Pharmacological Telomerase Inhibition can Sensitize Drug-Resistant and Drug-Sensitive Cells to Chemotherapeutic Treatment.

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Preface

This M.Sc. thesis is written in accordance with the McGill University Faculty of Graduate and Postdoctoral Studies thesis preparation guidelines. I have exercised the option of submitting a manuscript-based thesis which includes modified versions of two original manuscripts (Chapters 1 and 2). A comprehensive review of the literature and a general discussion are presented in Chapters 1 and 3. References for all chapters appear in one consolidated section found at the end of the thesis.

Published Manuscripts Included in this Thesis

Chapter 1- A modified version of the text that appears in: Cerone, M.A.*, Ward, R*. & Autexier. C. (2005) "**Telomere and telomerase based therapies.**" In Aging Interventions & Therapies. (Ed. S.I.S. Rattan). World Scientific Publishers, Singapore. http://www.worldscibooks.com/lifesci/5690.html **Co-first authors.*

Chapter 2- A modified version of the text that appears in: Ward, R.J. & Autexier, C. Pharmacological Telomerase Inhibition can Sensitize Drug-Resistant and Drug-Sensitive Cells to Chemotherapeutic Treatment. Mol Pharmacol. 2005 Jun 6; [Epub ahead of print] PMID: 15939802

Contributions of Authors

Chapter 1- The section of the published manuscript included in this thesis was written by the candidate and edited by the co-authors listed above. Sections of the published manuscript not written by the candidate have been excluded from this thesis.

Chapter 2- All the work presented in this chapter (data and text) was performed by the candidate under the supervision of Dr. C. Autexier.

Contribution to Original Knowledge

The work presented in this thesis is the first to report:

1. Basal telomere length and telomerase activity of paired drug-sensitive and drug-resistant human leukemia (HL60/WT and etoposide resistant HL60/MX2 respectively) and breast cancer (MCF-7/WT, melphalan resistant MCF-7/Mln^R, and doxorubicin resistant MCF-7/Adr^R) cell lines;
2. Telomerase activity of HL60/WT and HL60/MX2 cells post treatment with IC_{50} etoposide treatment;
3. The effect of treating HL60 and MCF-7 cells with a combination of BIBR1532 and etoposide, melphalan, or doxorubicin.

Abstract

Telomerase is the ribonucleoprotein enzyme whose principal function is to catalyze the *de novo* addition of telomeric repeats to the ends of linear chromosomes. Telomerase activity is predominantly observed in cancerous tissues and rarely in normal somatic cells making telomerase an attractive anticancer target. BIBR1532 is a highly selective pharmacological inhibitor of telomerase catalytic activity and induces telomere shortening and eventual growth arrest. We treated drug-sensitive and drug-resistant human leukemia and breast cancer cells lines with BIBR1532 and observed telomere shortening and a progressive decrease in proliferative capacity or colony forming ability. This effect was increased with the co-treatment of traditional chemotherapeutics, suggesting that pharmacological telomerase inhibition in combination with chemotherapeutics may be a valid strategy for the treatment of both drug-resistant and drug-sensitive cancers. Finally, our results support further investigation and development of pharmacological inhibitors of telomerase catalytic activity.

Resumé

La télomérase est une ribonucléoprotéine qui permet la synthèse *de novo* de répétitions télomériques à l'extrémité des chromosomes linéaires. L'activité télomérase est observée majoritairement dans les tissus cancéreux et rarement dans les cellules somatiques normales, faisant de la télomerase une cible anticancéreuse de choix. BIBR1532 est un inhibiteur pharmacologique hautement sélectif de l'activité télomérase et induit le raccourcissement des télomères et un éventuel arrêt de croissance. Nous avons traité des lignées cellulaires sensibles ou non aux drogues, établies à partir de leucémie humaine et de cancer du sein, avec BIBR1532 et nous avons observé un raccourcissement des télomères et une diminution progressive de la capacité prolifération ou de la capacité de former les colonies. Cet effet est augmenté lors d'un co-traitement avec des chimiothérapies traditionnelles, suggérant que l'inhibition pharmacologique de la télomérase couplée avec la chimiothérapie pourrait constituer une stratégie pour le traitement des cancers résistants ou sensibles aux drogues. Par conséquent, nos résultats renforcent la recherche et le développement d'inhibiteurs pharmacologiques de la télomérase.

Acknowledgments

"If you want to make God laugh, tell him about your plans." -Woody Allen

I am grateful to my supervisor Dr. Chantal Autexier. Chantal, your openness, availability, excellent scientific and life guidance, and infinite support truly set you apart from your colleagues. I know first hand that many students are envious of those who work in the Autexier lab, a testament to your friendly, approachable, and caring supervision. I wish you the very best fortune in everything your future holds.

I thank research assistants, students, post-doctoral fellows and scientists in Experimental Medicine and at the Lady Davis Institute who have contributed to my education, especially Dr.s Moulay Alaoui-Jamali, Lorraine Chalifour, Martin Loignon and other members of my advisory committees. Additionally, thanks to Lucy Badolato and Dominique Besso for continued support at the LDI and McGill.

My deepest thanks to everyone in the Autexier laboratory past and present. Thanks to Maria Antonietta. You are an excellent scientist, teacher and friend. Thanks to Tara Moriarty, Tamara Marie-Egyptienne, Laetitia Fragnet, Johans Fakhoury, Pooja Jain and Annie Dulude for training, guidance, laughs and friendly conversations. Additional thanks to Pooja for ensuring the safe arrival of this thesis. To new members of the lab, I wish you the best of luck. May your time in CA426 be as excellent as mine.

Most importantly to my family, with all my heart, Thank You. In everything I do I stand on your shoulders. My time in Montréal has seen the miraculous arrival of a niece and a nephew, and the passing of two grandparents. I will always love and remember. To Alexandra, the *only* reason this thesis has been written, I hope my actions speak louder than any words ever could.

Table of Contents:

Chapter 1- Introduction & Literature Review

1.1 Historical Background

The German cytologist Hermann J. Muller was one of the first to propose the idea that the ends of chromosomes were in someway different than the rest (Blackburn and Greider 1995). In 1938, Muller spoke of two types of genes; those that were unipolar with only one neighbouring gene due to their location at the end of the chromosome, and those that were bipolar with neighbouring genes on both sides and located throughout the chromosome. These unipolar genes he called telomeres (telos meaning end, and meros meaning part) and attributed to them the special function of sealing the chromosomes, a function that could not be reproduced by simply tearing two bipolar genes apart. In the late 1920's Muller had produced the first experimentally induced mutations by X-ray irradiating *Drosophila*. His observations, for which he was later awarded the Nobel Prize, allowed him to note that many mutations were associated with chromosomal rearrangements that involved large translocations between non-homologous chromosomes or inversions in the same chromosome. Muller hypothesized that these rearrangements were the consequence of two independent breaks and the exchange or inversion of the broken ends. Initially these observations were made by genetic analysis of irradiated *Drosophila*, however the examination of the large polytene chromosome allowed Muller to directly observe this phenomenon. All together, Muller's experiments enabled him to deduce that the rearrangements were the consequence of joining two newly broken chromosomes, and not the attachment of a broken end to an originally free end (the telomere) (Blackburn and Greider 1995).

Around the same time, Barbara McClintock's work in maize closely supported Muller's observations by demonstrating that, independent of how the break in the chromosome occurred, broken chromosome ends were extremely 'sticky' and would readily join to other broken ends (Blackburn and Greider 1995). In the system that she established, a breakage-fusion-bridge cycle occurred where dicentric chromosomes (containing two centromeres) were pulled to both sides of the mitotic spindle creating a chromosome bridge. Though this bridge

was broken during anaphase or telophase to create broken chromosomal ends, McClintock noted that the broken ends of the sister chromatids fused to one another before the next mitosis and thus generated a new dicentric chromosome to further continue the breakage-fusion-bridge cycle (Blackburn and Greider 1995). From this work, she went on to other studies that led to her theory that spontaneous chromosomal breaks could sometimes be generated by the existence of transposable elements, work for which she was eventually awarded the Nobel Prize. Thus, both Muller and McClintock recognized that broken chromosomal ends were highly unstable, preferring to join with other broken ends as compared to normal telomeres, which were stable and in some way sealed.

1.2 The End Replication Problem

Though the importance of telomeres was understood from the work described above, their role was not fully appreciated until the elucidation of DNA structure and the mechanisms by which it is replicated. It was recognized that the terminal ends of the chromosome would be incompletely replicated at each round of cellular division due to the 5' to 3' directionality of the conventional DNA replication machinery, and the use of short RNA primers (Watson 1972; Olovnikov 1973). Leading strand synthesis is continuous from the origin of replication to the end of the chromosome. The discontinuous nature of lagging strand synthesis and the use of RNA primers for the initiation of replication generate a region of unreplicated DNA at the most 5' end of the lagging strand (Figure 1). With successive rounds of cell division, this unreplicated region would become larger and eventually erode the ends of the chromosomes potentially leading to genomic instability.

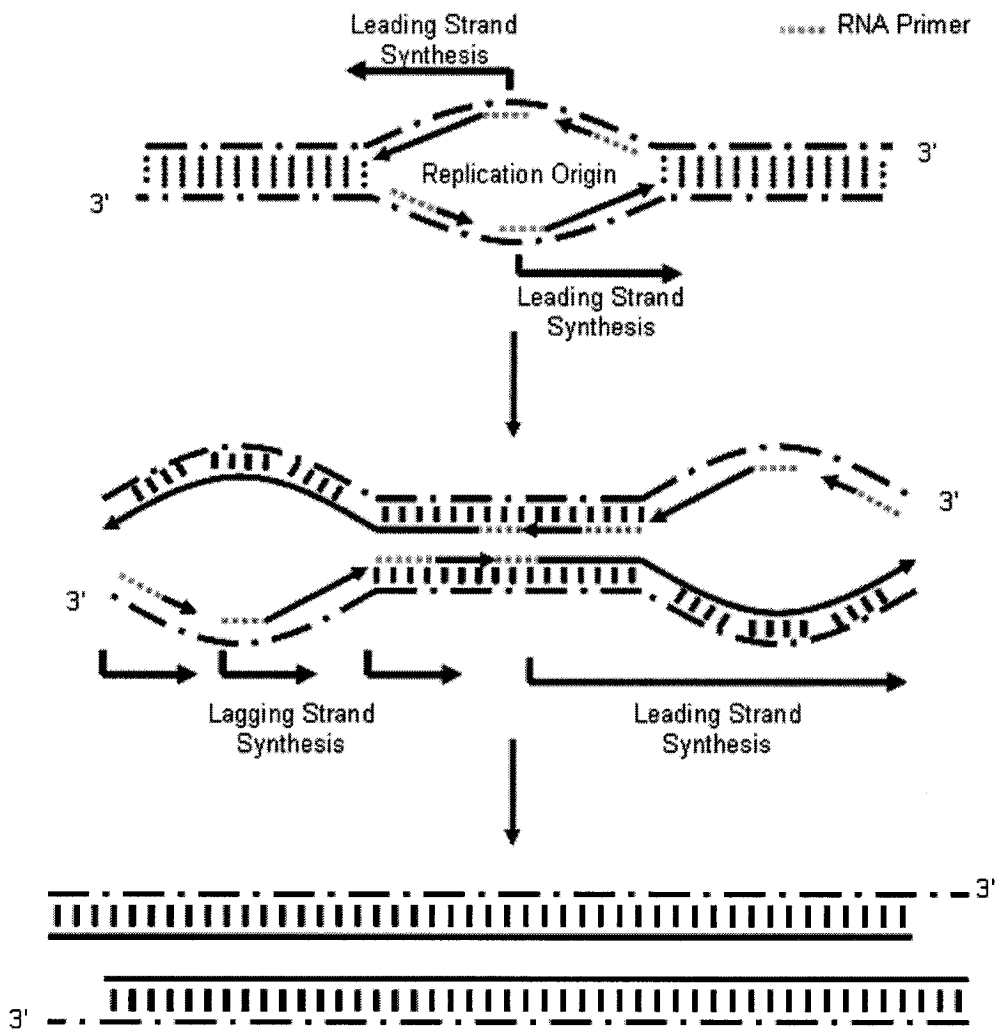

Leading Strand
Synthesis
RNA Primer
3'
Replication Origin
3'
Leading Strand
Synthesis
3'
3'
Lagging Strand
Synthesis
Leading Strand
Synthesis
3'
3'

Figure 1- DNA synthesis remains incomplete at the 5' end after lagging strand synthesis. Replication begins at the replication origin and progresses distally to the end of the DNA. Leading strand synthesis is continuous, however the discontinuous nature of lagging strand synthesis generates Okazaki fragments separated by short RNA primers. When the RNA primers are removed and replaced by DNA, replication of the 5' lagging strand end remains incomplete. Without a mechanism to counter this effect, cells lose small amounts of DNA at each round of replication, a phenomenon known as the End Replication Problem.

1.3 Telomere Structure & Function

Telomeres are most simply defined as the terminal ends of linear chromosomes. In humans this DNA sequence usually ranges from 2 to 15 kilobase pairs (kb) in length (Wellinger and Sen 1997); however great length variation exists between organisms, for example the telomeres of the ciliate *Euplotes aediculatus* are less than 50 base pairs (Klobutcher, Swanton et al. 1981) versus that of the mouse which can be 60 kb (Zijlmans, Martens et al. 1997). Functionally, telomeres are stretches of non-coding, repetitive DNA that serve to protect the ends of the chromosomes from being recognized by the cellular machinery as broken or damaged DNA, from non-homologous end joining, end-to-end fusions or endogenous exonucleases (Blackburn 2001). In humans, telomeres consist of the hexanucleotide repeat TTAGGG on the 3' strand (termed the G-rich strand, or G-strand) and AATCCC on the 5' strand (termed the C-rich strand, or C-strand) (Moyzis, Buckingham et al. 1988; Kipling and Cooke 1990) (Figure 2). Though the telomeric sequence can differ between organisms, in general telomeres are G/C rich structures that terminate in a single-stranded 3' G-strand overhang (Wright, Tesmer et al. 1997). It is now thought that this overhang provides a structure by which chromosomes and telomeres are protected or 'capped'. Electron microscopy of telomeric DNA and purified de-proteinated telomeres from a variety of organisms has revealed that the 3' overhang can loop and invade the double-stranded telomere (Griffith, Comeau et al. 1999; Murti and Prescott 1999). This generates the telomere-loop (t-loop), and the displacement-loop (d-loop) at the region of invasion (Figure 2) (Griffith, Comeau et al. 1999). These loops can form in the absence of interacting proteins or cross-linking agents however their formation is greatly increased in the presence of telomere binding proteins (discussion to follow) (Yoshimura, Maruyama et al. 2004).

Further characterization of the G-rich DNA tracks suggests that telomeric DNA may exist in higher structural forms. NMR spectroscopy and X-ray crystallography of telomeric DNA sequences demonstrated that the guanine-rich sequences can form G-quartet structures in which multiple guanines hydrogen bond to form stacking structures in either parallel or anti-parallel conformation

(Neidle and Parkinson 2003) (Figure 3). Primarily observed *in vitro* there is now some evidence that G-quartet structures can form *in vivo* (Schaffitzel, Berger et al. 2001; Chang, Kuo et al. 2004) and data from recent *in vitro* studies suggest that these structures may involve the telomere binding proteins TRF1 and TRF2 (Yoshimura, Maruyama et al. 2004). Though direct evidence of the existence of G-quartet structures in human cells is still lacking, and what their exact orientation and structure might be, they do provide a model whereby the single stranded G-rich overhang might be further protected and stabilized.

1.4 Telomere Binding Proteins

A number of proteins have been shown to localize to telomeres, either directly by binding telomeric DNA, or indirectly by associating with proteins found on the telomeres. The best characterized telomere binding proteins in humans are TRF1, TRF2 and POT1 (de Lange 2002). TRF1 and TRF2 both localize to the double-stranded telomeric DNA, with TRF2 preferentially found at the junction between the double-stranded telomere and the invasion of the single-stranded 3' overhang, while POT1 is found at single-stranded telomeric DNA (Baumann and Cech 2001) (Figure 2). All appear to bind the telomeres as dimers (and in the case of TRF1 and TRF2 perhaps amalgamated tetramers), and participate in the stabilization of the t-loop structure (Yoshimura, Maruyama et al. 2004), aid in the loading of other telomere binding proteins (Iwano, Tachibana et al. 2004), and play roles in telomere length maintenance (Loayza and De Lange 2003; Yoshimura, Maruyama et al. 2004). Overexpression of both TRF1 and TRF2 (van Steensel and de Lange 1997) has negative regulatory effects on telomere length, suggesting that when bound by these proteins, telomeres are stabilized and their elongation is prevented. TRF1 null cells exhibit a growth defect with an extended population doubling time, chromosomal instability in the form of end-to-end sister chromatid fusions, and decreased association of TRF2 and the telomere binding protein TIN2 with the telomeres (Iwano, Tachibana et al. 2004). Expression of a mutant TRF2 protein that is defective in its ability to interact with telomeric DNA induces rapid onset of apoptosis or senescence

(depending on the particular cell line used) in an ATM/p53- or p16/RB-dependent fashion, end-to-end fusions and chromosomal instability, and erosion of the 3' overhang (Karlseder, Broccoli et al. 1999; Smogorzewska, van Steensel et al. 2000). Our understanding of TRF1 and TRF2 suggests that the two telomere binding proteins act to stabilize the structure of telomeric DNA preventing it from being recognized as damaged DNA, from joining to other chromosomal ends, and to sequester the 3' end from the active telomerase enzyme (de Lange 2002; Iwano, Tachibana et al. 2004). Additionally there is now evidence that telomere binding proteins may play active roles in the DNA damage response. TRF2 has recently been shown to rapidly (within seconds) associate with photo-induced double-stranded DNA breaks in non-telomeric human DNA (Bradshaw, Stavropoulos et al. 2005). Further, its overexpression inhibits break-induced phosphorylation of ATM signalling targets (Bradshaw, Stavropoulos et al. 2005). This association may serve to protect double-stranded breaks from the action of exonucleases or may inhibit the activation of the ATM kinase and the subsequent DNA damage response. It is also possible the other telomere binding proteins mediate non-telomeric function like TRF2. Accordingly studies are underway to in investigate novel roles of TRF1, Pot1 and other telomere binding proteins.

