon and S. I. Do (1999). Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J. Biol. Chem.* **274**(19): 13085-90.
- Karlseder, J., A. Smogorzewska and T. de Lange (2002). Senescence induced by altered telomere state, not telomere loss. *Science* **295**(5564): 2446-9.
- Kelland, L. R., S. Y. Sharp, P. M. Rogers, T. G. Myers and P. Workman (1999). DT-Diaphorase expression and tumor cell sensitivity to 17-allylamino, 17-demethoxygeldanamycin, an inhibitor of heat shock protein 90. *J. Natl. Cancer Inst.* **91**(22): 1940-9.

- Keppeler, B. R. and M. B. Jarstfer (2004). Inhibition of telomerase activity by preventing proper assemblage. *Biochemistry* **43**(2): 334-43.
- Kha, H., W. Zhou, K. Chen, B. Karan-Tamir, T. San Miguel, L. Zeni, K. Kearns, A. Mladenovic, B. Rasnow, M. Robinson and R. C. Wahl (2004). A telomerase enzymatic assay that does not use polymerase chain reaction, radioactivity, or electrophoresis. *Anal. Biochem.* **331**(2): 230-4.
- Kharbanda, S., V. Kumar, S. Dhar, P. Pandey, C. Chen, P. Majumder, Z. M. Yuan, Y. Whang, W. Strauss, T. K. Pandita, D. Weaver and D. Kufe (2000). Regulation of the hTERT telomerase catalytic subunit by the c-Abl tyrosine kinase. *Curr. Biol.* **10**(10): 568-75.
- Kilian, A., D. D. Bowtell, H. E. Abud, G. R. Hime, D. J. Venter, P. K. Keese, E. L. Duncan, R. R. Reddel and R. A. Jefferson (1997). Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum. Mol. Genet.* **6**(12): 2011-9.
- Kim, J. H., S. M. Park, M. R. Kang, S. Y. Oh, T. H. Lee, M. T. Muller and I. K. Chung (2005). Ubiquitin ligase MKRN1 modulates telomere length homeostasis through a proteolysis of hTERT. *Genes Dev.* **19**(7): 776-81.
- Kim, M. Y., H. Vankayalapati, K. Shin-Ya, K. Wierzba and L. H. Hurley (2002). Telomestatin, a potent telomerase inhibitor that interacts quite specifically with the human telomeric intramolecular g-quadruplex. *J. Am. Chem. Soc.* **124**(10): 2098-9.
- Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich and J. W. Shay (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**(5193): 2011-5.
- Kim, N. W. and F. Wu (1997). Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res.* **25**(13): 2595-7.
- Kimmins, S. and T. H. MacRae (2000). Maturation of steroid receptors: an example of functional cooperation among molecular chaperones and their associated proteins. *Cell Stress Chaperones* **5**(2): 76-86.
- Klingelhutz, A. J., S. A. Foster and J. K. McDougall (1996). Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature* **380**(6569): 79-82.
- Koeppel, F., J. F. Riou, A. Laoui, P. Mailliet, P. B. Arimondo, D. Labit, O. Petitgenet, C. Helene and J. L. Mergny (2001). Ethidium derivatives bind to G-quartets, inhibit



- telomerase and act as fluorescent probes for quadruplexes. *Nucleic Acids Res.* **29**(5): 1087-96.
- Koide, A., S. Abbatiello, L. Rothgery and S. Koide (2002). Probing protein conformational changes in living cells by using designer binding proteins: application to the estrogen receptor. *Proc. Natl. Acad. Sci. U S A* **99**(3): 1253-8.
- Kondo, S., Y. Kondo, G. Li, R. H. Silverman and J. K. Cowell (1998). Targeted therapy of human malignant glioma in a mouse model by 2-5A antisense directed against telomerase RNA. *Oncogene* **16**(25): 3323-30.
- Konigsberg, W. H. (1995). Limited proteolysis of DNA polymerases as probe of functional domains. *Methods Enzymol.* **262**: 331-46.
- Ku, W. C., A. J. Cheng and T. C. Wang (1997). Inhibition of telomerase activity by PKC inhibitors in human nasopharyngeal cancer cells in culture. *Biochem. Biophys. Res. Commun.* **241**(3): 730-6.
- Kusumoto, M., T. Ogawa, K. Mizumoto, H. Ueno, H. Niiyama, N. Sato, M. Nakamura and M. Tanaka (1999). Adenovirus-mediated p53 gene transduction inhibits telomerase activity independent of its effects on cell cycle arrest and apoptosis in human pancreatic cancer cells. *Clin. Cancer Res.* **5**(8): 2140-7.
- Kyo, S., M. Takakura, T. Kanaya, W. Zhuo, K. Fujimoto, Y. Nishio, A. Orimo and M. Inoue (1999). Estrogen activates telomerase. *Cancer Res.* **59**(23): 5917-21.
- Kyo, S., M. Takakura, T. Taira, T. Kanaya, H. Itoh, M. Yutsudo, H. Ariga and M. Inoue (2000). Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). *Nucleic Acids Res.* **28**(3): 669-77.
- Lai, B. T., N. W. Chin, A. E. Stanek, W. Keh and K. W. Lanks (1984). Quantitation and intracellular localization of the 85K heat shock protein by using monoclonal and polyclonal antibodies. *Mol. Cell. Biol.* **4**(12): 2802-10.
- Lee, A. R., W. L. Wu, W. L. Chang, H. C. Lin and M. L. King (1987). best n and bioactivity of new tanshinones. *J. Nat. Prod.* **50**(2): 157-60.
- Leeper, T. C. and G. Varani (2005). The structure of an enzyme-activating fragment of human telomerase RNA. *RNA* **11**(4): 394-403.
- Lei, M., A. J. Zaug, E. R. Podell and T. R. Cech (2005). Switching human telomerase on and off with hPOT1 protein in vitro. *J. Biol. Chem.* **280**(21): 20449-56.
- Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell* **88**(3): 323-31.

- Lewis, J., A. Devin, A. Miller, Y. Lin, Y. Rodriguez, L. Neckers and Z. G. Liu (2000). Disruption of hsp90 function results in degradation of the death domain kinase, receptor-interacting protein (RIP), and blockage of tumor necrosis factor-induced nuclear factor-kappaB activation. *J. Biol. Chem.* **275**(14): 10519-26.
- Li, H., L. L. Zhao, J. W. Funder and J. P. Liu (1997). Protein phosphatase 2A inhibits nuclear telomerase activity in human breast cancer cells. *J. Biol. Chem.* **272**(27): 16729-32.
- Li, H., L. Zhao, Z. Yang, J. W. Funder and J. P. Liu (1998). Telomerase is controlled by protein kinase Calpha in human breast cancer cells. *J. Biol. Chem.* **273**(50): 33436-42.
- Liao, S. and R. A. Hiipakka (1995). Selective inhibition of steroid 5 alpha-reductase isozymes by tea epicatechin-3-gallate and epigallocatechin-3-gallate. *Biochem. Biophys. Res. Commun.* **214**(3): 833-8.
- Lin, S. Y. and S. J. Elledge (2003). Multiple tumor suppressor pathways negatively regulate telomerase. *Cell* **113**(7): 881-9.
- Lin, Y. L. and J. K. Lin (1997). (-)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-kappaB. *Mol. Pharmacol.* **52**(3): 465-72.
- Lingner, J., L. L. Hendrick and T. R. Cech (1994). Telomerase RNAs of different ciliates have a common secondary structure and a permuted template. *Genes Dev.* **8**(16): 1984-98.
- Liu, K., M. M. Schoonmaker, B. L. Levine, C. H. June, R. J. Hodes and N. P. Weng (1999). Constitutive and regulated expression of telomerase reverse transcriptase (hTERT) in human lymphocytes. *Proc. Natl. Acad. Sci. U S A* **96**(9): 5147-52.
- Liu, K., R. J. Hodes and N. Weng (2001). Cutting edge: telomerase activation in human T lymphocytes does not require increase in telomerase reverse transcriptase (hTERT) protein but is associated with hTERT phosphorylation and nuclear translocation. *J. Immunol.* **166**(8): 4826-30.
- Loayza, D. and T. De Lange (2003). POT1 as a terminal transducer of TRF1 telomere length control. *Nature* **423**(6943): 1013-8.
- Ly, H., E. H. Blackburn and T. G. Parslow (2003a). Comprehensive structure-function analysis of the core domain of human telomerase RNA. *Mol. Cell. Biol.* **23**(19): 6849-56.

- Ly, H., L. Xu, M. A. Rivera, T. G. Parslow and E. H. Blackburn (2003b). A role for a novel 'trans-pseudoknot' RNA-RNA interaction in the functional dimerization of human telomerase. *Genes Dev.* **17**(9): 1078-83.
- Lyu, S. Y., S. H. Choi and W. B. Park (2002). Korean mistletoe lectin-induced apoptosis in hepatocarcinoma cells is associated with inhibition of telomerase via mitochondrial controlled pathway independent of p53. *Arch. Pharm. Res.* **25**(1): 93-101.
- Machida, H., Y. Matsumoto, M. Shirai and N. Kubota (2003). Geldanamycin, an inhibitor of Hsp90, sensitizes human tumour cells to radiation. *Int. J. Radiat. Biol.* **79**(12): 973-80.
- Marcu, M. G., A. Chadli, I. Bouhouche, M. Catelli and L. M. Neckers (2000a). The heat shock protein 90 antagonist novobiocin interacts with a previously unrecognized ATP-binding domain in the carboxyl terminus of the chaperone. *J. Biol. Chem.* **275**(47): 37181-6.
- Marcu, M. G., T. W. Schulte and L. Neckers (2000b). Novobiocin and related coumarins and depletion of heat shock protein 90-dependent signaling proteins. *J. Natl. Cancer Inst.* **92**(3): 242-8.
- Martin-Rivera, L. and M. A. Blasco (2001). Identification of functional domains and dominant negative mutations in vertebrate telomerase RNA using an in vivo reconstitution system. *J. Biol. Chem.* **276**(8): 5856-65.
- Maruya, M., M. Sameshima, T. Nemoto and I. Yahara (1999). Monomer arrangement in HSP90 dimer as determined by decoration with N and C-terminal region specific antibodies. *J. Mol. Biol.* **285**(3): 903-7.
- Masutomi, K. and W. C. Hahn (2003). Telomerase and tumorigenesis. *Cancer Lett.* **194**(2): 163-72.
- Masutomi, K., E. Y. Yu, S. Khurts, I. Ben-Porath, J. L. Currier, G. B. Metz, M. W. Brooks, S. Kaneko, S. Murakami, J. A. DeCaprio, R. A. Weinberg, S. A. Stewart and W. C. Hahn (2003). Telomerase maintains telomere structure in normal human cells. *Cell* **114**(2): 241-53.
- Matts, R. L. and R. Hurst (1989). Evidence for the association of the heme-regulated eIF-2 alpha kinase with the 90-kDa heat shock protein in rabbit reticulocyte lysate in situ. *J. Biol. Chem.* **264**(26): 15542-7.
- Maxam, A. M. and W. Gilbert (1977). A new method for sequencing DNA. *Proc. Natl. Acad. Sci. U S A* **74**(2): 560-4.

- McDonough, H. and C. Patterson (2003). CHIP: a link between the chaperone and proteasome systems. *Cell Stress Chaperones* **8**(4): 303-8.
- McElligott, R. and R. J. Wellinger (1997). The terminal DNA structure of mammalian chromosomes. *Embo J.* **16**(12): 3705-14.
- McLaughlin, S. H., H. W. Smith and S. E. Jackson (2002). Stimulation of the weak ATPase activity of human hsp90 by a client protein. *J. Mol. Biol.* **315**(4): 787-98.
- Melana, S. M., J. F. Holland and B. G. Pogo (1998). Inhibition of cell growth and telomerase activity of breast cancer cells in vitro by 3'-azido-3'-deoxythymidine. *Clin. Cancer Res.* **4**(3): 693-6.
- Meyer, P., C. Prodromou, B. Hu, C. Vaughan, S. M. Roe, B. Panaretou, P. W. Piper and L. H. Pearl (2003). Structural and functional analysis of the middle segment of hsp90: implications for ATP hydrolysis and client protein and cochaperone interactions. *Mol. Cell* **11**(3): 647-58.
- Meyerson, M., C. M. Counter, E. N. Eaton, L. W. Ellisen, P. Steiner, S. D. Caddle, L. Ziaugra, R. L. Beijersbergen, M. J. Davidoff, Q. Liu, S. Bacchetti, D. A. Haber and R. A. Weinberg (1997). hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell* **90**(4): 785-95.
- Minami, Y., H. Kawasaki, Y. Miyata, K. Suzuki and I. Yahara (1991). Analysis of native forms and isoform compositions of the mouse 90-kDa heat shock protein, HSP90. *J. Biol. Chem.* **266**(16): 10099-103.
- Minamino, T., S. A. Mitsialis and S. Kourembanas (2001). Hypoxia extends the life span of vascular smooth muscle cells through telomerase activation. *Mol. Cell. Biol.* **21**(10): 3336-42.
- Misiti, S., S. Nanni, G. Fontemaggi, Y. S. Cong, J. Wen, H. W. Hirte, G. Piaggio, A. Sacchi, A. Pontecorvi, S. Bacchetti and A. Farsetti (2000). Induction of hTERT expression and telomerase activity by estrogens in human ovary epithelium cells. *Mol. Cell. Biol.* **20**(11): 3764-71.
- Mitchell, J. R., J. Cheng and K. Collins (1999). A box H/ACA small nucleolar RNA-like domain at the human telomerase RNA 3' end. *Mol. Cell. Biol.* **19**(1): 567-76.
- Mitchell, J. R. and K. Collins (2000). Human telomerase activation requires two independent interactions between telomerase RNA and telomerase reverse transcriptase. *Mol. Cell* **6**(2): 361-71.
- Mokbel, K. (2003). The evolving role of telomerase inhibitors in the treatment of cancer. *Curr. Med. Res. Opin.* **19**(6): 470-2.