In contrast, Pot1 has been shown to bind the single-stranded G-rich sequence, but not the C-rich sequence, consistent with the hypothesis that it preferentially binds the single-stranded 3' overhang portion of the telomere (Baumann and Cech 2001). Overexpression of Pot1 induces telomere lengthening in a telomerase-dependent way, and accordingly its inhibition by antisense oligonucleotides induces telomere shortening (Colgin, Baran et al. 2003), reduces 3' overhang signal, and increases the frequency of anaphase bridges (Kondo, Oue et al. 2004). The X-ray crystallographic structure of the N-terminal half of human POT1 (hPOT1) bound to a telomeric decanucleotide has been elucidated and demonstrated that the protein binds telomeric DNA via two binding folds, one of which forms a pocket which buries the terminal 3' guanine base to physically cap the end of the chromosome (Lei, Podell et al. 2004). Pot1 has been shown to functionally regulate telomerase's access to the end of the telomere. When

capped, the DNA-protein structure inhibits association of telomerase to the telomere. However, when hPOT1 is bound one telomeric repeat before the 3'-end an 8-nucleotide telomeric sequence is exposed permitting telomerase's access to the telomere and allowing elongation (Lei, Zaug et al. 2005). Therefore, depending on the location of hPOT1 binding on the telomere, it can either inhibit telomere elongation or generate a substrate for telomerase (Lei, Zaug et al. 2005).

The disruption of telomere binding proteins causes telomere dysfunction in a length-independent way. Uncapped telomeres have now been associated with DNA damage response factors such as 53BP-1, γ-H2AX, Rad17, ATM and Mre11 (Takai, Smogorzewska et al. 2003). The binding proteins cap the telomere in dynamic ways to prevent genomic instability and to regulate the activity of telomerase. Uncapping of the telomeres, either by critical shortening or inhibition of binding proteins induces a DNA damage response (de Lange 2002).

As mentioned, at each round of cellular replication DNA lagging strand synthesis remains incomplete. Telomeres cap the ends of linear chromosomes with non-coding sequences, providing a strategy to ensure that coding sequences are not lost, but they do not resolve the issue of incomplete DNA replication. Accordingly, small amounts of telomeric DNA are lost at each round of cellular division, and thus telomeres shorten with every round of replication (Harley, Futcher et al. 1990).

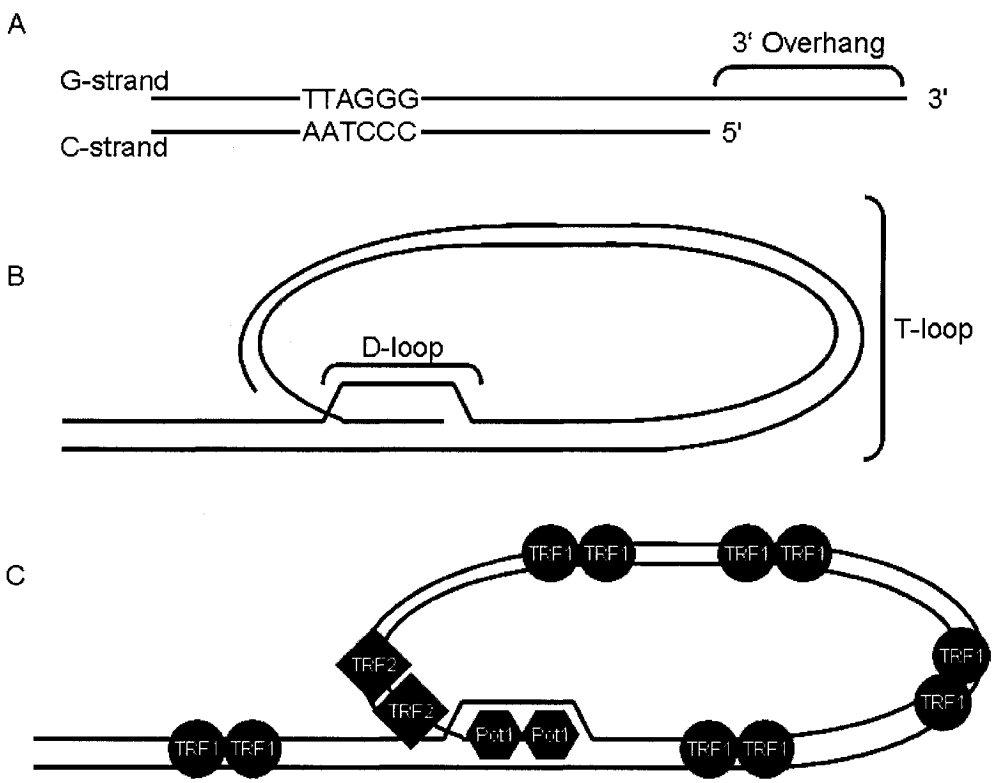

A
3' Overhang
G-strand
TTAGGG
3'
C-strand
AATCCC
5'
B
D-loop
T-loop
C
TRF1
TRF2
Pot1

Figure 2- General Telomere Structure. A, Telomeres are non-coding DNA sequences that cap the ends of chromosomes. B, The G-strand terminates in a 3-prime overhang that loops and invades double stranded telomeric DNA generating the telomere-loop (t-loop) and displacement-loop (d-loop). C, Telomere binding proteins can induce and further stabilize this looped structure, TRF1 localizes to double stranded telomeres, TRF2 localizes preferentially to the junction at d-loop invasion, and Pot1 found at the single stranded G-strand overhand.

This continual and gradual loss of telomeric DNA has been proposed to be a mechanism by which cells can 'count' their number of divisions: a putative 'Replicometer'. This idea was first proposed by Leonard Hayflick who is credited with the observation that normal human cells, when grown *in vitro*, have a finite replicative lifespan. As a student, Hayflick demonstrated that cells, when explanted from tissues and grown in cell culture conditions did not (as widely thought at the time) have infinite growth capacity (Hayflick and Moorhead 1961). Rather the cells in his experiments grew for a reproducible number of population doublings before they stopped dividing. Even cryogenically frozen cells that were thawed and returned to tissue culture systems retained their limited capacity for the same number of cell divisions suggesting that their limited growth potential was not determined by the simple lapse of time. We now call the point at which normal cells cease proliferating the Hayflick limit, and associate it with a cellular state called senescence. Primary human cells will proliferate in cell culture conditions for a certain number of population doublings, progressively losing telomeric DNA (Harley, Futcher et al. 1990). Eventually, telomeres shorten to a point where they are recognized as damaged DNA and cells stop proliferating. This proliferative arrest is characterized by the stabilization of p53, the activation of the DNA damage response mechanisms, and the hyperphosphorylation of the cell cycle control protein Rb (Sherr and McCormick 2002; Smogorzewska and de Lange 2002). Inactivation of cell cycle control checkpoints allows further proliferation beyond the Hayflick limit and generates dysfunctional telomeres which, as well as being recognized as DNA damage, are prone to G-overhang loss, end-to-end chromosomal fusions and general genomic instability (Wright, Pereira-Smith et al. 1989; Shay, Pereira-Smith et al. 1991; Counter, Avilion et al. 1992). Therefore, without the presence or activation of a mechanism to either maintain or elongate telomeres, cells cannot grow indefinitely. Interestingly, there does exist a highly conserved mechanism by which cells can maintain their telomeres, that being the enzyme telomerase. As discussed in the next section, telomerase has the ability to elongate telomeres and can, in fact, impart cellular

immortality when transfected into certain cell types (Bodnar, Ouellette et al. 1998).

1.5 Telomerase

The enzyme that maintains telomeres was first discovered in the ciliate *Tetrahymena* by Carol Greider and Elizabeth Blackburn (Greider and Blackburn 1985). In subsequent studies the enzyme initially termed 'telomere terminal transferase' but now known as telomerase, was identified as a ribonucleoprotein consisting of a reverse transcriptase protein component (denoted as TERT, telomerase reverse transcriptase, hTERT in humans) and an RNA template component (denoted as TR, telomerase RNA, hTR in humans). Within a few years telomerase enzymes were discovered in numerous other organisms including humans (Morin 1989).

The principle function of telomerase is to catalyze *de novo* telomeric repeat synthesis at the 3' end of DNA (Greider and Blackburn 1987). Telomerase elongates the G-strand overhang by reverse transcribing the nucleotide template sequence encoded by its TR (Yu, Bradley et al. 1990). In humans, telomerase synthesizes multiple TTAGGG sequence repeats in a reiterative way: binding the 3' end of the DNA substrate, elongating the DNA, translocating to the newly synthesized 3' end, and repeating the process (Greider 1991). Telomerase activity can be characterized into two types of enzymatic processivity: type I and type II. Type I processivity refers the addition of individual nucleotides within a single telomeric repeat (for example, in vertebrates the addition of individual nucleotides within the TTAGGG repeat) (Peng, Mian et al. 2001). Type II processivity refers to the unique ability of telomerase to add multiple and sequential telomeric repeats to the end of the telomere (for example, in vertebrates the ability of telomerase to add multiple T_2AG_3 sequences) (Morin 1989; Greider 1991; Hardy, Schultz et al. 2001). Once telomerase has elongated telomeres, traditional DNA polymerases can then copy the G-stand in the 5' to 3' direction (Autexier and Greider 1996). Finally, processing of both C- and G-strands by unidentified

nucleases appears to occur to generate the structurally important G-overhang (Sfeir, Chai et al. 2005).

Telomerase activity can be measured in the laboratory using a polymerase chain reaction-based assay called the Telomeric Repeat Amplification Protocol (TRAP) (Kim, Piatyszek et al. 1994). Using this sensitive assay, limited numbers of cells and amounts of tissue can be screened for the presence of telomerase activity (Kim and Wu 1997). What was exciting to discover was that most normal human tissues do not display telomerase activity, whereas a large percentage of all cancer tissues and cell lines tested demonstrate activity (Kim, Piatyszek et al. 1994). Accordingly, much interest has been generated in using telomerase as an anti-cancer target in humans, or telomerase activity as a marker for the early detection of cancers (as further discussed below).

Results from two studies now suggest that telomerase mediates elongation of the shortest telomeres within a chromosome population. In the first study, late generation TR (mTR in mice) knockout mice that display short telomeres were crossed with a mTR heterozygous strain with long wild-type telomeres (Hemann, Strong et al. 2001). Crossing the strains to reintroduce an active telomerase enzyme did not induce global telomere elongation, but rather a reduction in the total number of telomeres that lacked telomeric signals indicating that telomerase was targeted to elongate the shortest telomeres. In the second study, telomerase RNA knockout yeasts with short telomeres were mated to yeasts expressing an active telomerase enzyme (Teixeira, Arneric et al. 2004). The direct analysis of individual telomere lengths demonstrated that telomerase does not act on every telomere during a particular cell cycle, but rather acts preferentially to elongate the shortest telomeres. Further, the frequency of elongation steadily increases as telomere length decreases. When the same experiments were performed with yeasts double deficient for telomere regulatory proteins and TR, telomeres of normal length were also elongated indicating a role for telomere binding proteins in modulating the accessibility of the telomerase enzyme to the telomeric substrate. Taken together, a model now exists whereby telomeres exist in one of two conformational states: telomerase extendible and non-extendible depending

on telomere length. When a telomere is of sufficient length, regulatory proteins, such as Pot1, and protective structures, such as the t-loop and/or G-quartets, inhibit the association of telomerase with the end of the telomere. In contrast, when telomeres are short they become accessible to the telomerase enzyme due to loss of the above mentioned structures or disassociation of telomere binding proteins or their regulators (Teixeira, Arneric et al. 2004).

The active *in vivo* telomerase complex has long been suspected to be a multimer of telomerase enzymes and associated proteins. Initial observations that affinity-purified catalytically active human telomerase sedimented at a molecular weight of ~550 kilodaltons (kDa) showed a striking difference to telomerase sedimented from nuclear extracts of HeLa cells which displayed a molecular weight of ~1000 kDa (Schnapp, Rodi et al. 1998). Coupled to the observation that hTERT and hTR, with a minimal molecular weight calculated at ~280kDa, are sufficient to reconstitute telomerase activity, argued strongly that *in vivo* the telomerase complex consists of multimerized hTERTs and hTRs and other associated proteins (Weinrich, Pruzan et al. 1997). Based on recent data we now have a working model whereby two hTERT-hTR complexes multimerize to generate an active telomerase complex (Beattie, Zhou et al. 2001; Wenz, Enenkel et al. 2001; Moriarty, Huard et al. 2002; Moriarty, Marie-Egyptienne et al. 2004). Other data indicate that hTERT and hTR can associate with numerous other proteins, some specific for telomerase and others common binding partners of many ribonucleoproteins. Disruption of these protein interactions with hTERT or hTR could have the consequences of improper enzyme assembly, telomerase mislocalization, and telomere length changes (Harrington 2003).

1.6 Alternative Mechanisms to Maintain Telomeres

Though the vast majority of human cells that maintain their telomeres do so by the expression of telomerase, it is not the only mechanism by which cells can solve the end replication problem. At least three other mechanisms exist: The first being the elimination of chromosome ends as exemplified by the circular chromosomes of bacteria. The second is the use of retrotransposable elements that

mobilize from more centromeric DNA sequences and translocate to the telomeres to template the addition of telomeric repeats (Biessmann and Mason 2003). Amazingly, this phenomenon has thus far only been observed in *Drosophila*, the model organism used by Muller in his original work to differentiate between newly broken DNA ends and the relatively stable telomeres.

A third mechanism of telomere maintenance was identified in the yeast *S. cerevisiae*. While yeasts normally express telomerase to maintain telomeres, telomerase inactivation results in cellular death and the rare emergence of survivors that are able to maintain telomeres through a recombination based mechanism (Teng and Zakian 1999; Teng, Chang et al. 2000; Chen, Ijpma et al. 2001). Based on the telomere structures, these survivors can be subdivided into two types: I and II. Type I survivors are characterized by short telomeres maintained via recombination in the subtelomeric region of the chromosome, while type II survivors demonstrate long and heterogeneous telomeres which are likely maintained by inter-telomeric recombination (Teng and Zakian 1999; Teng, Chang et al. 2000; Chen, Ijpma et al. 2001). Though ~85% of tumour cells maintain their telomeres through the expression of telomerase, 20-30% of human immortalized cell lines and 10-15% of human tumours maintain their telomeres by a different mechanism, referred to as alternative lengthening of telomeres (ALT) (Bryan, Englezou et al. 1995; Bryan, Englezou et al. 1997; Bryan and Reddel 1997). Characterization of Human ALT cells reveals similarities with yeast type II survivors; their telomeres are very long (up to 50kb) and heterogeneous with rapid telomeric shortening and lengthening events (Murnane, Sabatier et al. 1994). They are further characterized by the presence of ALT-associated promyelocytic leukemia (PML) body (APB) nuclear structures which contain telomeric DNA, proteins involved in recombination, the PML protein, and the telomere-binding proteins TRF1 and TRF2 (Yeager, Neumann et al. 1999; Grobelny, Godwin et al. 2000; Wu, Lee et al. 2000). Finally, evidence exists suggesting that human ALT cells maintain their telomeres by homologous recombination, inter- and/or intra-telomere coping, or a roll-and-spread mechanism involving extrachromosomal telomeric DNA circles (Dunham,

Neumann et al. 2000; Cesare and Griffith 2004). Whether these mechanisms are exclusive or occur in a co-ordinated fashion is still unclear.

1.7 Telomerase and Cancer

The fact that telomerase activity is predominantly observed in cancer cells has generated a lot of excitement regarding the use of telomerase activity as a clinical marker of cancer, or targeting telomerase for highly selective anti-cancer treatment (Autexier and Greider 1996; Huard and Autexier 2002). Unfortunately, to date anti-telomerase therapies remain experimentally and clinically unproven; however the possibility of targeting telomerase to treat cancer remains a very exciting therapeutic strategy.

Of principal interest in cancer research is finding exploitable differences between normal tissues and cancer cells that may lead to better, more targeted anti-cancer therapies. One such difference is unlimited proliferative capacity (referred to as cellular immortality) characteristic of cancer cells compared to the limited capacity (referred to as cellular mortality) of most normal cells. Another difference is the ability of cancer cells versus the inability of mortal cells to maintain telomeres. Accordingly, telomeres of mortal cells shorten by 50-250 bps at each round of replication due to the end replication problem. In contrast, telomere length and integrity of cancer cells is maintained. In some mortal cell lines, expression of telomerase alone is sufficient to immortalize cells and impart to them unlimited proliferative capacity (Bodnar, Ouellette et al. 1998; Vaziri and Benchimol 1998). Together with the observation that all cancer cells activate a mechanism to maintain telomeres (either by telomerase expression or ALT) suggests that telomere maintenance is a requisite for the development of cancer. Therefore, inhibiting/inactivating telomerase may reverse cellular immortality and provide an effective strategy to selectivity inhibit cancer cell growth. Further, this anti-cancer strategy should have relatively minor side-effects as the normal cells which display telomerase activity (stem cells, germline cells, and a small percentage of somatic cells) have longer telomeres and slower cellular doubling rates (Shay and Bacchetti 1997).

A number of strategies to inhibit telomerase have been tested, including the use of reverse transcriptase inhibitors, nucleotide analogues, antisense oligodeoxynucleotides (ODN) or siRNA against hTERT or hTR, catalytically inactive dominant negative (DN) hTERT variants, or treating cells with highly selective pharmacological telomerase inhibitors (Corey 2002). The results from these experiments demonstrate that inhibiting/inactivating telomerase induces telomere shortening to a critical length, the activation of DNA damage responses, cellular senescence, and/or apoptosis (Parkinson 2003). Unfortunately these effects are often only observed after prolonged anti-telomerase treatment (sometimes after as many as 120 population doublings) (Damm, Hemmann et al. 2001), and in cells with initially short telomeres (Hahn, Stewart et al. 1999; Zhang, Mar et al. 1999; Delhommeau, Thierry et al. 2002; Bechter, Zou et al. 2004). The lag-time required for anti-telomerase therapy to exert its effects is exemplified by mTR knockout mice strains, which do not display any phenotype until generations 4 to 6 (Blasco 2002). Though laboratory mice telomeres are much longer than most human telomeres, the results indicate that telomere shortening can be tolerated for numerous population doublings until telomeres become short, dysfunctional, and elicit a DNA damage response (Blasco 2002). Similar observations have been made from individuals with defects in telomere maintenance where disease anticipation occurs in successive generations of individuals harbouring the genetic defect (Vulliamy, Marrone et al. 2004). Further, the selective pressure for telomere maintenance is strong and cellular survivors often emerge after prolonged anti-telomerase treatment (Zhang, Mar et al. 1999; Delhommeau, Thierry et al. 2002). Therefore, anti-telomerase therapies alone are not likely to serve as effective treatments against cancer; however anti-telomerase therapy in combination with traditional chemotherapeutics does hold the possibility for effective and targeted anti-cancer treatment (Parkinson 2003).

Evidence to support this idea comes from experiments demonstrating that cellular sensitivity towards DNA damaging agents, UV irradiation, serum deprivation and signal transduction inhibitors is increased upon telomerase inhibition/inactivation (Kondo, Kondo et al. 1998; Goytisolo, Samper et al. 2000;

Wong, Chang et al. 2000; Lee, Rudolph et al. 2001; Ludwig, Saretzki et al. 2001; Tauchi, Nakajima et al. 2002; Chen, Koeneman et al. 2003; Tentori, Portarena et al. 2003). Further, data from these experiments demonstrates that the lag time required for anti-telomerase therapy to exert its effects is much reduced (in as little time as 14 days) (Chen, Koeneman et al. 2003). Unfortunately, the major drawback of these experiments is the strategy by which telomerase is inhibited. Though antisense ODNs are promising therapeutic compounds, the drug category as a whole remains largely unproven clinically (Lane 2005). This point is exemplified by the fact that there still only exists one antisense compound approved for clinical use: Vitravene®, an antisense compound used for the treatment of cytomegalovirus retinitis, and which is applied directly as eye drops. This route of administration avoids the significant difficulties of stability and cellular uptake which commonly hinder the activity of antisense compounds used to treat diseases that require systemic routes of delivery (Lane 2005). Therefore, the search for small molecule inhibitors of telomerase was undertaken by a number of groups leading to the discovery and characterization of a variety of different pharmacological agents (Damm, Hemmann et al. 2001).

1.8 G-quadruplex Interacting/Stabilizing Agents

The ability of G-rich DNA to adopt non-Watson-Crick G-quadruplex conformations has lead to the development of compounds that selectively target and stabilize these G-quartet structures. In theory, if G-quadruplexes exist at telomeres *in vivo* then their structural stabilization may render the telomere inaccessible to telomerase, may hinder ALT pathways of telomere maintenance, or obstruct DNA polymerases from copying the entire chromosome (Riou 2004). This class of compound should be relatively selective for cancer cells, as G-quadruplex stabilization should inhibit telomere maintenance in telomerase positive cells, or hinder DNA replication of cancer cells (versus slow or non-replicating normal somatic cells). A number of natural and synthetic G-quadruplex interacting compounds have now been isolated and the consequence of treating cells with these compounds can be telomere shortening, reduced

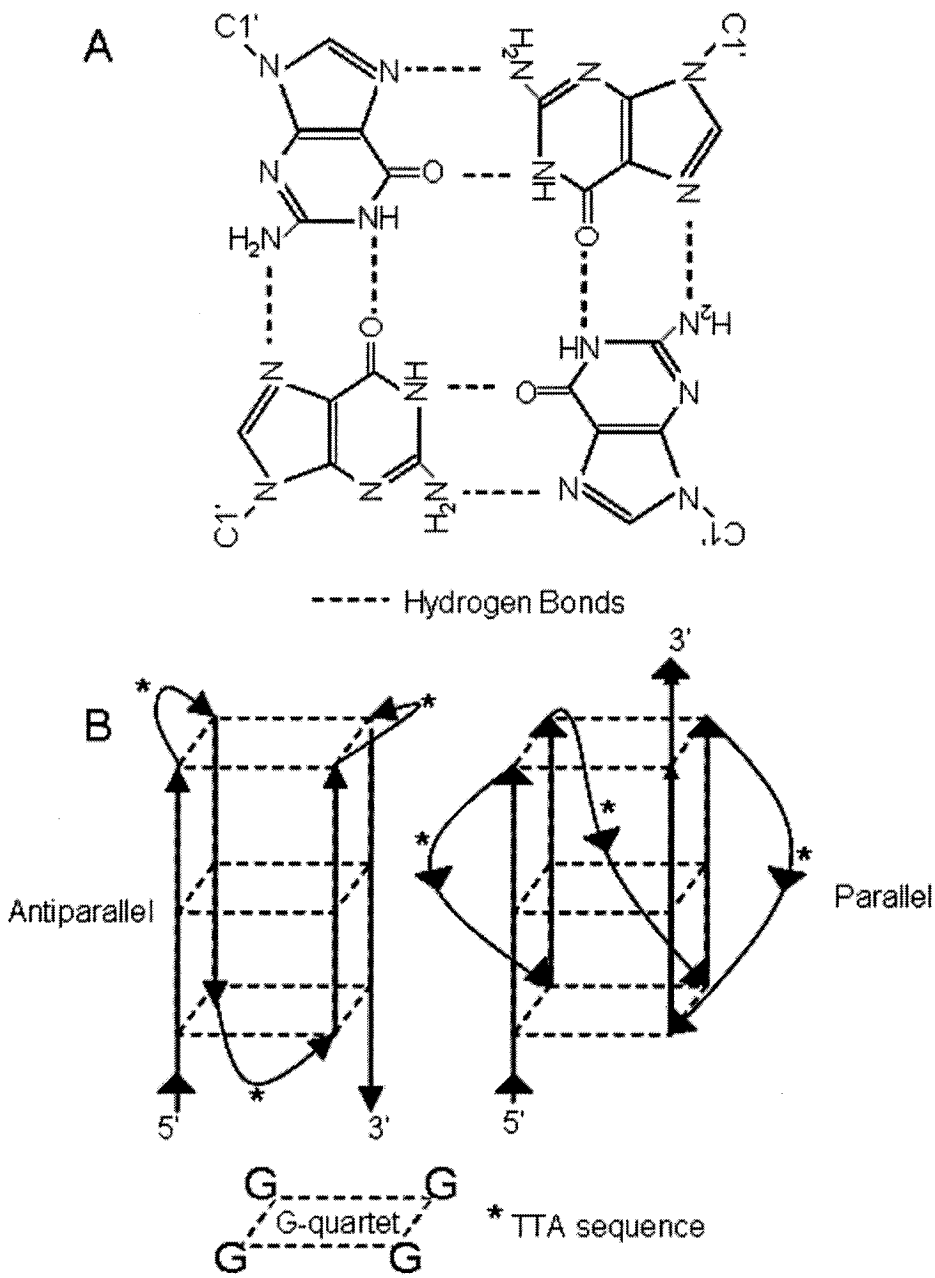

A
C1'
N
O
NH
H_2N
HN
N^2H
H_2N
Hydrogen Bonds
B
3'
5'
Antiparallel
Parallel
G
G-quartet
* TTA sequence

Figure 3- Structure of G-quadruplexes. A, Spatial arrangement of 4 guanines hydrogen bonding in a G-quartet. B, Putative antiparallel and parallel conformations of G-quadruplexes. Boxes with dashed lines depict the G-quartet and solid lines depict the DNA backbone with arrows showing 5' to 3' directionality. * represents TTA sequence of telomeric repeat not involved in G-quartet hydrogen bonding.

proliferative capacity and apoptosis in telomerase positive cells demonstrating that G-quadruplex stabilizing agents can target telomeres and inhibit telomerase (Riou 2004). However, a number of studies demonstrate that G-quadruplex interacting agents can reduce cellular proliferation and viability independent of telomere length and telomerase expression. Notably, ALT cells are susceptible to G-quadruplex stabilizing agent treatment without detectable telomere shortening (Pennarun, Granotier et al. 2005). Further, it now appears likely that G-quartet structures can also form in non-telomeric DNA including the promoter regions of oncogenes such as c-*myc*, (Ambrus, Chen et al. 2005). G-quadruplex formation and stabilization in these promoter regions leads to transcriptional inhibition of these oncogenes, decreased proliferative capacity, and apoptosis (Siddiqui-Jain, Grand et al. 2002). Therefore, current data suggests that G-quadruplex stabilizing agents can target telomeres and inhibit telomerase, but can also target non-telomeric DNA to inhibit oncogene expression.

1.9 BIBR1532 and Small Molecule Inhibitors of Telomerase Catalysis

Telomerase catalytic activity can be characterized by two types of enzymatic processivity: type I which is common to all DNA and RNA polymerases, and type II which is unique to telomerases. Nucleoside analogues hinder type I processivity, and therefore result in generalized inhibition of DNA and RNA synthesis (Pandit and Bhattacharyya 1998). Inhibiting type II processivity however, would result in highly selective inhibition of telomerase activity without affecting other DNA or RNA polymerases. This type of inhibition, in theory would be highly selective for telomerase positive cells and therefore targeted towards cancer cells (Shay and Bacchetti 1997). A number of such compounds exist, including the Boerhinger Ingelheim compound BIBR1532 and related analogues (Figure 4). BIBR1532 is a highly selective inhibitor of telomerase type II processivity, demonstrating *in vitro* IC_{50} concentrations in the nanomolar range, and very little enzymatic inhibition against a panel of DNA and RNA polymerases, helicases and the HIV-1 reverse transcriptase (Damm,

Hemmann et al. 2001). Treatment of telomerase positive cells with BIBR1532 leads to telomere shortening and eventual growth arrest, an effect that is reversible upon release from treatment (Damm, Hemmann et al. 2001). The mode of action of BIBR1532 has been investigated. While the exact drug binding site on the telomerase RNP is still unknown, BIBR1532 demonstrates properties of a non-competitive inhibitor of telomerase activity suggesting a drug-binding site distinct from those of the deoxyribonucleotides and the DNA primer (Pascolo, Wenz et al. 2002). Finally, unlike G-quadruplex interacting agents, BIBR1532 treatment of normal human lung fibroblasts and ALT cell lines does not result in telomere shortening or proliferative arrest demonstrating that BIBR1532 is a highly selective inhibitor of telomerase catalytic activity (Damm, Hemmann et al. 2001).

Unfortunately, like all other methods of telomerase inhibition the effects of BIBR1532 treatment are only apparent after prolonged treatment, exemplified by the fact that when fibrosarcoma, breast and prostate carcinoma cell lines were treated with BIBR1532, a minimum of 75 population doublings of treatment were required before cellular proliferation slowed or stopped (Damm, Hemmann et al. 2001). This result indicates that, like other telomerase inhibitors, BIBR1532 will likely not serve as a useful compound for single agent anti-cancer therapies. However, unlike antisense or gene therapy strategies of telomerase inhibition, BIBR1532 is potentially more clinically relevant as small molecule enzymatic inhibitors are successfully used for the treatment of many diseases, though to date there are no studies addressing the stability, toxicity or bioavailability of BIBR1532 in humans (Lane 2005).

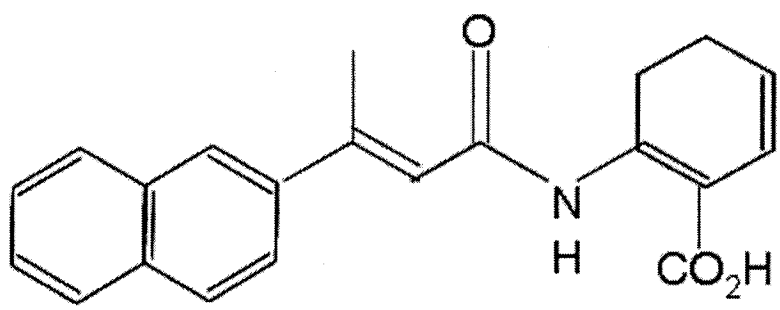

O
N
H
CO2H

Figure 4- Chemical structure of the small molecule inhibitor of telomerase type II processivity: BIBR1532 (Boehringer Ingelheim, Austria). BIBR1532 is a highly selective inhibitor of human telomerase catalytic activity and demonstrates very little inhibition against a panel of human DNA and RNA polymerases, and the HIV-1 reverse transcriptase (Damm, Hemmann et al. 2001).

1.10 Telomerase, other functions?

Recent evidence suggests strongly that telomerase mediates other effects in addition to telomere maintenance. Overexpression of hTERT protects cells against DNA damaging agents, TERT mRNA is induced in post mitotic rat neurons upon cerebral artery occlusion, and neurons of transgenic animals overexpressing TERT are protected from ischemia and N-methyl-D-aspartate (NMDA)-induced neurotoxicity (Akiyama, Yamada et al. 2002; Kang, Choi et al. 2004). In yeast, a direct role for telomerase in the DNA damage response has been determined, with telomerase having the ability to 'heal' double stranded DNA breaks with telomeric repeats (Kramer and Haber 1993). Further, constitutive TERT expression in mouse thymocytes generates increased incidence and dissemination of T-cell lymphomas, which infiltrate non-lymphoid organs (including the liver, lungs and kidneys) in experimental but not control mice. Transgenic and control animals showed intact telomere function and similar telomere length, suggesting that this phenotype was not telomere length-specific (Canela, Martin-Caballero et al. 2004). Finally, GM847 ALT cells, which maintain telomeres by a telomerase-independent mechanism, are capable of sub-cutaneous tumour formation in mice when expressing both the oncogene H-RASV12 and wild-type (wt) hTERT. Importantly, an HA-tagged hTERT variant, which is biologically defective for telomere maintenance, also conferred tumour growth capacity to GM847 H- RASV12 cells, whereas no tumour formation was observed after the injection of either GM847 or GM847 H-RASV12 cells (Stewart, Hahn et al. 2002). Recently similar experiments have contradicted these results demonstrating that the ALT cells incapable of generating tumours in sub-cutaneous implantation models were, in fact, capable of growing into tumours when implanted beneath the kidney capsule of immunodeficient mice (Sun, Chen et al. 2005). hTERT likely promotes the initial survival of sub-cutaneously injected cells because the authors were able to demonstrate that hTERT-negative sub-cutaneous tumours developed when cells were serially transplanted from a tumour growing in the kidney (Sun, Chen et al. 2005). Finally, a noncanonical function of TERT has now been identified in epithelial stem cell proliferation in

the skin of transgenic mice. Importantly, inducible expression of mTERT in mTR+/+, mTR+/- *and* mTR-/- genetic backgrounds drove quiescent, multipotent stem cells in the hair follicle bulge region into the cell cycle demonstrating that the template RNA component is not required for TERT to promote stem cell proliferation (Sarin, Cheung et al. 2005). Therefore, experimental results suggest that telomerase expression confers increased protection from DNA damaging agents, and increases metastatic potential, survival and proliferation *in vivo* via a mechanism which may be independent of its telomere maintenance function. If this hypothesis is true, than targeting telomerase by antisense ODNs, siRNA or pharmacological inhibitors of catalytic activity may further serve to sensitize telomerase positive tumour cells to treatment or reduce their metastatic and invasive potential. Support for this hypothesis comes from recent findings which demonstrate that sustained inhibition of hTERT expression alters the chromatin state to a configuration that hinders the activation of the DNA damage response and sensitizes cells to ionizing radiation. These results suggest that hTERT mediates an important function in the response to, and repair of, genotoxic damage (Masutomi, Possemato et al. 2005).

1.11 Chemotherapeutic Drug Resistance

The single largest obstacle to the effective treatment of most cancers is chemotherapeutic drug resistance. Drug resistance is universally fatal once developed, and usually occurs after initial rounds of treatment. Unfortunately, drug-resistant cancers are often resistant to the compounds to which they were exposed, and also to a broad range of chemically related and unrelated drugs (Baird and Kaye 2003). While mutations in cellular targets that confer drug-resistance against specific compounds have been discovered, it is thought that the majority of cancers achieve drug-resistance by increasing drug-efflux, decreasing drug influx, and activating detoxification systems (Baird and Kaye 2003). Drug-resistance is correlated with increased expression of transmembrane ATP-dependent efflux pumps, such as p-glycoprotein (Pgp) and multidrug resistance-associated protein (MRP) (Leslie, Deeley et al. 2005), and with increased

glutathione S-transferase (GST) activity (Salinas and Wong 1999). GST enzymes catalyze the conjugation of the glutathione thiol-group with reactive sites of drugs to generate conjugated products which are less biologically active, more water soluble and easily excluded from the cell via transport pumps (including MRP) (Stavrovskaya 2000).

Drug-resistant reversal strategies are actively being investigated; however the promise of single agent approaches to sensitize drug-resistant cells to treatment has not been fulfilled (Baird and Kaye 2003). The discovery that efflux pumps could be inhibited with high or low selectivity by small interfering molecules lead to the development of hundreds of such compounds with the capacity to inhibit Pgp. Unfortunately none of these compounds demonstrated significant effects in clinical trials and to our knowledge there are currently no reversal agents clinically available. It is now thought that the mechanisms that lead to drug-resistance are redundant and sensitization of cells will only occur if multiple cellular processes are simultaneously targeted (Baird and Kaye 2003).

1.12 Drug-Resistance and the Role of Telomeres and Telomerase

In the context of cellular survival, a number of manuscripts have reported that drug-resistant and radio-resistant phenotypes correlate with changes in basal telomerase activity and/or basal telomere length (further discussed in Chapter 2). Additionally, there are now at least three reports that describe increased telomerase activity upon cellular treatment of DNA damaging agents, though other reports make contrasting observations (Sato, Mizumoto et al. 2000; Moriarty, Dupuis et al. 2002; Klapper, Qian et al. 2003; Jeyapalan, Leake et al. 2004). The possibility exists that in human cells telomerase mediates a protective role against the effects of DNA damage and that changes in telomerase and telomere regulation may aid in the development or maintenance of a drug-resistant phenotype. Therefore, we were interested in studying the role telomerase mediates in cellular resistance to cell death. To achieve this, we characterized basal telomere length and telomerase activity in paired drug-sensitive and drug-resistant cell lines (including parental HL60/WT and etoposide resistant

HL60/MX2 human leukemia cells, and parental MCF-7/WT, adriamycin resistant MCF-7/Adr^R, and melphalan resistant MCF-7/Mln^R human breast cancer cells), characterized telomerase activity of HL60/WT and HL60/MX2 cells in response to chemotherapeutic treatment at equivalent doses; and determined if treatment with BIBR1532 rendered the drug-sensitive and resistant cells more susceptible to chemotherapeutic treatment by monitoring proliferative capacity and colony forming ability of BIBR1532-treated cells.

We observed that pharmacological telomerase inhibition can sensitize drug-resistant and drug-sensitive cells to chemotherapeutic treatment in a telomere length dependent fashion. Continuous treatment of BIBR1532 inhibited telomerase activity, induced progressive telomere shortening, and progressively decreased the proliferative capacity, or colony forming ability of all cell lines tested except MCF-7/Adr^R. These cells were insensitive to BIBR1532 treatment and accordingly did not demonstrate any telomere shortening or increased cellular sensitivity to doxorubicin treatment. We investigated whether this insensitivity to BIBR1532 was mediated by a change in the telomerase enzyme of these cells, however BIBR1532 effectively inhibited telomerase activity when added *in vitro* to both MCF-7/WT and MCF-7/Adr^R cell extracts suggesting that MCF-7/Adr^R cells are likely resistant to BIBR1532 via a mechanism involving increased drug-efflux or metabolism, or decreased drug-influx. Our novel results suggest that, in a telomere length dependent fashion, pharmacological telomerase inhibition may aid in the clinical treatment of both drug-sensitive and drug-resistant malignancies. Finally, our observations suggest that this class of pharmacological agent merits further investigation against both solid and haematological tumours.