- Monia, B. P., E. A. Lesnik, C. Gonzalez, W. F. Lima, D. McGee, C. J. Guinasso, A. M. Kawasaki, P. D. Cook and S. M. Freier (1993). Evaluation of 2'-modified oligonucleotides containing 2'-deoxy gaps as antisense inhibitors of gene expression. *J. Biol. Chem.* **268**(19): 14514-22.
- Morishima, Y., K. C. Kanelakis, P. J. Murphy, D. S. Shewach and W. B. Pratt (2001). Evidence for iterative ratcheting of receptor-bound hsp70 between its ATP and ADP conformations during assembly of glucocorticoid receptor.hsp90 heterocomplexes. *Biochemistry* **40**(4): 1109-16.
- Munster, P. N., M. Srethapakdi, M. M. Moasser and N. Rosen (2001). Inhibition of heat shock protein 90 function by ansamycins causes the morphological and functional differentiation of breast cancer cells. *Cancer Res.* **61**(7): 2945-52.
- Murakami, J., N. Nagai, K. Shigemasa and K. Ohama (1999). Inhibition of telomerase activity and cell proliferation by a reverse transcriptase inhibitor in gynaecological cancer cell lines. *Eur. J. Cancer* **35**(6): 1027-34.
- Murphy, M., J. Ahn, K. K. Walker, W. H. Hoffman, R. M. Evans, A. J. Levine and D. L. George (1999). Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. *Genes Dev.* **13**(19): 2490-501.
- Murphy, P. J., K. C. Kanelakis, M. D. Galigniana, Y. Morishima and W. B. Pratt (2001). Stoichiometry, abundance, and functional significance of the hsp90/hsp70-based multiprotein chaperone machinery in reticulocyte lysate. *J. Biol. Chem.* **276**(32): 30092-8.
- Naasani, I., H. Seimiya and T. Tsuruo (1998). Telomerase inhibition, telomere shortening, and senescence of cancer cells by tea catechins. *Biochem. Biophys. Res. Commun.* **249**(2): 391-6.
- Naasani, I., H. Seimiya, T. Yamori and T. Tsuruo (1999). FJ5002: a potent telomerase inhibitor identified by exploiting the disease-oriented screening program with COMPARE analysis. *Cancer Res.* **59**(16): 4004-11.
- Nakamura, T. M., G. B. Morin, K. B. Chapman, S. L. Weinrich, W. H. Andrews, J. Lingner, C. B. Harley and T. R. Cech (1997). Telomerase catalytic subunit homologs from fission yeast and human. *Science* **277**(5328): 955-9.
- Nakamura, T. M. and T. R. Cech (1998). Reversing time: origin of telomerase. *Cell* **92**(5): 587-90.
- Nakayama, J., H. Tahara, E. Tahara, M. Saito, K. Ito, H. Nakamura, T. Nakanishi, T. Ide and F. Ishikawa (1998). Telomerase activation by hTERT in human normal fibroblasts and hepatocellular carcinomas. *Nat. Genet.* **18**(1): 65-8.

- Nakayama, Y., H. Sakamoto, K. Satoh and T. Yamamoto (2000). Tamoxifen and gonadal steroids inhibit colon cancer growth in association with inhibition of thymidylate synthase, survivin and telomerase expression through estrogen receptor beta mediated system. *Cancer Lett.* **161**(1): 63-71.
- Neckers, L., T. W. Schulte and E. Mimnaugh (1999). Geldanamycin as a potential anti-cancer agent: its molecular target and biochemical activity. *Invest. New Drugs* **17**(4): 361-73.
- Neidle, S. and M. A. Read (2000). G-quadruplexes as therapeutic targets. *Biopolymers* **56**(3): 195-208.
- Nemoto, T., Y. Ohara-Nemoto, M. Ota, T. Takagi and K. Yokoyama (1995). Mechanism of dimer formation of the 90-kDa heat-shock protein. *Eur. J. Biochem.* **233**(1): 1-8.
- Nguyen, D. C. and D. L. Crowe (1999). Intact functional domains of the retinoblastoma gene product (pRb) are required for downregulation of telomerase activity. *Biochim. Biophys. Acta* **1445**(2): 207-15.
- Nishimoto, A., N. Miura, I. Horikawa, H. Kugoh, Y. Murakami, S. Hirohashi, H. Kawasaki, A. F. Gazdar, J. W. Shay, J. C. Barrett and M. Oshimura (2001). Functional evidence for a telomerase repressor gene on human chromosome 10p15.1. *Oncogene* **20**(7): 828-35.
- Norton, J. C., M. A. Piatyszek, W. E. Wright, J. W. Shay and D. R. Corey (1996). Inhibition of human telomerase activity by peptide nucleic acids. *Nat. Biotechnol.* **14**(5): 615-9.
- Obermann, W. M., H. Sondermann, A. A. Russo, N. P. Pavletich and F. U. Hartl (1998). In vivo function of Hsp90 is dependent on ATP binding and ATP hydrolysis. *J. Cell Biol.* **143**(4): 901-10.
- Oh, S., Y. Song, J. Yim and T. K. Kim (1999). The Wilms' tumor 1 tumor suppressor gene represses transcription of the human telomerase reverse transcriptase gene. *J. Biol. Chem.* **274**(52): 37473-8.
- Oh, S., Y. H. Song, J. Yim and T. K. Kim (2000). Identification of Mad as a repressor of the human telomerase (hTERT) gene. *Oncogene* **19**(11): 1485-90.
- Oh, S. T., S. Kyo and L. A. Laimins (2001). Telomerase activation by human papillomavirus type 16 E6 protein: induction of human telomerase reverse transcriptase expression through Myc and GC-rich Sp1 binding sites. *J. Virol.* **75**(12): 5559-66.