Chapter 2- Pharmacological Telomerase Inhibition can Sensitize Drug-Resistant and Drug-Sensitive Cells to Chemotherapeutics

2.1 Abstract

Effective strategies to reverse or prevent chemotherapeutic resistance are required before cancer therapies can be curative. Telomerase is the ribonucleoprotein responsible for *de novo* synthesis and maintenance of telomeres, and its activity is predominantly observed in cancer cells. The telomerase enzyme has been successfully inhibited or inactivated to sensitize cells to cellular stresses; however no studies have yet determined the effect of combining a pharmacological inhibitor of telomerase catalysis and traditional chemotherapeutics for the treatment of drug-sensitive or drug-resistant cancers. Here we describe the effect of BIBR1532, a small molecule inhibitor of telomerase catalytic activity, on drug-resistant leukemia and breast cancer cells and their parental counterparts when treated in combination with chemotherapeutics. We observed that BIBR1532 treated cells show progressive telomere shortening, decreased proliferative capacity and sensitization to chemotherapeutic treatment. These effects are telomere length dependent as cells insensitive to BIBR1532, or cells released from telomerase inhibition did not demonstrate changes in growth ability or drug sensitivity. Our novel observations suggest that pharmacological telomerase inhibition in combination therapy may be a valid strategy for the treatment of both drug-sensitive and drug-resistant cancers.

2.2 Introduction

Chemotherapeutic drug resistance remains a major obstacle to the effective treatment and cure of almost all cancers (Baird and Kaye 2003). Numerous strategies to overcome drug resistance are currently being explored but none have demonstrated success in the clinic (Robert and Jarry 2003). Sensitization of resistant tumours to drug treatment will likely require interference with multiple cellular processes (Mattern 2003). Therefore, the combination of resistant reversal strategies may have substantial impact on the overall survival of many cancers.

Telomerase is the reverse-transcriptase enzyme whose primary function is the maintenance and *de novo* synthesis of telomeres at the ends of linear chromosomes (Cech 2004). Telomerase activity is rarely present in normal somatic cells, but is observed in ~85% of all cancer cells tested making the telomerase enzyme an attractive target for anticancer therapeutics (Corey 2002; Cech 2004). Our lab, and others, have reported that telomerase activity is upregulated in human cell lines upon treatment with DNA damaging chemotherapeutics suggesting that telomerase may play an active role in the response to DNA damaging agents (Sato, Mizumoto et al. 2000; Moriarty, Dupuis et al. 2002; Klapper, Qian et al. 2003; Jeyapalan, Leake et al. 2004). Furthermore, telomerase expression in normal human fibroblasts enhances DNA repair activities (Shin, Kang et al. 2004). Telomerase has also been implicated in mediating other effects in addition to telomere maintenance, such as suppression of apoptosis (Zhang, Chan et al. 2003), promotion of *in vivo* proliferative capacity (Stewart, Hahn et al. 2002), and protection against ischemia and NMDA-induced neurotoxicity (Kang, Choi et al. 2004).

The consequence of telomerase inhibition in immortal human cells is telomere shortening and eventual growth arrest and/or apoptosis (Hahn, Stewart et al. 1999; Corey 2002). Unfortunately, these effects are often observed only in cells with initially short telomeres, and after prolonged anti-telomerase treatment. Further, strong selective pressure to maintain telomeres can lead to the reactivation of telomerase, either by transcriptional upregulation or loss of the

inhibitor (Zhang, Mar et al. 1999; Delhommeau, Thierry et al. 2002), or the activation of an alternative lengthening of telomere (ALT) mechanism (Bechter, Zou et al. 2004). These observations suggest that telomerase inhibition alone may not be an effective strategy for cancer treatment (Corey 2002). However, combining telomerase inhibition with chemotherapeutic treatment may prove more effective than either approach on its own. Indeed, studies have demonstrated that telomerase inhibition or inactivation generates increased cellular sensitivity to UV irradiation (Goytisolo, Samper et al. 2000; Wong, Chang et al. 2000), DNA damaging agents (Kondo, Kondo et al. 1998; Lee, Rudolph et al. 2001; Ludwig, Saretzki et al. 2001; Chen, Koeneman et al. 2003; Tentori, Portarena et al. 2003), and the tyrosine kinase inhibitor imatinib (Tauchi, Nakajima et al. 2002). Conversely, telomerase overexpression confers cellular protection from apoptosis by serum deprivation (Akiyama, Yamada et al. 2002) and DNA damaging agents (Lu, Fu et al. 2001).

Telomerase inhibition is usually achieved via gene knockout, antisense oligonucleotides or dominant-negative forms of the telomerase enzyme. While these strategies are specific, one major drawback to these approaches is their clinical feasibility and/or utility (Lane 2005). Therefore, we were interested in determining the effect of combining the treatment of a small molecule pharmacological inhibitor of telomerase catalytic activity, BIBR1532, and traditional chemotherapeutics on paired drug-sensitive and drug-resistant cell lines. BIBR1532 is a highly selective non-competitive, non-nucleoside pharmacological inhibitor of telomerase catalytic activity, demonstrating *in vitro* IC_{50} concentrations in the nanomolar range (Damm, Hemmann et al. 2001; Pascolo, Wenz et al. 2002).

This is the first study to address the consequence of using a highly selective and potent pharmacological inhibitor of telomerase catalytic activity in combination with chemotherapeutics; moreover, there have been no reports addressing the feasibility of using such a pharmacological telomerase inhibitor to sensitize drug-resistant cells to traditional therapies. We hypothesized that BIBR1532 would inhibit telomerase and sensitize both drug-sensitive and drug-

resistant cell lines to traditional chemotherapeutics such as etoposide, melphalan or doxorubicin; three commonly prescribed DNA damage inducing chemotherapeutics used for the treatment of cancers of the bone, lung, breast, brain, blood and ovaries (http://www.nlm.nih.gov/medlineplus/druginformation.html). To test this, we first characterized basal telomerase activity and basal telomere length in drug-resistant human promyelocytic leukemia (HL60/MX2) and breast cancer (MCF-7/Mln^{R} and MCF-7/Adr^{R}) cell lines and their drug-sensitive parental (WT) counterparts (HL60/WT and MCF-7/WT respectively). We examined telomere length, growth capacity, and chemotherapeutic sensitivity in parental and drug-resistant cell lines treated with BIBR1532. We found that BIBR1532 reduced growth capacity and enhanced chemotherapeutic sensitivity in both drug-sensitive and drug-resistant cell lines in a telomere length dependent manner. These novel observations suggest that pharmacological telomerase inhibitors may aid in the treatment of both drug-sensitive and drug-resistant malignancies.

2.3 Materials and Methods

Cell lines & reagents

HL60/WT and etoposide-resistant HL60/MX2 cells (purchased from ATCC, USA) were grown in RPMI 1640 (GIBCO, Canada) supplemented with antibiotics and 10% FBS (WISENT, Canada) and at each passage were counted by hemacytometer and strictly maintained at a cell density between $2x10^5$ and $1.2x10^6$ cells/ml. MCF-7/WT, melphalan-resistant MCF-7/Mln^R (acquired from Dr Moulay Alaoumi-Jamali, McGill University, Canada) and doxorubicin-resistant MCF-7/Adr^R (from Dr. Michael Pollack, McGill University, Canada) cells were grown in MEM supplemented with antibiotics and 10% FBS (WISENT, Canada) and were routinely passed 1 in 4 upon reaching 80-90% confluency. Where indicated, cells were grown continuously in media containing 2.5μM BIBR1532 (a gift from Dr. Jacques van Meel, Boehringer Ingelheim, Austria) suspended in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, USA). Etoposide, melphalan and doxorubicin were purchased from Sigma-Aldrich (St. Louis, USA) dissolved in DMSO, 95% ethanol or H_2O respectively, and stored in aliquots at -20°C. Where indicated, vehicle refers to the respective solvent.

Telomerase activity (TRAP) Assay

Cells were collected and stored at -80°C until extracts were prepared in NP-40 lysis buffer. Telomeric repeat amplification protocol (TRAP) reactions were performed with 10ng of protein extract per reaction (unless otherwise indicated), incubated at 30°C for 30 min, and telomerase elongation products were amplified by PCR (Moriarty, Dupuis et al. 2002). Telomerase activity was quantified relative to the internal PCR control, and this ratio was expressed relative to the same ratio calculated for the indicated reference samples.

To determine telomerase activity upon IC_{50} etoposide treatment, HL60/WT and HL60/MX2 cells were plated in 6-well plates in a final volume of 5ml, and at a starting density of $2x10^5$cells/ml. Cells were treated with vehicle or IC_{50} concentrations of etoposide (0.35μM for HL60/WT and 9.84μM for HL60/MX2

cells). 24, 48, 72, and 96h post treatment, cells were collected and processed for TRAP as described. To verify the effect of etoposide treatment, cells were plated in parallel for an MTT assay which was performed at the 48h time point. For *in vitro* determination of BIBR1532 mediated telomerase inhibition, 2.5μM BIBR1532 or an equal volume of DMSO was added to TRAP reactions containing 40ng or 20ng of untreated MCF-7/WT and MCF-7/Adr^R cell extracts. Reactions were incubated at 30°C for 30 min and processed as described above.

Telomere Length Analysis

Telomere length was determined by terminal restriction fragment (TRF) analysis (Cerone, Londono-Vallejo et al. 2001). Genomic DNA was extracted by standard procedure and digested with HinfI and RsaI. Equal amounts of digested DNA were separated by pulse-field gel electrophoresis (PFGE), gels were then partially dried, denatured and neutralized, and incubated for a minimum of 12 hours with a [γ^{32}P]ATP 5'end-labelled telomeric probe. Telomeric signals were visualized after exposure of gels to phosphoimager or X-ray films (Kodak). Hybridization signals were quantified with ImageQuant (Molecular Dynamics) from at least two independent DNA extracts subjected to elecrophoresis on two separate gels (minimally), and mean telomere length was determined and expressed +/- standard deviation as previously described (Cerone, Londono-Vallejo et al. 2001).

Cell Viability (MTT) Assay

In 96 well flatbottom microtest plates, HL60/WT and all MCF-7 cell lines were plated in triplicate, in a final volume of 200μl media, and at a cell density of $1x10^4$ cells/well. HL60/MX2 cells were plated at a starting density of $2x10^4$ cells/well. On the same day of plating for HL60 cell lines, and the next day for MCF-7 cell lines, cells were treated in triplicate as indicated. 48hrs post treatment, MTT assays were performed as described (Christodoulopoulos, Malapetsa et al. 1999). All MTT assays were performed at least two independent times.

Proliferation Assay

In 24 well plates, and in a final well volume of 1ml, HL60/WT and HL60/MX2 cells were plated at a starting density of $4x10^5$ cells/ml and treated with IC_{25} etoposide (0.117μM for HL60/WT and for 3.21μM HL60/MX2). 24hrs post treatment, cells were diluted in a final volume of 5ml media (1:20 for HL60/WT, 1:10 for HL60/MX2), allowed to proliferate (5 days HL60/WT, 6 days HL60/MX2) and counted with trypan blue. Viable cells counts were normalized and expressed relative to that of the non-treated controls.

FACS Analysis

Cells treated in the same manner as in Proliferation Assays were taken 24, 48, and 72 hours after treatment, and DNA content was determined by fluorescent-activated cell sorting (FACS) analysis by propidium iodide staining as previously described (Aloyz, Xu et al. 2002). Cell cycle analysis was performed using an EPICS XL-MCL fluorescent-activated cell sorter (Beckman Coulter, Fullerton, CA). The percentages represent the mean value and the standard deviation of two independent experiments.

Colony Forming Assay

MCF-7 cell lines were plated at a density of $2x10^5$ cells/ well in six well plates the day before treatment. MCF-7/WT cells and MCF-7/Mln^R cells were treated with 1μM and 5μM melphalan respectively. MCF-7/WT and MCF-7/Adr^R cells were treated with 0.125μM and 100μM doxorubicin respectively. Twenty-four hours post treatment, cells were diluted (1:500 for non-chemotherapeutic treated cells or 1:10 for chemotherapeutic treated cells) into $10cm^2$ plates and allowed to proliferate until control cells had grown into clearly visible colonies (1-3 weeks depending on cell lines and treatments). Plates were then stained with crystal violet, colonies were counted and normalized to the number observed for controls. Minimally, two plates of the same treatment were counted for each colony forming assay, and graphs represent experiments from at least two independent

experiments. For accuracy, only colony forming assays that gave greater than twenty colonies in control plates were used when calculating averages.

Statistical and Data Analysis

Data was analyzed and graphed using Microsoft Excel. Statistical analysis was performed by t test using the online statistical software GraphPad Quickcalcs (http://www.graphpad.com/quickcalcs/ttest1.cfm). Statistical probability in figures is expressed as $*p<0.05$, and $**p<0.01$.

2.4 Results

Drug-resistant and drug-sensitive cell lines display different basal telomere lengths and basal telomerase activities.

A number of reports have indicated that drug-resistant cell lines have different levels of telomerase activity or exhibit changes in telomere length compared to drug-sensitive controls (Park, Rha et al. 1998; Kuranaga, Shinomiya et al. 2001; Kim, Lee et al. 2002; Incles, Schultes et al. 2003; Deschatrette, Ng et al. 2004). Therefore, we characterized basal telomere length and telomerase activity in parental and drug-resistant cell lines. HL60/MX2 cells displayed 28 fold resistance to etoposide compared to parental HL60/WT cells (Figure 6A) (Harker, Slade et al. 1989). MCF-7/Mln^R cells and MCF-7/Adr^R cells are 5 fold and over 200 fold resistant to melphalan (Yen, Woo et al. 1995) and doxorubicin (Alaoui-Jamali, Schecter et al. 1993) respectively. In all cases, we observed differences in basal telomere length between parental and drug-resistant cells (Figure 5A). HL60/MX2 cells have longer telomeres (mean TRF length 5.73 +/- 0.15 Kb) compared to those of HL60/WT cells (mean TRF length 3.66 +/- 0.29 Kb). Conversely, telomere lengths were shorter in both MCF-7/Mln^R and MCF-7/Adr^R (mean TRF length 4.15 +/- 0.21 Kb and 4.66 +/- 0.68 Kb respectively) compared to those of MCF-7/WT (mean TRF length 7.06 +/- 0.6 Kb). Similarly, basal telomerase activity of HL60/MX2 and MCF-7/Mln^R cells differed from that of the WT cell lines as measured by TRAP. HL60/MX2 showed lower basal telomerase activity, MCF-7/Mln^R cells demonstrated higher basal telomerase activity, however MCF-7/Adr^R cells did not display any change in activity compared to that of the parental cell line (Figure 5B & 5C).

HL60/MX2 cells do not demonstrate any significant difference in telomerase activity compared to HL60/WT cells when treated with IC_{50} concentrations of etoposide.

A number of studies have reported that telomerase activity increases upon cellular treatment with certain chemotherapeutic drugs (Sato, Mizumoto et al. 2000; Moriarty, Dupuis et al. 2002; Klapper, Qian et al. 2003; Jeyapalan, Leake et al.

2004). Therefore, we assessed telomerase activity of HL60/WT and HL60/MX2 cells at different time points following treatment with IC_{50} concentrations of etoposide (Figure 6A). In both HL60/WT and HL60/MX2 cell lines, we observed a decrease in telomerase activity 72 and 96h post treatment (Figure 6B), but did not observe any statistically significant differences between cell lines. Finally, at the etoposide concentrations and time points analyzed, we did not observe any statistically significant upregulation of telomerase activity after treatment.

BIBR1532 inhibits telomerase activity, induces telomere shortening, and reduces proliferative capacity in both drug-sensitive HL60/WT and drug-resistant HL60/MX2 cell lines.

Telomerase inhibition or inactivation has been reported to increase sensitivity to chemotherapeutic treatment (Corey 2002). However, there have been no studies to date that have addressed the usefulness of a pharmacological inhibitor of telomerase catalytic activity in combination strategies. BIBR1532 is an effective small molecule inhibitor of the human telomerase enzyme, demonstrating high potency and selectivity *in vitro* (Damm, Hemmann et al. 2001). We first determined if telomerase inhibition sensitizes HL60 cell lines to etoposide treatment. Both HL60/WT and HL60/MX2 cell lines were treated with 2.5μM BIBR1532; higher concentrations inhibited cell growth (Figure 7A) likely due to non-specific cytotoxicity. We observed progressive telomere shortening upon prolonged and continued growth of HL60/WT and HL60/MX2 cells in the presence of BIBR1532 (Figure 7B). This effect was reversible as late PD BIBR1532-treated HL60/MX2 cells released from telomerase inhibition and allowed to proliferate an additional 30 PDs (hereafter referred to as HL60/MX2 Rel) displayed lengthened telomeres (Figure 7B, Rel). Despite progressive telomere shortening, we did not observe any major defect in cellular proliferation of mass cultures, as shown by similar slopes of growth curves (Figure 7C). However, when HL60/WT and HL60/MX2 cells were diluted to low densities (1:20 or 1:10 respectively) and allowed to proliferate for longer periods of time (5 or 6 days respectively) without allowing the cultures to become overconfluent, we

observed decreased proliferative capacity of late PD BIBR1532-treated cells (Figure 7D and 3E). Proliferative capacity progressively declined as the duration of BIBR1532 treatment increased. We observed that the reduced proliferative capacity of telomerase-inhibited cells was reversible and returned to baseline levels observed for controls following release from BIBR1532 treatment (Figure 7E: compare NT and Rel samples.) Next, we treated both HL60/WT and HL60/MX2 cells with IC_{25} etoposide for 24h and performed proliferation assays to analyze the effect of combining BIBR1532 and etoposide treatment. We observed that IC_{25} etoposide treatment significantly inhibited cellular proliferation (Figure 7D and 7E: compare NT and Cont samples). Finally, we observed that BIBR1532 pretreatment further sensitized both drug-sensitive and drug-resistant HL60 cell lines to IC_{25} etoposide treatment (Figure 7D and 7E: compare Cont (treated with etoposide only) to samples treated with both etoposide and BIBR1532).