- Olovnikov, A. M. (1971). [Principle of marginotomy in template synthesis of polynucleotides]. *Dokl. Akad. Nauk SSSR* **201**(6): 1496-9.
- Oshimura, M. and J. C. Barrett (1997). Multiple pathways to cellular senescence: role of telomerase repressors. *Eur. J. Cancer* **33**(5): 710-5.
- Palmquist, K., B. Riis, A. Nilsson and O. Nygard (1994). Interaction of the calcium and calmodulin regulated eEF-2 kinase with heat shock protein 90. *FEBS Lett.* **349**(2): 239-42.
- Panaretou, B., C. Prodromou, S. M. Roe, R. O'Brien, J. E. Ladbury, P. W. Piper and L. H. Pearl (1998). ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. *Embo J.* **17**(16): 4829-36.
- Panaretou, B., G. Siligardi, P. Meyer, A. Maloney, J. K. Sullivan, S. Singh, S. H. Millson, P. A. Clarke, S. Naaby-Hansen, R. Stein, R. Cramer, M. Mollapour, P. Workman, P. W. Piper, L. H. Pearl and C. Prodromou (2002). Activation of the ATPase activity of hsp90 by the stress-regulated cochaperone aha1. *Mol. Cell* **10**(6): 1307-18.
- Pandey, P., A. Saleh, A. Nakazawa, S. Kumar, S. M. Srinivasula, V. Kumar, R. Weichselbaum, C. Nalin, E. S. Alnemri, D. Kufe and S. Kharbanda (2000). Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *Embo J.* **19**(16): 4310-22.
- Papazisis, K. T., G. D. Geromichalos, K. A. Dimitriadis and A. H. Kortsaris (1997). Optimization of the sulforhodamine B colorimetric assay. *J. Immunol. Methods* **208**(2): 151-8.
- Pascolo, E., C. Wenz, J. Lingner, N. Hael, H. Priepke, I. Kauffmann, P. Garin-Chesa, W. J. Rettig, K. Damm and A. Schnapp (2002). Mechanism of human telomerase inhibition by BIBR1532, a synthetic, non-nucleosidic drug candidate. *J. Biol. Chem.* **277**(18): 15566-72.
- Paull, K. D., R. H. Shoemaker, L. Hodes, A. Monks, D. A. Scudiero, L. Rubinstein, J. Plowman and M. R. Boyd (1989). Display and analysis of patterns of differential activity of drugs against human tumor cell lines: development of mean graph and COMPARE algorithm. *J. Natl. Cancer Inst.* **81**(14): 1088-92.
- Perry, P. J., M. A. Read, R. T. Davies, S. M. Gowan, A. P. Reszka, A. A. Wood, L. R. Kelland and S. Neidle (1999). 2,7-Disubstituted amidofluorenone derivatives as inhibitors of human telomerase. *J. Med. Chem.* **42**(14): 2679-84.
- Perry, P. J. and T. C. Jenkins (2001). DNA tetraplex-binding drugs: structure-selective targeting is critical for antitumour telomerase inhibition. *Mini Rev. Med. Chem.* **1**(1): 31-41.

- Pitts, A. E. and D. R. Corey (1998). Inhibition of human telomerase by 2'-O-methyl-RNA. *Proc. Natl. Acad. Sci. U S A* **95**(20): 11549-54.
- Pratt, W. B. (1993). The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. *J. Biol. Chem.* **268**(29): 21455-8.
- Pratt, W. B. and D. O. Toft (2003). Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp. Biol. Med. (Maywood)* **228**(2): 111-33.
- Prodromou, C., S. M. Roe, R. O'Brien, J. E. Ladbury, P. W. Piper and L. H. Pearl (1997). Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* **90**(1): 65-75.
- Prodromou, C., B. Panaretou, S. Chohan, G. Siligardi, R. O'Brien, J. E. Ladbury, S. M. Roe, P. W. Piper and L. H. Pearl (2000). The ATPase cycle of Hsp90 drives a molecular 'clamp' via transient dimerization of the N-terminal domains. *Embo J.* **19**(16): 4383-92.
- Pruzan, R., K. Pongracz, K. Gietzen, G. Wallweber and S. Gryaznov (2002). Allosteric inhibitors of telomerase: oligonucleotide N3'-->P5' phosphoramidates. *Nucleic Acids Res.* **30**(2): 559-68.
- Pugh, B. F. and R. Tjian (1991). Transcription from a TATA-less promoter requires a multisubunit TFIID complex. *Genes Dev.* **5**(11): 1935-45.
- Qu, Y., S. Q. Liu, W. Z. Peng and B. L. Liu (2002). [Inhibition of telomerase activity by ribozyme targeted to human telomerase transcriptase]. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)* **34**(3): 323-8.
- Rahman, R., L. Latonen and K. G. Wiman (2004). hTERT antagonizes p53-induced apoptosis independently of telomerase activity. *Oncogene*.
- Ramachandran, C. and W. You (1999). Differential sensitivity of human mammary epithelial and breast carcinoma cell lines to curcumin. *Breast Cancer Res. Treat.* **54**(3): 269-78.
- Ramachandran, C., H. B. Fonseca, P. Jhabvala, S. J. Melnick and E. Escalon (2001). Inhibition of human telomerase reverse transcriptase (hTERT) and telomerase activity by curcumin in human mammary epithelial and breast carcinoma cell lines. *Scientific World Journal* **1**(1 Suppl 3): 109.
- Ramachandran, C., H. B. Fonseca, P. Jhabvala, E. A. Escalon and S. J. Melnick (2002). Curcumin inhibits telomerase activity through human telomerase reverse transcriptase in MCF-7 breast cancer cell line. *Cancer Lett.* **184**(1): 1-6.