To determine weather decreased proliferative capacity of BIBR1532 treated cells was a result of increased cell cycle length and/or apoptosis, we performed cell cycle analysis of HL60/WT and HL60/MX2 cells treated in the same way as in our proliferation assays. Twenty-four, 48 and 72 hours after treatment cells were collected and DNA content was analyzed by propidium iodide staining and FACS analysis. However, we were unable to identify any statistically significant differences in the percentage of cells in sub-G1, G1, S or G2/M cell cycle phases between BIBR1532-treated and untreated cells (see Appendix A).

BIBR1532 inhibits telomerase activity and induces telomere length shortening and chemotherapeutic sensitization in MCF-7/WT and MCF-7/Mln^R but not MCF-7/Adr^R.

As for the HL60 cell lines, treatment of MCF-7 cell lines with BIBR1532 doses greater than 2.5μM impaired short term cell viability as measured by the MTT assay (Figure 8A); therefore, cells were treated with 2.5μM BIBR1532. After continuous treatment with BIBR1532, we did not observe any difference in

cellular growth of treated versus untreated cultures (Figure 8C), but did observe telomere shortening in MCF-7/WT and MCF-7/Mln^R cell lines but not MCF-7/Adr^R cells (Figure 8B). BIBR1532, when used alone, significantly inhibited the colony forming ability of MCF-7/WT cells and reduced the number of MCF-7/Mln^R colonies counted (Figure 9A). Interestingly only MCF-7/WT and MCF-7/Mln^R cells demonstrated decreased colony forming ability after continuous growth in BIBR15323 followed by combination treatment with melphalan or doxorubicin (Figure 9B&C). This effect was progressive and dependent on duration of BIBR1532 treatment. Sensitization to chemotherapeutics occurred in both drug-sensitive MCF-7/WT and drug-resistant MCF-7/Mln^R BIBR1532-treated cell lines (Figure 9B&C). MCF-7/Adr^R cells did not show telomere shortening (despite prolonged growth in BIBR1532 (Figure 8B)), nor did they demonstrate any sensitization toward doxorubicin treatment (Figure 9C).

This apparent difference between MCF-7/WT, MCF-7/Mln^R and MCF-7/Adr^R cells with respect to BIBR1532 sensitivity prompted us to ask whether the telomerase enzyme of the MCF-7/Adr^R cells was resistant to BIBR1532. We assessed the sensitivity of the telomerase enzyme *in vitro* by adding 2.5μM BIBR1532 to TRAP reactions of untreated MCF-7/WT and MCF-7/Adr^R cell extracts. We observed similar inhibition of telomerase activity from extracts of both cell lines upon addition of BIBR1532, indicating that the telomerase enzyme in MCF-7/Adr^R cells is not resistant to BIBR-mediated catalytic inhibition (Figure 10).

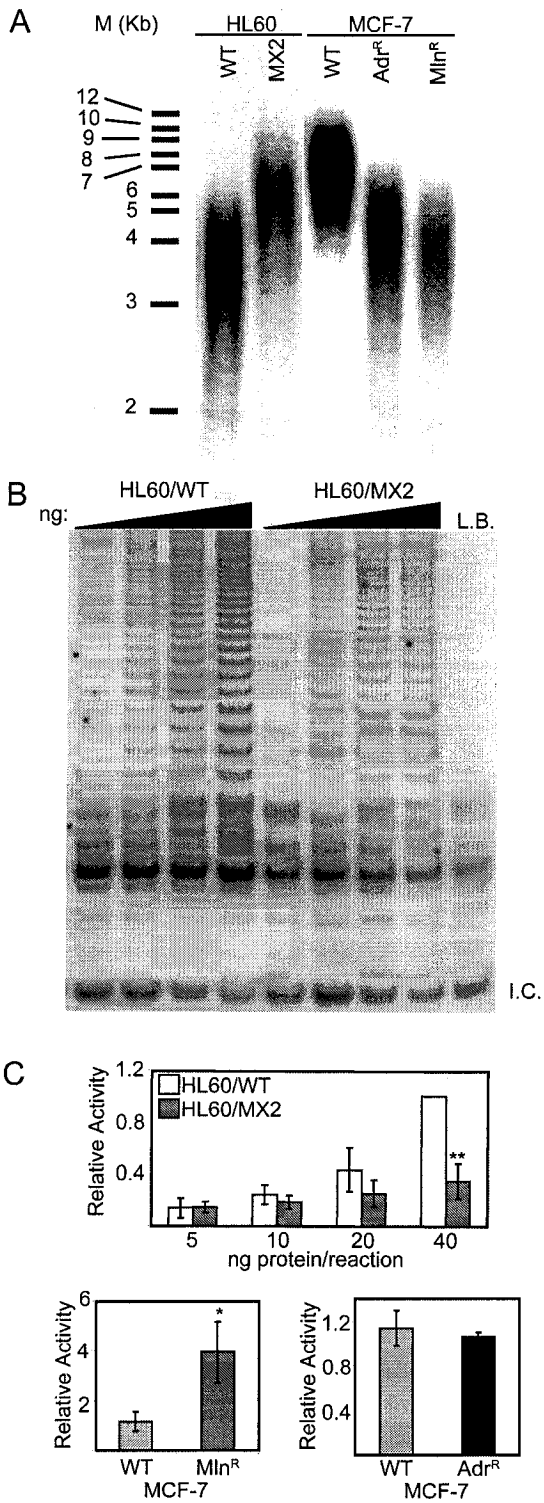
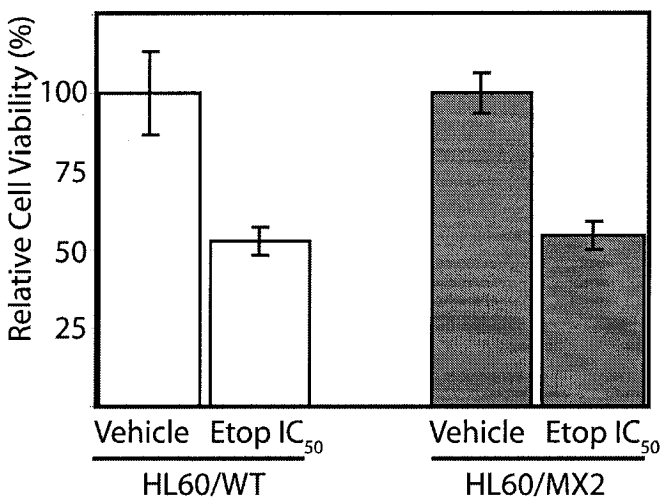

A
M (Kb)
HL60
MCF-7
WT
MX2
WT
Adr^R
Mln^R
12
10
9
8
7
6
5
4
3
2
B
ng:
HL60/WT
HL60/MX2
L.B.
I.C.
C
Relative Activity
1.2
0.8
0.4
HL60/WT
HL60/MX2
**
5
10
20
40
ng protein/reaction
Relative Activity
6
4
2
*
WT
Mln^R
MCF-7
Relative Activity
1.2
0.8
0.4
WT
Adr^R
MCF-7

Figure 5- Basal telomere length and telomerase activity of paired drug-resistant and drug-sensitive cell lines. A, TRF analysis of basal telomere length. M, DNA marker (Kb). B, Representative gel showing basal telomerase activity of 5, 10, 20, or 40 nanograms (ng) (◢) of HL60/WT or HL60/MX2 protein extract per TRAP reaction. L.B. lysis buffer. I.C. PCR internal control. C, Quantification of basal telomerase activity from 3 independent extract preparations, expressed relative to the telomerase activity/PCR internal control ratio of the 40ng reaction, +/- standard deviation. *$p<0.05$, and **$p<0.01$ compared to relative activity of parental cell line.

A


Relative Cell Viability (%)
100
75
50
25
Vehicle
Etop IC50
Vehicle
Etop IC50
HL60/WT
HL60/MX2

B

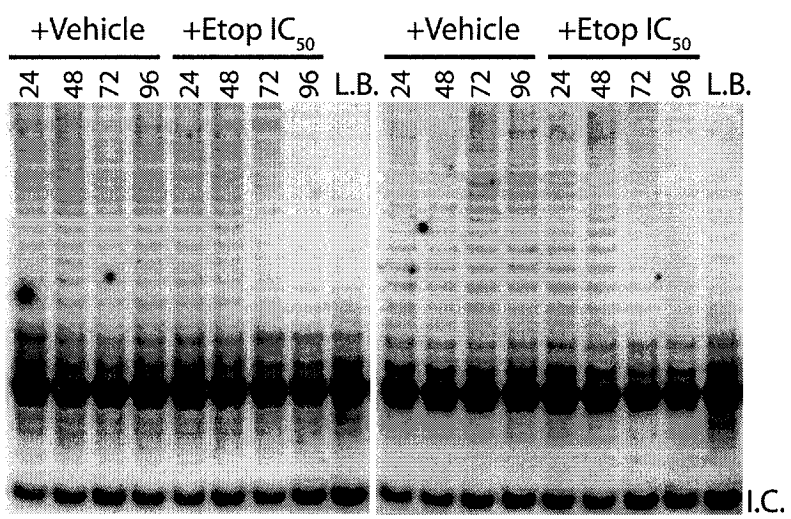

+Vehicle
+Etop IC50
+Vehicle
+Etop IC50
24
48
72
96
24
48
72
96
L.B.
24
48
72
96
24
48
72
96
L.B.
I.C.

Figure 6- HL60/WT and HL60/MX2 telomerase activity post treatment at IC_{50} etoposide concentrations. A, Cellular viability is inhibited by 50% 48hrs post etoposide treatment (0.35μM for HL60/WT cells, 9.84μM for HL60/MX2 cells) as measured by MTT assay. Graph represents data from 3 independent treatments +/- standard error. B, Representative data showing HL60/WT and HL60/MX2 telomerase activity 24 to 96hrs post vehicle or IC_{50} etoposide treatment. L.B. lysis buffer, I.C. Internal PCR control.

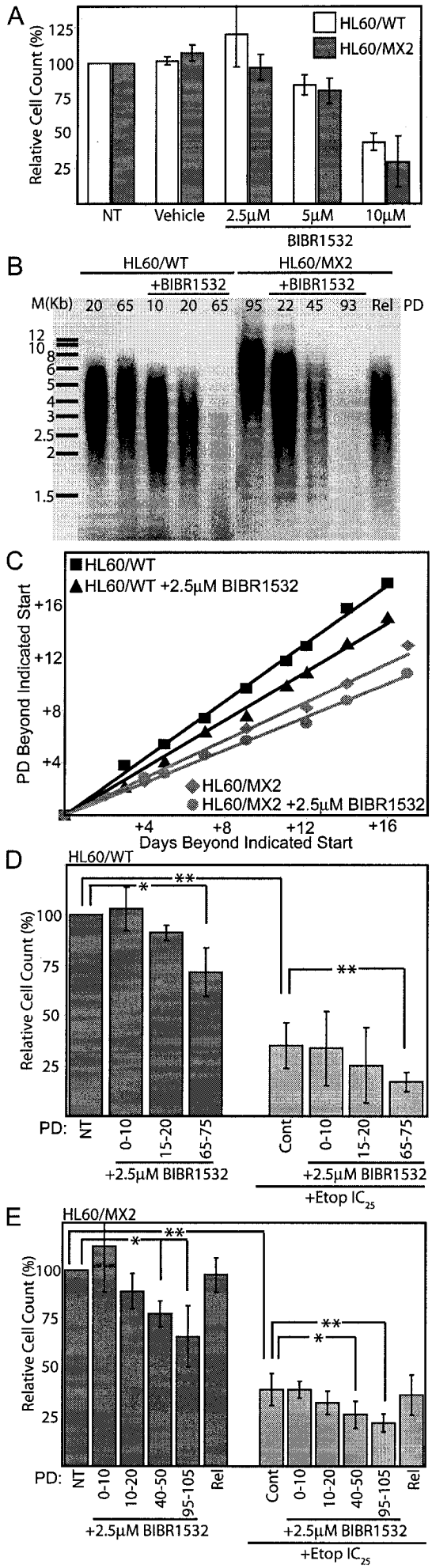

A
HL60/WT
HL60/MX2
Relative Cell Count (%)
125
100
75
50
25
NT
Vehicle
2.5μM
5μM
10μM
BIBR1532
B
HL60/WT
+BIBR1532
HL60/MX2
+BIBR1532
M(Kb) 20 65 10 20 65 95 22 45 93 Rel PD
12
10
8
6
5
4
3
2.5
2
1.5
C
HL60/WT
HL60/WT +2.5μM BIBR1532
HL60/MX2
HL60/MX2 +2.5μM BIBR1532
PD Beyond Indicated Start
+16
+12
+8
+4
+4
+8
+12
+16
Days Beyond Indicated Start
D
HL60/WT
Relative Cell Count (%)
100
75
50
25
PD: NT 0-10 15-20 65-75 Cont 0-10 15-20 65-75
+2.5μM BIBR1532
+2.5μM BIBR1532
+Etop IC25
E
HL60/MX2
Relative Cell Count (%)
100
75
50
25
PD: NT 0-10 10-20 40-50 95-105 Rel Cont 0-10 10-20 40-50 95-105 Rel
+2.5μM BIBR1532
+2.5μM BIBR1532
+Etop IC25

Figure 7- 2.5μM BIBR1532 inhibits telomerase, induces telomere shortening, reduces proliferative capacity and sensitizes HL60/WT and HL60/MX2 to etoposide (Etop) treatment. A, Cellular growth 72hrs post treatment with 2.5μM, 5μM or 10μM BIBR1532. $2x10^5$ cells/ml were plated, treated as indicated and counted by hemocytometer and trypan blue exclusion method 72hrs later. Cell counts were normalized to those of non-treated (NT) controls. Graphs represent 3 independent experiments +/- standard deviation. B, TRF analysis of HL60/WT and HL60/MX2 cells untreated or treated with 2.5μM BIBR1532 for the indicated number of population doublings (PD). Rel indicates late passage BIBR1532-treated HL60/MX2 cells released from treatment and allowed to proliferate for an additional 30PD. M, DNA marker (Kb). C, Growth analysis of cells in mass culture at the time of proliferation assays. Graph shows HL60/WT BIBR1532-treated cells starting from PD60 and HL60/MX2 BIBR1532-treated cells starting from PD93. D&E, Proliferative capacity of HL60/WT (7D) and HL60/MX2 (7E) +/- IC_{25} etoposide treatment as measured by 5 or 6 day proliferation assay. PD indicates the number of population doublings the cells were treated with BIBR1532 before experiments were performed. Bars represent values from at least 3 independent proliferation assays +/- standard deviation. $*p<0.05$, and $**p<0.01$ compared to the indicated control.

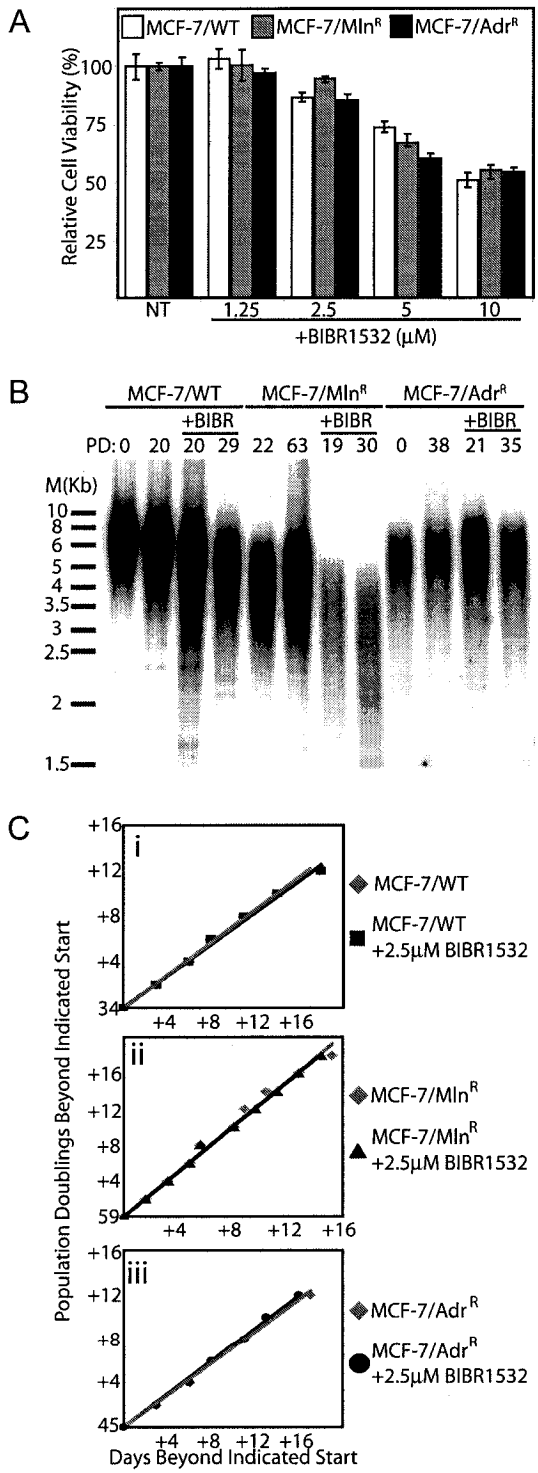

A
MCF-7/WT
MCF-7/MIn^R
MCF-7/Adr^R
Relative Cell Viability (%)
100
75
50
25
NT
1.25
2.5
5
10
+BIBR1532 (μM)
B
MCF-7/WT
MCF-7/MIn^R
MCF-7/Adr^R
+BIBR
PD: 0 20 20 29 22 63 19 30 0 38 21 35
M(Kb)
10
8
6
5
4
3.5
3
2.5
2
1.5
C
i
ii
iii
+16
+12
+8
+4
34
59
45
+4 +8 +12 +16
MCF-7/WT
MCF-7/WT
+2.5μM BIBR1532
MCF-7/MIn^R
MCF-7/MIn^R
+2.5μM BIBR1532
MCF-7/Adr^R
MCF-7/Adr^R
+2.5μM BIBR1532
Population Doublings Beyond Indicated Start
Days Beyond Indicated Start

Figure 8- 2.5μM BIBR1532 inhibits telomerase, and induces telomere shortening, in MCF-7/WT and MCF-7/Mln^R treated cells without affecting mass culture growth. A, Cell viability 72hrs post treatment of MCF-7 cells with 1.25, 2.5, 5 or 10μM BIBR1532 as measured by MTT assay. Values were normalized to those of non-treated (NT) controls and graphs represent 2 independent experiments +/- standard error. B, TRF analysis of MCF-7 cells untreated or treated with 2.5μM BIBR1532 (+BIBR) for the indicated number of population doublings (PD). M, DNA marker (Kb). C, Growth analysis of cells in mass culture at the time of colony forming assays. Graphs show (i) MCF-7/WT and MCF-7/WT BIBR1532-treated cells starting from PD34, (ii) MCF-7/Mln^R and MCF-7/Mln^R BIBR1532-treated cells starting from PD59, and (iii) MCF-7/Adr^R and MCF-7/Adr^R BIBR1532-treated cells starting from PD45.