- Rangan, A., O. Y. Fedoroff and L. H. Hurley (2001). Induction of duplex to G-quadruplex transition in the c-myc promoter region by a small molecule. *J. Biol. Chem.* **276**(7): 4640-6.
- Read, M., R. J. Harrison, B. Romagnoli, F. A. Tanious, S. H. Gowan, A. P. Reszka, W. D. Wilson, L. R. Kelland and S. Neidle (2001). Structure-based design of selective and potent G quadruplex-mediated telomerase inhibitors. *Proc. Natl. Acad. Sci. U S A* **98**(9): 4844-9.
- Read, M. A., A. A. Wood, J. R. Harrison, S. M. Gowan, L. R. Kelland, H. S. Dosanjh and S. Neidle (1999). Molecular modeling studies on G-quadruplex complexes of telomerase inhibitors: structure-activity relationships. *J. Med. Chem.* **42**(22): 4538-46.
- Reddel, R. R. (2003). Alternative lengthening of telomeres, telomerase, and cancer. *Cancer Lett.* **194**(2): 155-62.
- Ren, X., G. Gavory, H. Li, L. Ying, D. Klenerman and S. Balasubramanian (2003). Identification of a new RNA:RNA interaction site for human telomerase RNA (hTR): structural implications for hTR accumulation and a dyskeratosis congenita point mutation. *Nucleic Acids Res.* **31**(22): 6509-15.
- Rhodes, D., L. Fairall, T. Simonsson, R. Court and L. Chapman (2002). Telomere architecture. *EMBO Rep.* **3**(12): 1139-45.
- Richter, K. and J. Buchner (2001). Hsp90: chaperoning signal transduction. *J. Cell. Physiol.* **188**(3): 281-90.
- Richter, K., J. Reinstein and J. Buchner (2002). N-terminal residues regulate the catalytic efficiency of the Hsp90 ATPase cycle. *J. Biol. Chem.* **277**(47): 44905-10.
- Roe, S. M., C. Prodromou, R. O'Brien, J. E. Ladbury, P. W. Piper and L. H. Pearl (1999). Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J. Med. Chem.* **42**(2): 260-6.
- Rohde, V., H. P. Sattler, T. Bund, H. Bonkhoff, T. Fixemer, C. Bachmann, R. Lensch, G. Unteregger, M. Stoeckle and B. Wullich (2000). Expression of the human telomerase reverse transcriptase is not related to telomerase activity in normal and malignant renal tissue. *Clin. Cancer Res.* **6**(12): 4803-9.
- Romero, D. P. and E. H. Blackburn (1991). A conserved secondary structure for telomerase RNA. *Cell* **67**(2): 343-53.

- Rose, D. W., R. E. Wettenhall, W. Kudlicki, G. Kramer and B. Hardesty (1987). The 90-kilodalton peptide of the heme-regulated eIF-2 alpha kinase has sequence similarity with the 90-kilodalton heat shock protein. *Biochemistry* **26**(21): 6583-7.
- Ryu, S. Y., C. O. Lee and S. U. Choi (1997). In vitro cytotoxicity of tanshinones from *Salvia miltiorrhiza*. *Planta Med.* **63**(4): 339-42.
- Saretzki, G. (2003). Telomerase inhibition as cancer therapy. *Cancer Lett.* **194**(2): 209-19.
- Sato, S., N. Fujita and T. Tsuruo (2000). Modulation of Akt kinase activity by binding to Hsp90. *Proc. Natl. Acad. Sci. U S A* **97**(20): 10832-7.
- Savoysky, E., K. Akamatsu, M. Tsuchiya and T. Yamazaki (1996). Detection of telomerase activity by combination of TRAP method and scintillation proximity assay (SPA). *Nucleic Acids Res.* **24**(6): 1175-6.
- Scheibel, T., T. Weikl and J. Buchner (1998). Two chaperone sites in Hsp90 differing in substrate specificity and ATP dependence. *Proc. Natl. Acad. Sci. U S A* **95**(4): 1495-9.
- Scheibel, T., H. I. Siegmund, R. Jaenicke, P. Ganz, H. Lilie and J. Buchner (1999). The charged region of Hsp90 modulates the function of the N-terminal domain. *Proc. Natl. Acad. Sci. U S A* **96**(4): 1297-302.
- Schnapp, G., H. P. Rodi, W. J. Rettig, A. Schnapp and K. Damm (1998). One-step affinity purification protocol for human telomerase. *Nucleic Acids Res.* **26**(13): 3311-3.
- Schneider, C., L. Sepp-Lorenzino, E. Nimmesgern, O. Ouerfelli, S. Danishefsky, N. Rosen and F. U. Hartl (1996). Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. *Proc. Natl. Acad. Sci. U S A* **93**(25): 14536-41.
- Schulte, T. W., M. V. Blagosklonny, C. Ingui and L. Neckers (1995). Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. *J. Biol. Chem.* **270**(41): 24585-8.
- Schulte, T. W., W. G. An and L. M. Neckers (1997). Geldanamycin-induced destabilization of Raf-1 involves the proteasome. *Biochem. Biophys. Res. Commun.* **239**(3): 655-9.
- Schulte, T. W. and L. M. Neckers (1998). The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. *Cancer Chemother. Pharmacol.* **42**(4): 273-9.

- Shay, J. W. (1999). Toward identifying a cellular determinant of telomerase repression. *J. Natl. Cancer Inst.* **91**(1): 4-6.
- Shay, J. W. and W. E. Wright (2002). Telomerase: a target for cancer therapeutics. *Cancer Cell* **2**(4): 257-65.
- Shi, D. F., R. T. Wheelhouse, D. Sun and L. H. Hurley (2001). Quadruplex-interactive agents as telomerase inhibitors: synthesis of porphyrins and structure-activity relationship for the inhibition of telomerase. *J. Med. Chem.* **44**(26): 4509-23.
- Shin-ya, K., K. Wierzba, K. Matsuo, T. Ohtani, Y. Yamada, K. Furihata, Y. Hayakawa and H. Seto (2001). Telomestatin, a novel telomerase inhibitor from *Streptomyces anulatus*. *J. Am. Chem. Soc.* **123**(6): 1262-3.
- Singer, M. S. and D. E. Gottschling (1994). TLC1: template RNA component of *Saccharomyces cerevisiae* telomerase. *Science* **266**(5184): 404-9.
- Smith, D. F. (1993). Dynamics of heat shock protein 90-progesterone receptor binding and the disactivation loop model for steroid receptor complexes. *Mol. Endocrinol.* **7**(11): 1418-29.
- Smith, D. F., L. Whitesell, S. C. Nair, S. Chen, V. Prapapanich and R. A. Rimerman (1995). Progesterone receptor structure and function altered by geldanamycin, an hsp90-binding agent. *Mol. Cell. Biol.* **15**(12): 6804-12.
- Smith, D. F. (2000). Chaperones in progesterone receptor complexes. *Semin. Cell Dev. Biol.* **11**(1): 45-52.
- Smogorzewska, A., B. van Steensel, A. Bianchi, S. Oelmann, M. R. Schaefer, G. Schnapp and T. de Lange (2000). Control of human telomere length by TRF1 and TRF2. *Mol. Cell. Biol.* **20**(5): 1659-68.
- Smucker, E. J. and J. J. Turchi (2001). TRF1 inhibits telomere C-strand DNA synthesis in vitro. *Biochemistry* **40**(8): 2426-32.
- Solit, D. B., F. F. Zheng, M. Drobnjak, P. N. Munster, B. Higgins, D. Verbel, G. Heller, W. Tong, C. Cordon-Cardo, D. B. Agus, H. I. Scher and N. Rosen (2002). 17-Allylamino-17-demethoxygeldanamycin induces the degradation of androgen receptor and HER-2/neu and inhibits the growth of prostate cancer xenografts. *Clin. Cancer Res.* **8**(5): 986-93.
- Soti, C., A. Racz and P. Csermely (2002). A Nucleotide-dependent molecular switch controls ATP binding at the C-terminal domain of Hsp90. N-terminal nucleotide binding unmasks a C-terminal binding pocket. *J. Biol. Chem.* **277**(9): 7066-75.

- Stancato, L. F., Y. H. Chow, K. A. Hutchison, G. H. Perdew, R. Jove and W. B. Pratt (1993). Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system. *J. Biol. Chem.* **268**(29): 21711-6.
- Stansel, R. M., T. de Lange and J. D. Griffith (2001). T-loop assembly in vitro involves binding of TRF2 near the 3' telomeric overhang. *Embo J.* **20**(19): 5532-40.
- Stebbins, C. E., A. A. Russo, C. Schneider, N. Rosen, F. U. Hartl and N. P. Pavletich (1997). Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell* **89**(2): 239-50.
- Steenbergen, R. D., D. Kramer, C. J. Meijer, J. M. Walboomers, D. A. Trott, A. P. Cuthbert, R. F. Newbold, W. J. Overkamp, M. Z. Zdzienicka and P. J. Snijders (2001). Telomerase suppression by chromosome 6 in a human papillomavirus type 16-immortalized keratinocyte cell line and in a cervical cancer cell line. *J. Natl. Cancer Inst.* **93**(11): 865-72.
- Stewart, S. A., W. C. Hahn, B. F. O'Connor, E. N. Banner, A. S. Lundberg, P. Modha, H. Mizuno, M. W. Brooks, M. Fleming, D. B. Zimonjic, N. C. Popescu and R. A. Weinberg (2002). Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. *Proc. Natl. Acad. Sci. U S A* **99**(20): 12606-11.
- Strahl, C. and E. H. Blackburn (1996). Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalized human cell lines. *Mol. Cell. Biol.* **16**(1): 53-65.
- Suganuma, H., M. Suganuma, E. Sueoka, N. Lida, A. Komori, S. J. Kim and H. Fujiki (1996). A new process of cancer prevention mediated through inhibition of tumor necrosis factor alpha expression. *Cancer Res.* **56**: 3711-3715.
- Sullivan, W., B. Stensgard, G. Caucutt, B. Barth, N. McMahon, E. S. Alnemri, G. Litwack and D. Toft (1997). Nucleotides and two functional states of hsp90. *J. Biol. Chem.* **272**(12): 8007-12.
- Sun, D., B. Thompson, B. E. Cathers, M. Salazar, S. M. Kerwin, J. O. Trent, T. C. Jenkins, S. Neidle and L. H. Hurley (1997). Inhibition of human telomerase by a G-quadruplex-interactive compound. *J. Med. Chem.* **40**(14): 2113-6.
- Sung, H. J., S. M. Choi, Y. Yoon and K. S. An (1999). Tanshinone IIA, an ingredient of *Salvia miltiorrhiza* BUNGE, induces apoptosis in human leukemia cell lines through the activation of caspase-3. *Exp. Mol. Med.* **31**(4): 174-8.
- Supko, J. G., R. L. Hickman, M. R. Grever and L. Malspeis (1995). Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. *Cancer Chemother. Pharmacol.* **36**(4): 305-15.

- Tahara, H., W. Yasui, E. Tahara, J. Fujimoto, K. Ito, K. Tamai, J. Nakayama, F. Ishikawa and T. Ide (1999). Immuno-histochemical detection of human telomerase catalytic component, hTERT, in human colorectal tumor and non-tumor tissue sections. *Oncogene* **18**(8): 1561-7.
- Takahashi, K., X. Ouyang, K. Komatsu, N. Nakamura, M. Hattori, A. Baba and J. Azuma (2002). Sodium tanshinone IIA sulfonate derived from Danshen (*Salvia miltiorrhiza*) attenuates hypertrophy induced by angiotensin II in cultured neonatal rat cardiac cells. *Biochem. Pharmacol.* **64**(4): 745-9.
- Takakura, M., S. Kyo, T. Kanaya, M. Tanaka and M. Inoue (1998). Expression of human telomerase subunits and correlation with telomerase activity in cervical cancer. *Cancer Res.* **58**(7): 1558-61.
- Takakura, M., S. Kyo, T. Kanaya, H. Hirano, J. Takeda, M. Yutsudo and M. Inoue (1999). Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. *Cancer Res.* **59**(3): 551-7.
- Takakura, M., S. Kyo, Y. Sowa, Z. Wang, N. Yatabe, Y. Maida, M. Tanaka and M. Inoue (2001). Telomerase activation by histone deacetylase inhibitor in normal cells. *Nucleic Acids Res.* **29**(14): 3006-11.
- Tanenbaum, D. M., Y. Wang, S. P. Williams and P. B. Sigler (1998). Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. *Proc. Natl. Acad. Sci. U S A* **95**(11): 5998-6003.
- Tesmer, V. M., L. P. Ford, S. E. Holt, B. C. Frank, X. Yi, D. L. Aisner, M. Ouellette, J. W. Shay and W. E. Wright (1999). Two inactive fragments of the integral RNA cooperate to assemble active telomerase with the human protein catalytic subunit (hTERT) in vitro. *Mol. Cell. Biol.* **19**(9): 6207-16.
- Theimer, C. A., C. A. Blois and J. Feigon (2005). Structure of the human telomerase RNA pseudoknot reveals conserved tertiary interactions essential for function. *Mol. Cell* **17**(5): 671-82.
- Tsao, D. A., C. W. Wu and Y. S. Lin (1998). Molecular cloning of bovine telomerase RNA. *Gene* **221**(1): 51-8.
- Ulaner, G. A., J. F. Hu, T. H. Vu, L. C. Giudice and A. R. Hoffman (1998). Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. *Cancer Res.* **58**(18): 4168-72.