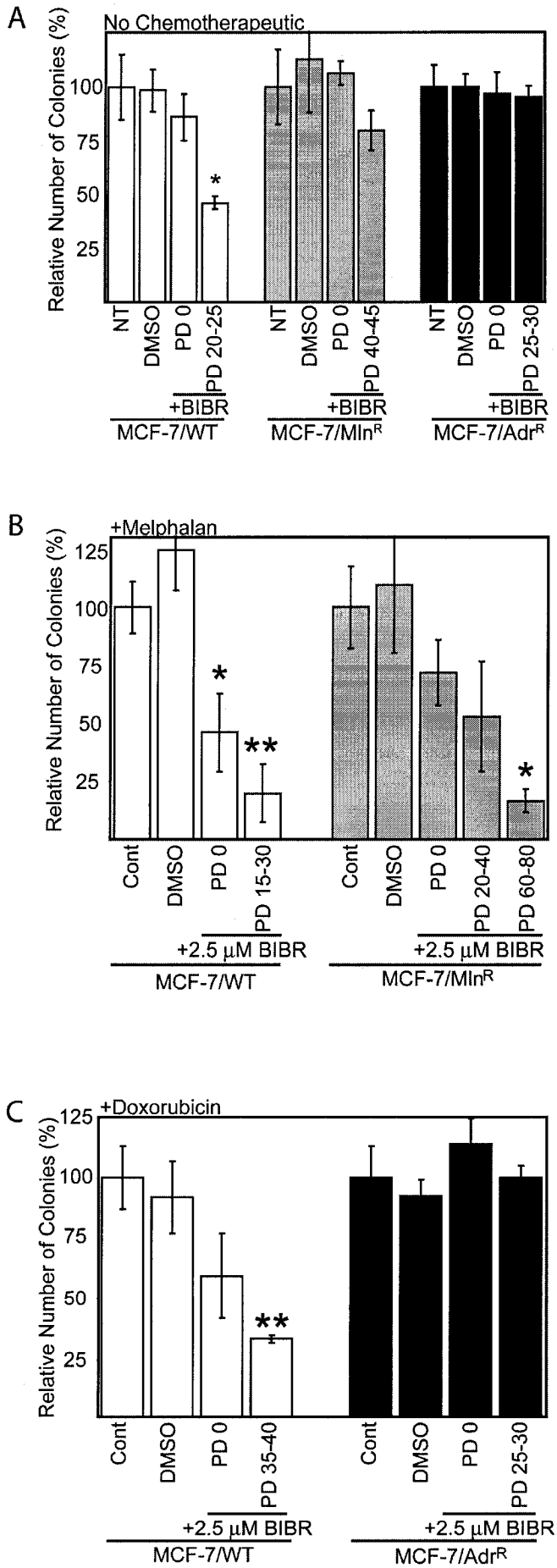

A
No Chemotherapeutic
Relative Number of Colonies (%)
100
75
50
25
*
NT
DMSO
PD 0
PD 20-25
+BIBR
MCF-7/WT
NT
DMSO
PD 0
PD 40-45
+BIBR
MCF-7/MlnR
NT
DMSO
PD 0
PD 25-30
+BIBR
MCF-7/AdrR
B
+Melphalan
Relative Number of Colonies (%)
125
100
75
50
25
*
**
*
Cont
DMSO
PD 0
PD 15-30
+2.5 μM BIBR
MCF-7/WT
Cont
DMSO
PD 0
PD 20-40
PD 60-80
+2.5 μM BIBR
MCF-7/MlnR
C
+Doxorubicin
Relative Number of Colonies (%)
125
100
75
50
25
**
Cont
DMSO
PD 0
PD 35-40
+2.5 μM BIBR
MCF-7/WT
Cont
DMSO
PD 0
PD 25-30
+2.5 μM BIBR
MCF-7/AdrR

Figure 9- 2.5μM BIBR1532 treatment reduces colony forming ability and sensitizes MCF-7/WT and MCF-7/Mln^R cells to melphalan and doxorubicin, or melphalan treatment respectively, in a telomere length dependent fashion. Colony forming assays of control (NT, non-treated; Cont, Control)), DMSO or BIBR1532-treated MCF-7 cells. PD indicates the number of population doublings cells were pretreated with 2.5μM BIBR1532 (+BIBR). A, Colony forming assays without chemotherapeutic co-treatment. B, Colony forming assays of MCF-7/WT and MCF-7/Mln^R with 1μM and 5 μM melphalan treatment respectively. C, Colony forming assays of MCF-7/WT and MCF-7/Adr^R with 0.125μM and 100μM doxorubicin treatment respectively. Bars represent relative number of colonies counted after treatment compared to controls +/- standard error. *p<0.05, and **p<0.01 compared to control (NT or Cont) of same cell line.

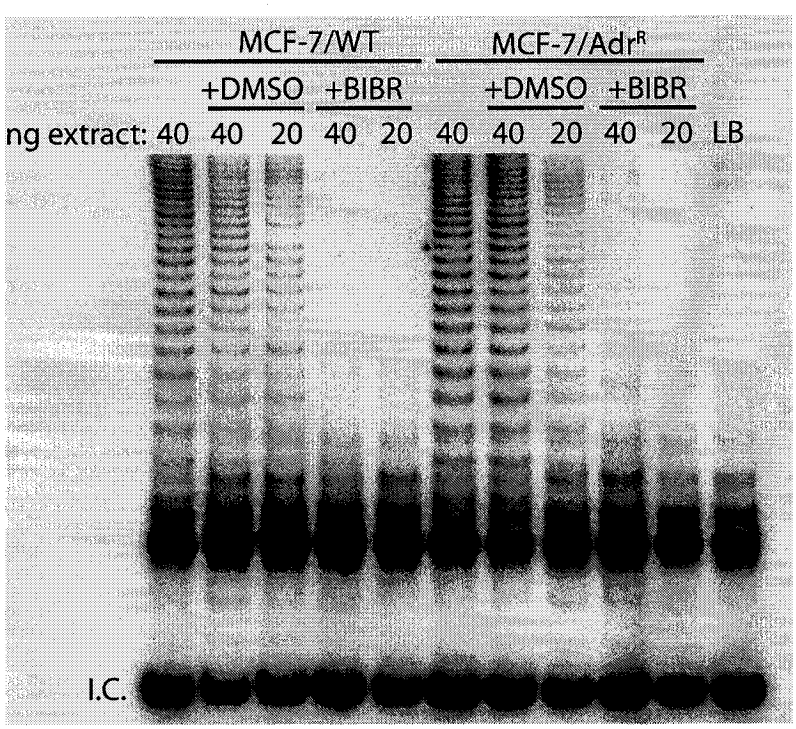

MCF-7/WT
MCF-7/Adr^R
+DMSO
+BIBR
+DMSO
+BIBR
ng extract: 40 40 20 40 20 40 40 20 40 20 LB
I.C.

Figure 10- Telomerase enzyme from MCF-7/Adr^R protein extracts is sensitive to BIBR1532 *in vitro*. TRAP assay of MCF-7/WT and MCF-7/Adr^R cell extracts without, or with *in vitro* addition of DMSO or 2.5μM BIBR1532 to TRAP reactions which were incubated for 30min at 30°C and processed as described in Materials and Methods. ng extract, nanograms protein extract per reaction, L.B. lysis buffer, I.C., PCR internal control.

2.5 Discussion

The clinical development of chemotherapeutic drug-resistance is universally fatal, and strategies to overcome this event are still required (Baird and Kaye 2003). Telomerase is an attractive anti-cancer target as its activity is predominantly observed in cancer cells (Cech 2004). In previous studies telomerase has been inhibited or inactivated via gene knock-out, antisense or dominant-negative strategies to induce growth arrest or apoptosis and to sensitize cells to various cellular stresses (Hahn, Stewart et al. 1999; Corey 2002). At least two such studies reported that treatment with antisense oligonucleotides targeting either hTERT or the hTR template sensitized human bladder and prostate cancer cells respectively to chemotherapeutic treatment (Chen, Koeneman et al. 2003; Kraemer, Fuessel et al. 2004). While gene therapy and antisense strategies hold great potential for the treatment of many human diseases, the feasibility of gene therapy and clinical utility of most antisense compounds remain to be proven (Dorsett and Tuschl 2004; Lane 2005). A potentially more practical strategy for anti-telomerase therapy in patients is the use of small molecule inhibitors of telomerase catalytic activity, like BIBR1532. Unfortunately, due to the time lag between the start of anti-telomerase treatment and growth arrest or apoptosis, telomerase inhibitors alone may not be effective anticancer agents (Corey 2002). Telomerase inhibition in combination with chemotherapeutics, however, does hold the potential for the treatment of many malignancies.

Our observations suggest that drug-sensitive and drug-resistant cell lines can be sensitized to chemotherapeutic treatment via pharmacological inhibition of telomerase catalytic activity in a telomere length dependent manner. We observed decreased proliferative capacity and sensitization to chemotherapeutic treatment only in cell lines that demonstrated telomere shortening and not in cells released from telomerase inhibition or in cells insensitive to BIBR1532 treatment. Sensitization was progressive with duration of BIBR1532 treatment, and analysis of colony forming ability demonstrates that as few as 20 PDs of telomerase inhibition are required to observe sensitization. Taken together, our novel

observations suggest that pharmacological anti-telomerase therapy may be effective in the treatment of both drug-resistant and drug-sensitive malignancies.

Drug-resistance correlates with changes in basal telomere length and basal telomerase activity.

We observed differences in both basal telomere length and telomerase activity of drug-resistant cells versus drug-sensitive parental cells. Specifically, HL60/MX2 cells have longer telomeres and lower basal telomerase activity compared to HL60/WT cells, MCF-7/Mln^R cells have shorter telomeres and higher basal telomerase activity, and MCF-7/Adr^R cells have shorter telomeres compared to parental cells. A number of other studies observed different telomere lengths and basal telomerase activity in drug-resistant cells compared to drug-sensitive controls (Park, Rha et al. 1998; Kuranaga, Shinomiya et al. 2001; Kim, Lee et al. 2002; Incles, Schultes et al. 2003; Deschatrette, Ng et al. 2004). Of note, one study that tracked telomere lengths and drug sensitivities of rat hepatoma cells reported that periods of drug-resistance to methotrexate or cisplatin correlated with either shortened or elongated telomeres respectively (Deschatrette, Ng et al. 2004). Another study observed that expression of multi-drug resistance genes, telomere length and telomerase activity were all increased upon long-term treatment of human colorectal carcinoma cells with cisplatin and 5-fluorouracil (Kuranaga, Shinomiya et al. 2001). Conversely, others observed that increased sensitivity of human cell lines to chemotherapeutics correlated with higher basal telomerase activity (Asai, Kiyozuka et al. 1998; Lin, Lim et al. 2001). These opposing correlations might be explained by differences in cell-type, drug-type, or levels of drug-resistance between studies. It is tempting to speculate that changes in basal telomere length or telomerase activity, in general, may contribute to the development or maintenance of chemotherapeutic resistance. However we cannot rule out the possibility that the observed differences in telomere length and basal telomerase activity in drug-resistant cells versus parental cells are due to clonal selection and expansion during the process of *in vitro* drug-resistance development. Indeed, when subclones of Hela and 293

cells were isolated and analyzed, the clones showed a range of mean telomere lengths and telomerase activity, and some differed dramatically from those of the mass culture (Bryan, Englezou et al. 1998). These results indicate that telomere length and telomerase activity heterogeneity exists within a mass population (Bryan, Englezou et al. 1998).

Telomerase activity of HL60/WT cells does not differ significantly from that of HL60/MX2 cells upon IC_{50} etoposide treatment

Previous studies have reported a time and concentration dependent upregulation of telomerase activity in human leukemia, neuroblastoma and pancreatic cells after treatment with etoposide (Sato, Mizumoto et al. 2000; Moriarty, Dupuis et al. 2002; Klapper, Qian et al. 2003; Jeyapalan, Leake et al. 2004). Other studies report no upregulation of telomerase activity after treatment, but share the observation that at later times after treatment telomerase activity is decreased (Park, Rha et al. 1998; Lin, Lim et al. 2001). As far as we are aware, no studies have compared telomerase activity in drug-resistant and parental cell lines after treatment with equivalent (IC_{50}) concentrations of drug. Two studies have reported that telomerase activity in doxorubicin-resistant cells is increased or maintained after doxorubicin treatment compared to drug-sensitive controls (Ishikawa, Kamiyama et al. 1999; Yoon, Ku et al. 2003). However, equal doxorubicin concentrations were used to treat drug-sensitive and drug-resistant cell lines in these experiments which did not take into account differences in drug sensitivities. As IC_{50} drug concentrations differ between drug-sensitive and drug-resistant cell lines, the observed differences in telomerase activity may simply reflect different levels of cell death. Therefore, we treated HL60/WT and HL60/MX2 cells with equivalent concentrations of etoposide (0.35μM and 9.84μM respectively) based on cell viability MTT measurements. We did not observe any significant difference in telomerase activity between the two cell lines after treatment, nor did we observe any significant upregulation of telomerase activity. For HL60/WT cells, the results are consistent with our previous observation that 0.5 μM etoposide treatment does not significantly alter

telomerase activity within a 48hr time course (Moriarty, Dupuis et al. 2002). In our current study we were interested in treating HL60/WT and HL60/MX2 cells with concentrations of etoposide similar to those observed clinically (0.5-5μM) (Moriarty, Dupuis et al. 2002). The lack of telomerase upregulation noted in our studies is consistent with our use of etoposide concentrations lower than those previously reported to increase telomerase activity in HL60 cells (1, 2 and 4μM for three to twenty-four hours) (Moriarty, Dupuis et al. 2002; Klapper, Qian et al. 2003).

Drug-sensitive and drug-resistant cells can be sensitized to chemotherapeutic treatment in a telomere length dependent manner.

BIBR1532 is a potent, selective and reversible non-nucleoside inhibitor of human telomerase catalytic activity that exhibits *in vitro* IC_{50} concentrations in the nanomolar range (Damm, Hemmann et al. 2001). In the original reports, proliferation was almost completely arrested in telomerase positive cell lines after prolonged (PD>120) treatment with 10μM BIBR1532 (Damm, Hemmann et al. 2001). BIBR1532 treatment should only inhibit telomerase activity, and telomerase inhibition and telomere shortening should be tolerated until telomeres become short and dysfunctional. Treatment of leukemia cells with high concentrations (30μM to 80μM) of BIBR1532 causes short term cytotoxicity in both telomerase-positive and telomerase-negative cells (El-Daly, Kull et al. 2004). These results suggest that short term anti-proliferative effects of BIBR1532 are likely telomerase independent. We performed our experiments using 2.5μM BIBR1532 after the initial observation that higher concentrations inhibited cell growth or viability in a relatively short time frame (72h). Continuous 2.5μM BIBR1532 treatment did not affect mass population growth, but inhibited telomerase and induced telomere shortening in all cell lines except MCF-7/Adr^R. This effect was progressive and dependent on duration of treatment. In agreement with previous reports, telomerase inhibition by BIBR1532 was reversible as HL60/MX2 Rel cells again displayed elongated telomeres (Damm, Hemmann et al. 2001). Importantly, when BIBR1532-treated HL60 cell lines were tested for

growth ability over a five or six day period, we observed decreased proliferative capacity of cells with short telomeres. When treated in combination with etoposide, cells with short telomeres demonstrated a further reduction in proliferative capacity compared to controls. This effect was likely telomere length dependent, since proliferative capacity declined with increasing number of population doublings in the presence of BIBR1532, and as release from BIBR1532 treatment restored growth ability to control levels.

We hypothesized that BIBR1532-treated HL60/WT and HL60/MX2 cells with shortened telomeres had activated DNA damage response pathways leading to either increased apoptosis or cell cycle length. To test this, we performed cell cycle FACS analysis on late passage BIBR1532-treated HL60/WT and HL60/MX2 cells collected from our proliferation assays. Unfortunately we were unable to observe any statistically significant differences in the cell cycle profiles of BIBR1532-treated and untreated cells (see Appendix A), with or without etoposide co-treatment. Therefore we are unable to conclude whether the decreased proliferative capacity of BIBR1532-treated cells is due to increased cell death or cell cycle length. More definitive results may have been obtained had our FACS analysis been conducted at earlier time points (<24hrs post treatment), and/or with cells with initially synchronized cell cycles (Davis, Ho et al. 2001).

Colony forming assays of MCF-7/WT and MCF-7/Mln^R cells revealed decreased colony forming ability of BIBR1532 treated cells. When a combination of BIBR1532 and chemotherapeutic treatment was used (melphalan or doxorubicin for MCF-7/WT, melphalan for MCF-7/Mln^R) both drug-sensitive MCF-7/WT and drug-resistant MCF-7/Mln^R cell lines exhibited a dramatically reduced capacity to recover from drug treatment. Again, this effect is most likely telomere length dependent, as continuous BIBR1532 treatment progressively sensitized MCF-7/WT and MCF-7/Mln^R cells to chemotherapeutics. Further, MCF-7/Adr^R cells did not demonstrate any change in telomere length (despite being grown for 35 population doublings in the presence of the BIBR1532) nor did they show any alteration in colony forming ability with or without high dose doxorubicin treatment. These results are consistent with recent observations

showing that telomerase mediated resistance to genotoxic stress is telomere length dependent, likely owing to the ability of telomerase to elongate short, presumably dysfunctional, telomeres (Rubio, Davalos et al. 2004). DNA damage responses are activated when telomeres become short or experimentally uncapped (Takai, Smogorzewska et al. 2003; Gire, Roux et al. 2004). We hypothesize that telomere shortening and DNA damage is the consequence of continued BIBR1532 treatment, and provides an explanation for the telomere length dependent reduction of HL60 proliferative capacity and MCF-7/WT and MCF-7/Mln^R colony forming ability.

MCF-7/Adr^R cell insensitivity to telomerase inhibition by BIBR1532 is likely telomerase independent. When BIBR1532 was added *in vitro* to cell extracts, telomerase activity of both MCF-7/WT and MCF-7/Adr^R cells was effectively inhibited. Therefore, cellular mechanisms involving BIBR1532 influx, efflux or metabolism may be responsible for MCF-7/Adr^R resistance to pharmacological telomerase inhibition.