- Ulaner, G. A., J. F. Hu, T. H. Vu, H. Oruganti, L. C. Giudice and A. R. Hoffman (2000). Regulation of telomerase by alternate splicing of human telomerase reverse transcriptase (hTERT) in normal and neoplastic ovary, endometrium and myometrium. *Int. J. Cancer* **85**(3): 330-5.
- Ulaner, G. A., J. F. Hu, T. H. Vu, L. C. Giudice and A. R. Hoffman (2001). Tissue-specific alternate splicing of human telomerase reverse transcriptase (hTERT) influences telomere lengths during human development. *Int. J. Cancer* **91**(5): 644-9.
- van Steensel, B. and T. de Lange (1997). Control of telomere length by the human telomeric protein TRF1. *Nature* **385**(6618): 740-3.
- van Steensel, B., A. Smogorzewska and T. de Lange (1998). TRF2 protects human telomeres from end-to-end fusions. *Cell* **92**(3): 401-13.
- Veldman, T., I. Horikawa, J. C. Barrett and R. Schlegel (2001). Transcriptional activation of the telomerase hTERT gene by human papillomavirus type 16 E6 oncoprotein. *J. Virol.* **75**(9): 4467-72.
- Vilenchik, M., D. Solit, A. Basso, H. Huezo, B. Lucas, H. He, N. Rosen, C. Spampinato, P. Modrich and G. Chiosis (2004). Targeting wide-range oncogenic transformation via PU24FCl, a specific inhibitor of tumor Hsp90. *Chem. Biol.* **11**(6): 787-97.
- Villa, R., M. Folini, C. D. Porta, A. Valentini, M. Pennati, M. G. Daidone and N. Zaffaroni (2003). Inhibition of telomerase activity by geldanamycin and 17-allylamino, 17-demethoxygeldanamycin in human melanoma cells. *Carcinogenesis* **24**(5): 851-9.
- Wan, M. S., P. L. Fell and S. Akhtar (1998). Synthetic 2'-O-methyl-modified hammerhead ribozymes targeted to the RNA component of telomerase as sequence-specific inhibitors of telomerase activity. *Antisense Nucleic Acid Drug Dev.* **8**(4): 309-17.
- Wang, J., L. Y. Xie, S. Allan, D. Beach and G. J. Hannon (1998). Myc activates telomerase. *Genes Dev.* **12**(12): 1769-74.
- Wang, Q., D. Dube, R. W. Friesen, T. G. LeRiche, K. P. Bateman, L. Trimble, J. Sanghara, R. Pollex, C. Ramachandran, M. J. Gresser and Z. Huang (2004). Catalytic inactivation of protein tyrosine phosphatase CD45 and protein tyrosine phosphatase 1B by polyaromatic quinones. *Biochemistry* **43**(14): 4294-303.
- Wang, X., X. Qian, H. C. Guo and J. Hu (2003). Heat shock protein 90-independent activation of truncated hepatitis B virus reverse transcriptase. *J. Virol.* **77**(8): 4471-80.

- Wang, X., K. F. Bastow, C. M. Sun, Y. L. Lin, H. J. Yu, M. J. Don, T. S. Wu, S. Nakamura and K. H. Lee (2004). Antitumor Agents. 239. Isolation, structure elucidation, total synthesis, and anti-breast cancer activity of neo-tanshinlactone from *Salvia miltiorrhiza*. *J. Med. Chem.* **47**(23): 5816-9.
- Wang, Z., S. Kyo, M. Takakura, M. Tanaka, N. Yatabe, Y. Maida, M. Fujiwara, J. Hayakawa, M. Ohmichi, K. Koike and M. Inoue (2000). Progesterone regulates human telomerase reverse transcriptase gene expression via activation of mitogen-activated protein kinase signaling pathway. *Cancer Res.* **60**(19): 5376-81.
- Wartmann, M. and R. J. Davis (1994). The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex. *J. Biol. Chem.* **269**(9): 6695-701.
- Watson, J. D. (1972). Origin of concatemeric T7 DNA. *Nat. New Biol.* **239**(94): 197-201.
- Wegele, H., L. Muller and J. Buchner (2004). Hsp70 and Hsp90--a relay team for protein folding. *Rev. Physiol. Biochem. Pharmacol.* **151**: 1-44.
- Weinrich, S. L., R. Pruzan, L. Ma, M. Ouellette, V. M. Tesmer, S. E. Holt, A. G. Bodnar, S. Lichtsteiner, N. W. Kim, J. B. Trager, R. D. Taylor, R. Carlos, W. H. Andrews, W. E. Wright, J. W. Shay, C. B. Harley and G. B. Morin (1997). Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTERT. *Nat. Genet.* **17**: 498-502.
- White, L. K., W. E. Wright and J. W. Shay (2001). Telomerase inhibitors. *Trends Biotechnol.* **19**(3): 114-20.
- Whitesell, L., E. G. Mimnaugh, B. De Costa, C. E. Myers and L. M. Neckers (1994). Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc. Natl. Acad. Sci. U S A* **91**(18): 8324-8.
- Williams, S. P. and P. B. Sigler (1998). Atomic structure of progesterone complexed with its receptor. *Nature* **393**(6683): 392-6.
- Won, J., S. Chang, S. Oh and T. K. Kim (2004). Small-molecule-based identification of dynamic assembly of E2F-pocket protein-histone deacetylase complex for telomerase regulation in human cells. *Proc. Natl. Acad. Sci. U S A* **101**(31): 11328-33.
- Workman, P. (2004). Altered states: selectively drugging the Hsp90 cancer chaperone. *Trends Mol. Med.* **10**(2): 47-51.