To our knowledge this report is the first to show that a pharmacological inhibitor of telomerase catalytic activity can sensitize cells to traditional drug treatment in a telomere length dependent fashion. Incles *et al.* (2003) previously demonstrated that a G-quadruplex interacting agent (BRACO-19) sensitized parental and flavopiridol-resistant human colon carcinoma cell lines over a 10-20 PD treatment period. While a longer time is required for BIBR1532 to exert its effects, the mechanism of action between the two pharmacological agents is also different. It is proposed that G-quadruplex interacting agents induce and/or stabilize the formation of non-Watson-Crick G-quadruplex DNA structures within G-rich sequences, such as telomeres (Hurley 2002). G-quadruplex structures are predicted to sequester single-stranded telomeric DNA that typically serves as a substrate for telomerase (Hurley 2002). Accordingly, 15 days of subcytotoxic BRACO-19 treatment induced telomere shortening, cellular senescence, complete proliferative arrest, and also decreased hTERT expression in a human uterus carcinoma cell line, demonstrating that BRACO-19 can target telomeres and inhibit telomerase (Burger, Dai et al. 2005). However, a number of other studies

have demonstrated that G-quadruplex interacting agents affect telomere stability and exert cytotoxic effects in the absence of telomere shortening or telomerase expression (notably in telomerase-negative ALT cell lines) (Gowan, Heald et al. 2001; Kim, Gleason-Guzman et al. 2003; Pennarun, Granotier et al. 2005). Further, G-quadruplex interacting agents can also inhibit transcription by stabilizing G-quartet structures in non-telomeric DNA such as the c-*myc* oncogene (Hurley 2002; Siddiqui-Jain, Grand et al. 2002). In contrast, BIBR1532 is a highly selective and potent inhibitor of telomerase that targets the enzyme's catalytic activity and demonstrates very little enzymatic inhibition against a panel of DNA and RNA polymerases, including HIV-1 reverse transcriptase, and no growth inhibition of telomerase-negative normal human fibroblasts, or SA0S-2 ALT cells (Damm, Hemmann et al. 2001). Accordingly, the only known mechanism of action for BIBR1532 is through telomerase inhibition and telomere shortening.

In conclusion, the results presented in this study suggest that pharmacological inhibition of telomerase catalytic activity may be a highly selective strategy of anti-cancer therapy that might aid in the treatment of both drug-resistant and drug-sensitive malignancies.

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Chapter 3- General Discussion & Conclusion

3.1 Discussion

Cancer is poised to overtake heart and cardiovascular disease as the number one cause of death in North America (Stewart, King et al. 2004). Despite recent advances in our understanding of the molecular mechanisms that generate the disease, effective strategies to combat and cure cancer are still required. These strategies will likely involve the targeted disruption of multiple cellular processes to selectively target and kill tumour cells. Further, as cancer is predominantly a disease of the elderly, and as treatment is often less well tolerated in older patients, clinical success must necessarily involve a tolerated level of treatment (be it surgery, chemotherapy, or radiation therapy, alone or in some combination) and if possible, further sensitization of cancer cells so as to reduce treatment burden and/or increase treatment efficacy. Of great interest is the identification of cellular mechanisms and/or processes which are necessary and unique for cancer cell survival. Further, as cancer is not a single disease but a group of diseases with similar properties, both at the cellular and clinical level (Hanahan and Weinberg 2000), the identification of mechanisms/processes that are unique and necessary to all cancers is of utmost importance. It is understandable why the discovery of telomerase, and the observation that its activity is present in the vast majority of cancer cells, generated so much interest regarding telomerase as a novel anti-cancer drug target (Greider and Blackburn 1996).

Our current understanding of the telomerase RNP is that its principal role is to maintain telomeres; by doing so it can impart cellular immortality to the cells in which it is expressed. Recent evidence suggests that telomerase may mediate other functions however, these activities are poorly understood and it remains unclear whether they are telomere-length dependent or not. Telomerase activity has been inactivated/inhibited by antisense ODN, siRNA, gene knockout, and pharmacological inhibitors with similar results: telomere shortening, eventual proliferative arrest, and cellular senescence or apoptosis. The major limitation to these experimental results is the amount of time required for anti-telomerase

treatment to exert its effects (Parkinson 2003). This is a general limitation for telomerase based therapies and detracts from the use of single agent anti-telomerase therapy for cancer treatment. Combining anti-telomerase compounds plus traditional chemotherapeutic treatments does however hold the potential for highly targeted anti-cancer therapy. Further, telomerase inhibition may render cells more susceptible to chemotherapeutic treatment and this might support its application for the treatment of drug-resistant malignancies or for the clinical treatment of cancer in individuals who cannot withstand high dose chemotherapeutics.

Our novel results suggest that BIBR1532, a highly selective pharmacological inhibitor of telomerase type II processivity, can render both drug-sensitive and drug-resistant cells to chemotherapeutic treatment in a telomere length dependent fashion. Our experiments demonstrate that pre-treatment of cells with BIBR1532 for 20-40 population doublings decreases proliferative capacity of promyelocytic leukemia HL60/WT and HL60/MX2 cells, and colony forming ability of breast carcinoma MCF-7/WT and MCF-7/Mln^R cells. Sensitization of cells to chemotherapeutic treatment correlates with telomere shortening and is likely due to the critical shortening of some telomeres within the cellular population such that DNA damage responses are activated and further enhanced by the addition of traditional DNA damaging chemotherapeutics.

Though pharmacological approaches to cure disease are pervasive and clinically attractive, other strategies to selectively target telomeres and telomerase in combination therapies may also prove clinically useful. The overexpression of an hTR encoding a mutant template in telomerase-positive cancer cells inhibits cellular proliferation and induces apoptosis after prolonged growth (Marusic, Anton et al. 1997; Kim, Rivera et al. 2001). Apoptosis occurs independent of wild-type p53 and telomere length, and in tumour xenograft models growth and disease progression were significantly decreased (Li, Rosenberg et al. 2004). Further in the absence of endogenous hTR, expression of the mutant hTR template and telomerase rapidly (within a few cell cycles) induced mitotic

catastrophe and chromosomal fusion demonstrating that the limited incorporation of mutant-hTR sequences can led to telomere malfunction and loss of cellular viability (Guiducci, Cerone et al. 2001). These effects are reminiscent of experiments performed with a dominant-negative TRF2 (DN-TRF2) protein deficient for telomere binding ability. In these experiments, the expression of DN-TRF2 rapidly induced apoptosis in HeLa cells demonstrating that the binding of TRF2 to the TTAGGG repeats is required to prevent chromosome ends from activating DNA damage response pathways leading to cell death (Karlseder, Broccoli et al. 1999). Therefore, it is likely that telomeric incorporation of mutant-hTR sequences disrupts the protein-telomere structure resulting in the activation of DNA damage responses and reduced cellular viability.

The expression of a mutant hTR template combined with traditional anti-cancer treatments may prove successful for targeted, rapid treatment of many cancers. This strategy, not unlike the expression of DN-hTERT or the use hTERT or hTR antisense, relies on gene therapy techniques which remain challenging and clinically unproven (Zhou, Liu et al. 2004; Lane 2005). Therefore, pharmacological interference with telomerase and/or telomeres remains an attractive therapeutic strategy.

An astute and fair criticism of the results presented in this thesis might be that 20-40 population doublings is not a reasonable time frame for the clinical treatment of cancer. However, recent studies now support the hypothesis that, at least for cancers of the brain, breast and blood, tumours are made up of a population of cells with heterogeneity in repopulation capacity (Al-Hajj, Wicha et al. 2003; Hope, Jin et al. 2004; Singh, Clarke et al. 2004). The bulk of primary tumour cells are incapable of recapitulating the tumour in *in vitro* and *in vivo* models. However, in the above mentioned cancers, rare stem cell-like cells that possess the capacity to regenerate a pathologically identical tumour *in vivo* have now been identified (Singh, Hawkins et al. 2004; Huntly and Gilliland 2005). These rare cells, referred to as cancer stem cells, are though to be long-lived, inherently resistant to treatment, and have the ability to become quiescent to evade the acute effects of replication based chemotherapeutics (Dean, Fojo et al.

2005). Against these cells prolonged anti-telomerase therapies may have significant impact. As telomerase inhibition is expected to produce few side effects, continuous treatment with compounds such as BIBR1532 may reduce the proliferative capacity and increase the sensitivity of cancer stem cells to acute chemotherapeutic treatment. Further, as new generation pharmacological telomerase inhibitors with higher efficacy and potency are developed, the amount of time required for continuous anti-telomerase therapy to exert its effects may be reduced. A recent report demonstrates the significant difference between treating cells with DN-hTERT and BIBR1532 as cells expressing the DN telomerase variant senesced after 20-28 population doublings versus >100 population doublings required for BIBR1532 to inhibit cellular proliferation (Pantic, Zimmermann et al. 2005).

Another criticism of this work might be the inherent contradiction of treating cells that are already resistant to chemical compounds with other drugs to reverse resistance or sensitize cells to treatment. While true, pharmacological approaches to treat disease remain some of the most pervasive types of therapies, and resistance reversal will likely require interference with multiple cellular processes. It should also be noted that in two of the three resistant cell lines, telomere shortening was observed. Therefore, based on our results it is not impossible to envisage a scenario where traditional chemotherapeutics are administered in combination with inhibitors of export pumps (like Pgp), detoxifying enzymes (like GST) and telomerase. As long as combination approaches are clinically tolerated, the successful treatment of drug-resistant malignancies may be achieved.

A final limitation of this work might be the fact that the proliferation of HL60 cells was only inhibited by 25-50% after prolonged BIBR1532 treatment, and colony forming ability of MCF-7/WT and MCF-7/Mln^R was only decreased by 50-75% after melphalan and/or doxorubicin treatment. The cell lines we assayed were 5, 30 and over 200 fold resistant to melphalan, etoposide, and doxorubicin respectively. The fold resistance observed in drug-resistant tumours can be small (<2-fold resistant) (Lee and Siemann 1989), or large (>1000-fold

resistant) (Keshelava, Seeger et al. 1997) depending on when tumour cells are tested (before, during or after multiple rounds of chemotherapy). While using BIBR1532 to sensitize cells to chemotherapeutics might not be an effective strategy for cancers that are greater than 1000-fold drug-resistant, it might prove useful for tumours that are two to five fold-resistant, especially when combined with other drug-resistance reversal strategies.

3.2 Future Directions

We observed a significant decrease in HL60/WT and HL60/MX2 cell proliferative capacity and MCF-7/WT and MCF-7/Mln^R colony forming ability after prolonged BIBR1532 treatment and telomere shortening. We hypothesize that these effects are mediated by shortened telomeres which elicit DNA damage responses (Saretzki, Sitte et al. 1999) which can be further increased by the co-treatment of chemotherapeutics. However, in the work presented above we have not addressed this question directly. Therefore, to identify the mechanism by which BIBR1532-induced shortened telomeres decrease proliferative capacity and colony forming ability it would be important to characterize the DNA damage response signalling pathways in untreated cells and BIBR1532-treated cells with short telomeres. This could be achieved by monitoring the phosphorylation status of p53, MDM2 and Rb. Further, the synchronization of cells prior to cell cycle analysis, and the analysis of earlier time points (<24hrs) may clarify whether cells with short telomeres undergo increased apoptosis, have a longer cell cycle, or both.

Finally, the ultimate goal of this research is to identify highly selective strategies to inhibit cancer cell growth *in vivo*. Therefore it would be important to determine the tumourigenicity and chemotherapeutic sensitivity of BIBR1532-treated cells in rodent models of human cancer. A straightforward experiment would be the sub-cutaneous implantation of untreated, and BIBR1532-treated cells into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. If the results from our proliferation assays and colony forming assays are representative of *in vivo* experiments, we hypothesize that BIBR1532-treated cells

with short telomeres would produce fewer and/or smaller tumours. Further, local or systemic treatment of mice with xenograft tumours from BIBR1532-treated cells should result in increased mouse lifespan or survival compared to mice with xenograft tumours from control (untreated) cells. Such results would promote the use of pharmacological telomerase inhibitors *in vivo* and in the clinic.

3.3 Conclusions

Our results demonstrate that BIBR1532 and future new-generation pharmacological inhibitors of telomerase catalytic activity are a promising class of compounds that may prove useful in combination therapies for the clinical treatment of both drug-sensitive and drug-resistant malignancies. Finally, pharmacological telomerase inhibitors may target cancer stem cells and render this newly discovered cell type more susceptible to acute anti-neoplastic treatment.

4- References

Akiyama, M., O. Yamada, et al. (2002). "Telomerase overexpression in K562 leukemia cells protects against apoptosis by serum deprivation and double-stranded DNA break inducing agents, but not against DNA synthesis inhibitors." Canc. Lett. **178**: 187-197.

Al-Hajj, M., M. S. Wicha, et al. (2003). "Prospective identification of tumorigenic breast cancer cells." Proc Natl Acad Sci U S A **100**(7): 3983-8.

Alaoui-Jamali, M. A., R. L. Schecter, et al. (1993). "In vivo reversal of doxorubicin resistance by a new tiapamil analog Ro11-2933." J Pharmacol Exp Ther **264**(3): 1299-304.

Aloyz, R., Z. Y. Xu, et al. (2002). "Regulation of cisplatin resistance and homologous recombinational repair by the TFIIH subunit XPD." Cancer Res **62**(19): 5457-62.

Ambrus, A., D. Chen, et al. (2005). "Solution structure of the biologically relevant G-quadruplex element in the human c-MYC promoter. Implications for G-quadruplex stabilization." Biochemistry **44**(6): 2048-58.

Asai, A., Y. Kiyozuka, et al. (1998). "Telomere length, telomerase activity and telomerase RNA expression in human esophageal cancer cells: correlation with cell proliferation, differentiation and chemosensitivity to anticancer drugs." Anticancer Res **18**(3A): 1465-72.

Autexier, C. and C. W. Greider (1996). "Telomerase and cancer: revisiting the telomere hypothesis." Trends Biochem. Sci. **21**: 387-391.

Baird, R. D. and S. B. Kaye (2003). "Drug resistance reversal--are we getting closer?" Eur J Cancer **39**(17): 2450-61.

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, et al. (2001). "Functional multimerization of the human telomerase reverse transcriptase." Mol. Cell. Biol. **21**: 6151-6160.

Bechter, O. E., Y. Zou, et al. (2004). "Telomeric recombination in mismatch repair deficient human colon cancer cells after telomerase inhibition." Cancer Res **64**(10): 3444-51.

Biessmann, H. and J. M. Mason (2003). "Telomerase-independent mechanisms of telomere elongation." Cell Mol Life Sci **60**(11): 2325-33.

Blackburn, E. H. (2001). "Switching and signaling at the telomere." Cell **106**: 661-673.

Blackburn, E. H. and C. W. Greider, Eds. (1995). Telomeres. Cold Spring Harbor, Cold Spring Harbor Laboratory Press.

Blasco, M. A. (2002). "Mouse models to study the role of telomeres in cancer, aging and DNA repair." Eur J Cancer **38**(17): 2222-8.

Bodnar, A. G., M. Ouellette, et al. (1998). "Extension of life-span by introduction of telomerase into normal human cells." Science **279**: 349-352.

Bodnar, A. G., M. Ouellette, et al. (1998). "Extension of life-span by introduction of telomerase into normal human cells." Science **279**(5349): 349-52.

Bradshaw, P. S., D. J. Stavropoulos, et al. (2005). "Human telomeric protein TRF2 associates with genomic double-strand breaks as an early response to DNA damage." Nat Genet **37**(2): 193-7.

Bryan, T. M., A. Englezou, et al. (1997). "Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor derived cell lines." Nat. Med. **3**: 1271-1274.

Bryan, T. M., A. Englezou, et al. (1998). "Telomere length dynamics in telomerase-positive immortal human cell populations." Exp Cell Res **239**(2): 370-8.

Bryan, T. M., A. Englezou, et al. (1995). "Telomere elongation in immortal human cells without detectable telomerase activity." EMBO J. **14**: 4240-4248.

Bryan, T. M. and R. R. Reddel (1997). "Telomere dynamics and telomerase activity in *in vitro* immortalised human cells." Eur. J. Cancer **33**: 767-773.

Burger, A. M., F. Dai, et al. (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.

Canela, A., J. Martin-Caballero, et al. (2004). "Constitutive expression of tert in thymocytes leads to increased incidence and dissemination of T-cell lymphoma in Lck-Tert mice." Mol Cell Biol **24**(10): 4275-93.

Cech, T. R. (2004). "Beginning to understand the end of the chromosome." Cell **116**(2): 273-9.

Cerone, M. A., J. A. Londono-Vallejo, et al. (2001). "Telomere maintenance by telomerase and by recombination can coexist in human cells." Hum. Mol. Genet. **10**: 1945-1952.

Cesare, A. J. and J. D. Griffith (2004). "Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops." Mol Cell Biol **24**(22): 9948-57.

Chang, C. C., I. C. Kuo, et al. (2004). "Detection of quadruplex DNA structures in human telomeres by a fluorescent carbazole derivative." Anal Chem **76**(15): 4490-4.

Chen, Q., A. Ijpma, et al. (2001). "Two survivor pathways that allow growth in the absence of telomerase are generated by distinct telomere recombination events." Mol.Cell. Biol. **21**: 1819-1827.

Chen, Z., K. S. Koeneman, et al. (2003). "Consequences of telomerase inhibition and combination treatments for the proliferation of cancer cells." Cancer Res **63**(18): 5917-25.

Christodoulopoulos, G., A. Malapetsa, et al. (1999). "Chlorambucil induction of HsRad51 in B-cell chronic lymphocytic leukemia." Clin Cancer Res **5**(8): 2178-84.

Colgin, L. M., K. Baran, et al. (2003). "Human POT1 facilitates telomere elongation by telomerase." Curr Biol **13**(11): 942-6.

Corey, D. R. (2002). "Telomerase inhibition, oligonucleotides, and clinical trials." Oncogene **21**: 631-637.

Counter, C. M., A. A. Avilion, et al. (1992). "Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity." EMBO J. **11**: 1921-1929.

Damm, K., U. Hemmann, et al. (2001). "A highly selective telomerase inhibitor limiting human cancer cell proliferation." EMBO J. **20**: 6958-6968.

Davis, P. K., A. Ho, et al. (2001). "Biological methods for cell-cycle synchronization of mammalian cells." Biotechniques **30**(6): 1322-6, 1328, 1330-1.

de Lange, T. (2002). "Protection of mammalian telomeres." Oncogene **21**: 532-540.