- Wright, W. E., J. W. Shay and M. A. Piatyszek (1995). Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. *Nucleic Acids Res.* **23**(18): 3794-5.
- Wright, W. E., D. Brasiskyte, M. A. Piatyszek and J. W. Shay (1996a). Experimental elongation of telomeres extends the lifespan of immortal x normal cell hybrids. *Embo J.* **15**(7): 1734-41.
- Wright, W. E., M. A. Piatyszek, W. E. Rainey, W. Byrd and J. W. Shay (1996b). Telomerase activity in human germline and embryonic tissues and cells. *Dev. Genet.* **18**(2): 173-9.
- Wright, W. E., V. M. Tesmer, K. E. Huffman, S. D. Levene and J. W. Shay (1997). Normal human chromosomes have long G-rich telomeric overhangs at one end. *Genes Dev.* **11**(21): 2801-9.
- Wu, K. J., C. Grandori, M. Amacker, N. Simon-Vermot, A. Polack, J. Lingner and R. Dalla-Favera (1999). Direct activation of TERT transcription by c-MYC. *Nat. Genet.* **21**(2): 220-4.
- Wu, W. L., W. L. Chang and C. F. Chen (1991). Cytotoxic activities of tanshinones against human carcinoma cell lines. *Am. J. Chin. Med.* **19**(3-4): 207-16.
- Xu, D., S. Erickson, M. Szeps, A. Gruber, O. Sangfelt, S. Einhorn, P. Pisa and D. Grander (2000a). Interferon alpha down-regulates telomerase reverse transcriptase and telomerase activity in human malignant and nonmalignant hematopoietic cells. *Blood* **96**(13): 4313-8.
- Xu, D., Q. Wang, A. Gruber, M. Bjorkholm, Z. Chen, A. Zaid, G. Selivanova, C. Peterson, K. G. Wiman and P. Pisa (2000b). Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells. *Oncogene* **19**(45): 5123-33.
- Xu, D., N. Popov, M. Hou, Q. Wang, M. Bjorkholm, A. Gruber, A. R. Menkel and M. Henriksson (2001). Switch from Myc/Max to Mad1/Max binding and decrease in histone acetylation at the telomerase reverse transcriptase promoter during differentiation of HL60 cells. *Proc. Natl. Acad. Sci. U S A* **98**(7): 3826-31.
- Xu, W., M. Marcu, X. Yuan, E. Mimnaugh, C. Patterson and L. Neckers (2002). Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *Proc. Natl. Acad. Sci. U S A* **99**(20): 12847-52.
- Yamaki, H., H. Suzuki, E. C. Choi and N. Tanaka (1982). Inhibition of DNA synthesis in murine tumor cells by geldanamycin, an antibiotic of the benzoquinoid ansamycin group. *J. Antibiot. (Tokyo)* **35**(7): 886-92.



- Yamori, T. (1997). [A human cell line panel for screening anti-cancer drugs]. *Gan To Kagaku Ryoho* **24**(2): 129-35.
- Yang, H., S. Kyo, M. Takatura and L. Sun (2001). Autocrine transforming growth factor beta suppresses telomerase activity and transcription of human telomerase reverse transcriptase in human cancer cells. *Cell Growth Differ.* **12**(2): 119-27.
- Yano, M., Z. Naito, S. Tanaka and G. Asano (1996). Expression and roles of heat shock proteins in human breast cancer. *Jpn. J. Cancer Res.* **87**(9): 908-15.
- Yen, G. C. and H. Y. Chen (1995). Antioxidant Activity of Various Tea Extracts in Relation to Their Antimutagenicity. *J. Agric. Food Chem.* **43**(1): 27-32.
- Yi, X., V. M. Tesmer, I. Savre-Train, J. W. Shay and W. E. Wright (1999). Both transcriptional and posttranscriptional mechanisms regulate human telomerase template RNA levels. *Mol. Cell. Biol.* **19**(6): 3989-97.
- Yokoyama, Y., Y. Takahashi, A. Shinohara, X. Wan, S. Takahashi, K. Niwa and T. Tamaya (2000). The 5'-end of hTERT mRNA is a good target for hammerhead ribozyme to suppress telomerase activity. *Biochem. Biophys. Res. Commun.* **273**(1): 316-21.
- Young, J. C., C. Schneider and F. U. Hartl (1997). In vitro evidence that hsp90 contains two independent chaperone sites. *FEBS Lett.* **418**(1-2): 139-43.
- Young, J. C., W. M. Obermann and F. U. Hartl (1998). Specific binding of tetratricopeptide repeat proteins to the C-terminal 12-kDa domain of hsp90. *J. Biol. Chem.* **273**(29): 18007-10.
- Young, J. C., V. R. Agashe, K. Siegers and F. U. Hartl (2004). Pathways of chaperone-mediated protein folding in the cytosol. *Nat. Rev. Mol. Cell Biol.* **5**(10): 781-91.
- Yu, C. C., S. C. Lo and T. C. Wang (2001). Telomerase is regulated by protein kinase C-zeta in human nasopharyngeal cancer cells. *Biochem. J.* **355**(Pt 2): 459-64.
- Yuan, S. L., Y. Q. Wei, X. J. Wang, F. Xiao, S. F. Li and J. Zhang (2004). Growth inhibition and apoptosis induction of tanshinone II-A on human hepatocellular carcinoma cells. *World J. Gastroenterol.* **10**(14): 2024-8.
- Yudoh, K., H. Matsuno, F. Nakazawa, R. Katayama and T. Kimura (2001). Reconstituting telomerase activity using the telomerase catalytic subunit prevents the telomere shorting and replicative senescence in human osteoblasts. *J. Bone Miner. Res.* **16**(8): 1453-64.

- Yun, B. G., W. Huang, N. Leach, S. D. Hartson and R. L. Matts (2004). Novobiocin induces a distinct conformation of Hsp90 and alters Hsp90-cochaperone-client interactions. *Biochemistry* **43**(25): 8217-29.
- Zaffaroni, N., S. Lualdi, R. Villa, D. Bellarosa, C. Cermele, P. Felicetti, C. Rossi, L. Orlandi and M. G. Daidone (2002). Inhibition of telomerase activity by a distamycin derivative: effects on cell proliferation and induction of apoptosis in human cancer cells. *Eur. J. Cancer* **38**(13): 1792-801.
- Zhang, J. H., T. D. Chung and K. R. Oldenburg (1999). A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screen.* **4**(2): 67-73.
- Zhang, X., V. Mar, W. Zhou, L. Harrington and M. O. Robinson (1999). Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev.* **13**(18): 2388-99.
- Zhao, C. and E. Wang (2004). Heat shock protein 90 suppresses tumor necrosis factor alpha induced apoptosis by preventing the cleavage of Bid in NIH3T3 fibroblasts. *Cell Signal.* **16**(3): 313-21.