Dean, M., T. Fojo, et al. (2005). "Tumour stem cells and drug resistance." Nat Rev Cancer **5**(4): 275-84.

Delhommeau, F., A. Thierry, et al. (2002). "Telomere dysfunction and telomerase reactivation in human leukemia cell lines after telomerase inhibition by the expression of a dominant-negative hTERT mutant." Oncogene **21**: 8262-8271.

Deschatrette, J., K. H. Ng, et al. (2004). "Telomere dynamics determine episodes of anticancer drug resistance in rat hepatoma cells." Anticancer Drugs **15**(7): 671-8.

Dorsett, Y. and T. Tuschl (2004). "siRNAs: applications in functional genomics and potential as therapeutics." Nat Rev Drug Discov **3**(4): 318-29.

Dunham, M. A., A. A. Neumann, et al. (2000). "Telomere maintenance by recombination in human cells." Nat. Genet. **26**: 447-450.

El-Daly, H., M. Kull, et al. (2004). "Selective cytotoxicity and telomere damage in leukemia cells using the telomerase inhibitor BIBR1532." Blood.

Gire, V., P. Roux, et al. (2004). "DNA damage checkpoint kinase Chk2 triggers replicative senescence." Embo J **23**(13): 2554-63.

Gowan, S. M., R. Heald, et al. (2001). "Potent inhibition of telomerase by small-molecule pentacyclic acridines capable of interacting with G-quadruplexes." Mol. Pharmacol. **60**: 981-988.

Goytisolo, F. A., E. Samper, et al. (2000). "Short telomeres result in organismal hypersensitivity to ionizing radiation in mammals." J. Exp. Med. **192**: 1625-1636.

Greider, C. W. (1991). "Telomerase is processive." Mol. Cell. Biol. **11**: 4572-4580.

Greider, C. W. and E. H. Blackburn (1985). "Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts." Cell **43**: 405-413.

Greider, C. W. and E. H. Blackburn (1987). "The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity." Cell **51**: 887-898.

Greider, C. W. and E. H. Blackburn (1996). "Telomeres, Telomerase and Cancer." Scientific American **274**: 80-85.

Griffith, J. D., L. Comeau, et al. (1999). "Mammalian telomeres end in a large duplex loop." Cell **97**: 503-514.

Grobelny, J. V., A. K. Godwin, et al. (2000). "ALT-associated PML bodies are present in viable cells and are enriched in cells in the G2/M phase of the cell cycle." J. Cell Sci. **113**: 4577-4585.

Guiducci, C., M. A. Cerone, et al. (2001). "Expression of mutant telomerase in immortal telomerase-negative human cells results in cell cycle deregulation, nuclear and chromosomal abnormalities and rapid loss of viability." Oncogene **20**: 714-725.

Hahn, W. C., S. A. Stewart, et al. (1999). "Inhibition of telomerase limits the growth of human cancer cells." Nature Med. **5**: 1164-1170.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hardy, C. D., C. S. Schultz, et al. (2001). "Requirements for the dGTP-dependent repeat addition processivity of recombinant *Tetrahymena* telomerase." J. Biol. Chem. **276**: 4863-4871.

Harker, W. G., D. L. Slade, et al. (1989). "Multidrug resistance in mitoxantrone-selected HL-60 leukemia cells in the absence of P-glycoprotein overexpression." Cancer Res **49**(16): 4542-9.

Harley, C. B., A. B. Futcher, et al. (1990). "Telomeres shorten during ageing of human fibroblasts." Nature **345**: 458-460.

Harrington, L. (2003). "Biochemical aspects of telomerase function." Canc. Lett. **194**: 139-154.

Hayflick, L. and P. S. Moorhead (1961). "The serial cultivation of human diploid strains." Exp. Cell Res. **25**: 585-621.

Hemann, M. T., M. A. Strong, et al. (2001). "The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability." Cell **107**(1): 67-77.

Hope, K. J., L. Jin, et al. (2004). "Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity." Nat Immunol **5**(7): 738-43.

Huard, S. and C. Autexier (2002). "Targeting human telomerase in cancer therapy." Curr. Med. Chem. - Anti-Cancer Agents **2**: 577-587.

Huntly, B. J. and D. G. Gilliland (2005). "Leukaemia stem cells and the evolution of cancer-stem-cell research." Nat Rev Cancer **5**(4): 311-21.

Hurley, L. H. (2002). "DNA and its associated processes as targets for cancer therapy." Nat Rev Cancer **2**(3): 188-200.

Incles, C. M., C. M. Schultes, et al. (2003). "Acquired cellular resistance to flavopiridol in a human colon carcinoma cell line involves up-regulation of the telomerase catalytic subunit and telomere elongation. Sensitivity of resistant cells to combination treatment with a telomerase inhibitor." Mol Pharmacol **64**(5): 1101-8.

Ishikawa, T., M. Kamiyama, et al. (1999). "Telomerase enzyme activity and RNA expression in adriamycin-resistant human breast carcinoma MCF-7 cells." Canc. Lett. **187-194**: 187-194.

Iwano, T., M. Tachibana, et al. (2004). "Importance of TRF1 for functional telomere structure." J Biol Chem **279**(2): 1442-8.

Jeyapalan, J., A. Leake, et al. (2004). "The role of telomeres in Etoposide induced tumor cell death." Cell Cycle **3**(9): 1169-76.

Kang, H. J., Y. S. Choi, et al. (2004). "Ectopic expression of the catalytic subunit of telomerase protects against brain injury resulting from ischemia and NMDA-induced neurotoxicity." J Neurosci **24**(6): 1280-7.

Karlseder, J., D. Broccoli, et al. (1999). "p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2." Science **283**: 1321-1325.

Keshelava, N., R. C. Seeger, et al. (1997). "Drug resistance in human neuroblastoma cell lines correlates with clinical therapy." Eur J Cancer **33**(12): 2002-6.

Kim, J. H., G. E. Lee, et al. (2002). "A novel telomere elongation in an adriamycin-resistant stomach cancer cell line with decreased telomerase activity." Mol Cells **13**(2): 228-36.

Kim, M. M., M. A. Rivera, et al. (2001). "A low threshold level of expression of mutant-template telomerase RNA inhibits human tumor cell proliferation." Proc. Natl. Acad. Sci USA **98**: 7982-7987.

Kim, M. Y., M. Gleason-Guzman, et al. (2003). "The different biological effects of telomestatin and TMPyP4 can be attributed to their selectivity for interaction with intramolecular or intermolecular G-quadruplex structures." Cancer Res **63**(12): 3247-56.

Kim, N. W., M. A. Piatyszek, et al. (1994). "Specific association of human telomerase activity with immortal cells and cancer." Science **266**: 2011-2015.

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**: 2595-2597.

Kipling, D. and H. J. Cooke (1990). "Hypervariable ultra-long telomeres in mice." Nature **347**: 400-402.

Klapper, W., W. Qian, et al. (2003). "DNA damage transiently increases TRF2 mRNA expression and telomerase activity." Leukemia **17**(10): 2007-15.

Klobutcher, L. A., M. T. Swanton, et al. (1981). "All gene-sized DNA molecules in four species of hypotrichs have the same terminal sequence and an unusual 3' terminus." Proc. Natl. Acad. Sci. USA **78**: 3015-3019.

Kondo, T., N. Oue, et al. (2004). "Expression of POT1 is associated with tumor stage and telomere length in gastric carcinoma." Cancer Res **64**(2): 523-9.

Kondo, Y., S. Kondo, et al. (1998). "Inhibition of telomerase increases the susceptibility of human malignant glioblastoma cells to cisplatin-induced apoptosis." Oncogene **16**: 2243-2248.

Kraemer, K., S. Fuessel, et al. (2004). "Chemosensitization of bladder cancer cell lines by human telomerase reverse transcriptase antisense treatment." J Urol **172**(5 Pt 1): 2023-8.

Kramer, K. M. and J. E. Haber (1993). "New telomeres in yeast are initiated with a highly selected subset of TG1-3 repeats." Genes. Dev. **7**: 2345-2356.

Kuranaga, N., N. Shinomiya, et al. (2001). "Long-term cultivation of colorectal carcinoma cells with anti-cancer drugs incudes drug resistance and telomere elongation: an in vitro study." BMC Cancer **1**: 10.

Lane, L. (2005). "Antisense and Sensibility in RNA Therapeutics." The Scientist **19**(1): 30.

Lee, F. Y. and D. W. Siemann (1989). "Isolation by flow cytometry of a human ovarian tumor cell subpopulation exhibiting a high glutathione content phenotype and increased resistance to adriamycin." Int J Radiat Oncol Biol Phys **16**(5): 1315-9.

Lee, K.-H., K. L. Rudolph, et al. (2001). "Telomere dysfunction alters the chemotherapeutic profile of transformed cells." Proc. Natl. Acad. Sci. USA **98**: 3381-3386.

Lei, M., E. R. Podell, et al. (2004). "Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection." Nat Struct Mol Biol **11**(12): 1223-9.

Lei, M., A. J. Zaug, et al. (2005). "Switching human telomerase on and off with hPOT1 protein in vitro." J Biol Chem **280**(21): 20449-56.

Leslie, E. M., R. G. Deeley, et al. (2005). "Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense." Toxicol Appl Pharmacol **204**(3): 216-37.

Li, S., J. E. Rosenberg, et al. (2004). "Rapid inhibition of cancer cell growth induced by lentiviral delivery and expression of mutant-template telomerase RNA and anti-telomerase short-interfering RNA." Cancer Res **64**(14): 4833-40.

Lin, Z., S. Lim, et al. (2001). "Down-regulation of telomerase activity in malignant lymphomas by radiation and chemotherapeutic agents." Am J Pathol **159**(2): 711-9.

Loayza, D. and T. De Lange (2003). "POT1 as a terminal transducer of TRF1 telomere length control." Nature **423**(6943): 1013-8.

Lu, C., W. Fu, et al. (2001). "Telomerase protects developing neurons against DNA damage-induced cell death." Devel. Brain Res. **131**: 167-171.

Ludwig, A., G. Saretzki, et al. (2001). "Ribozyme cleavage of telomerase mRNA sensitizes breast epithelial cells to inhibitors of topoisomerase." Cancer Res. **61**: 3053-3061.

Marusic, L., M. Anton, et al. (1997). "Reprogramming of telomerase by expression of mutant telomerase RNA template in human cells leads to altered telomeres that correlate with reduced cell viability." Mol. Cell. Biol. **17**: 6394-6401.

Masutomi, K., R. Possemato, et al. (2005). "The telomerase reverse transcriptase regulates chromatin state and DNA damage responses." Proc Natl Acad Sci U S A **102**(23): 8222-7.

Mattern, J. (2003). "Drug resistance in cancer: a multifactorial problem." Anticancer Res **23**(2C): 1769-72.

Moriarty, T., S. Dupuis, et al. (2002). "Rapid up-regulation of telomerase activity in human leukemia HL-60 cells treated with the DNA-damaging drug etoposide." Leukemia **16**: 1112-1120.

Moriarty, T., S. Huard, et al. (2002). "Functional multimerization of human telomerase requires an RNA interaction domain in the N-terminus of the catalytic subunit." Mol. Cell. Biol. **22**: 1253-1265.

Moriarty, T. J., D. T. Marie-Egyptienne, et al. (2004). "Functional organization of repeat addition processivity and DNA synthesis determinants in the human telomerase multimer." Mol Cell Biol **24**(9): 3720-33.

Morin, G. B. (1989). "The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats." Cell **59**: 521-529.

Moyzis, R. K., J. M. Buckingham, et al. (1988). "A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes." Proc. Natl. Acad. Sci. USA **85**: 6622-6626.

Murnane, J. P., L. Sabatier, et al. (1994). "Telomere dynamics in an immortal human cell line." EMBO J. **13**: 4953-4962.

Murti, K. G. and D. M. Prescott (1999). "Telomeres of polytene chromosomes in a ciliated protozoan terminate in duplex DNA loops." Proc Natl Acad Sci U S A **96**(25): 14436-9.

Neidle, S. and G. N. Parkinson (2003). "The structure of telomeric DNA." Curr Opin Struct Biol **13**(3): 275-83.

Olovnikov, A. M. (1973). "A theory of marginotomy." J. Theor. Biol. **41**: 181-190.

Pandit, B. and N. P. Bhattacharyya (1998). "Detection of Telomerase Activity in Chinese Hamster V79 Cells and Its Inhibition by 7-Deaza-deoxy Guanosine Triphosphate and $(TTAGGG)_4$ *in Vitro*." Biochem. BioPhys. Res. Comm. **251**: 620-624.

Pantic, M., S. Zimmermann, et al. (2005). "The level of telomere dysfunction determines the efficacy of telomerase-based therapeutics in a lung cancer cell line." Int J Oncol **26**(5): 1227-32.

Park, K. H., S. Y. Rha, et al. (1998). "Telomerase activity and telomere lengths in various cell lines: changes of telomerase activity can be another method for chemosensitivity evaluation." Int J Oncol **13**(3): 489-95.

Parkinson, E. K. (2003). "Telomerase as a novel and potentially selective target for cancer chemotherapy." Ann Med **35**(7): 466-75.

Pascolo, E., C. Wenz, et al. (2002). "Mechanism of human telomerase inhibition by BIBR1532, a synthetic, non-nucleosidic drug candidate." J. Biol. Chem. **277**(18): 15566-72.

Peng, Y., S. I. Mian, et al. (2001). "Analysis of telomerase processivity: mechanistic similarity to HIV-1 reverse transcriptase and role in telomere maintenance." Mol. Cell **7**: 1201-1211.

Pennarun, G., C. Granotier, et al. (2005). "Apoptosis related to telomere instability and cell cycle alterations in human glioma cells treated by new highly selective G-quadruplex ligands." Oncogene **24**(18): 2917-28.

Pennarun, G., C. Granotier, et al. (2005). "Apoptosis related to telomere instability and cell cycle alterations in human glioma cells treated by new highly selective G-quadruplex ligands." Oncogene.

Riou, J. F. (2004). "G-quadruplex interacting agents targeting the telomeric G-overhang are more than simple telomerase inhibitors." Curr Med Chem Anti-Canc Agents **4**(5): 439-43.

Robert, J. and C. Jarry (2003). "Multidrug resistance reversal agents." J Med Chem **46**(23): 4805-17.

Rubio, M. A., A. R. Davalos, et al. (2004). "Telomere length mediates the effects of telomerase on the cellular response to genotoxic stress." Exp Cell Res **298**(1): 17-27.

Salinas, A. E. and M. G. Wong (1999). "Glutathione S-transferases--a review." Curr Med Chem **6**(4): 279-309.

Saretzki, G., N. Sitte, et al. (1999). "Telomere shortening triggers a p53-dependent cell cycle arrest via accumulation of G-rich single stranded DNA fragments." Oncogene **18**: 5148-5158.

Sarin, K. Y., P. Cheung, et al. (2005). "Conditional telomerase induction causes proliferation of hair follicle stem cells." Nature **436**(7053): 1048-52.

Sato, N., K. Mizumoto, et al. (2000). "Up-regulation of telomerase activity in human pancreatic cancer cells after exposure to etoposide." Br. J. Cancer **82**: 1819-1826.

Schaffitzel, C., I. Berger, et al. (2001). "In vitro generated antibodies specific for telomeric guanine-quadruplex DNA react with Stylonychia lemnae macronuclei." Proc Natl Acad Sci U S A **98**(15): 8572-7.

Schnapp, G., H.-P. Rodi, et al. (1998). "One-step affinity purification protocol for human telomerase." Nucl. Acids Res. **26**: 3311-3313.

Sfeir, A. J., W. Chai, et al. (2005). "Telomere-end processing the terminal nucleotides of human chromosomes." Mol Cell **18**(1): 131-8.

Shay, J. W. and S. Bacchetti (1997). "A survey of telomerase activity in human cancer." Eur. J. Cancer **33**: 787-791.

Shay, J. W., O. M. Pereira-Smith, et al. (1991). "A role for both RB and p53 in the regulation of human cellular senescence." Exp. Cell Res. **196**: 33-39.

Sherr, C. J. and F. McCormick (2002). "The RB and p53 pathways in cancer." Cancer Cell **2**(2): 103-12.

Shin, K. H., M. K. Kang, et al. (2004). "Introduction of human telomerase reverse transcriptase to normal human fibroblasts enhances DNA repair capacity." Clin Cancer Res **10**(7): 2551-60.

Siddiqui-Jain, A., C. L. Grand, et al. (2002). "Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription." Proc Natl Acad Sci U S A **99**(18): 11593-8.

Singh, S. K., I. D. Clarke, et al. (2004). "Cancer stem cells in nervous system tumors." Oncogene **23**(43): 7267-73.

Singh, S. K., C. Hawkins, et al. (2004). "Identification of human brain tumour initiating cells." Nature **432**(7015): 396-401.

Smogorzewska, A. and T. de Lange (2002). "Different telomere damage signaling pathways in human and mouse cells." Embo J **21**(16): 4338-48.

Smogorzewska, A., B. van Steensel, et al. (2000). "Control of Human Telomere Length by TRF1 and TRF2." Mol. Cell. Biol. **20**: 1659-1668.

Stavrovskaya, A. A. (2000). "Cellular mechanisms of multidrug resistance of tumor cells." Biochemistry (Mosc) **65**(1): 95-106.

Stewart, S. A., W. C. Hahn, et al. (2002). "Telomerase contributes to tumorigenesis by a telomere length-independent mechanism." Proc. Natl. Acad. Sci. USA.

Stewart, S. L., J. B. King, et al. (2004). "Cancer mortality surveillance--United States, 1990-2000." MMWR Surveill Summ **53**(3): 1-108.

Sun, B., M. Chen, et al. (2005). "Immortal ALT+ human cells do not require telomerase reverse transcriptase for malignant transformation." Cancer Res **65**(15): 6512-5.

Takai, H., A. Smogorzewska, et al. (2003). "DNA damage foci at dysfunctional telomeres." Curr Biol **13**(17): 1549-56.

Tauchi, T., A. Nakajima, et al. (2002). "Inhibition of human telomerase enhances the effect of the tyrosine kinase inhibitor, imatinib, in BCR-ABL-positive leukemia cells." Clin. Canc. Res. **8**: 3341-3347.

Teixeira, M. T., M. Arneric, et al. (2004). "Telomere length homeostasis is achieved via a switch between telomerase- extendible and -nonextendible states." Cell **117**(3): 323-35.

Teng, S. C., J. Chang, et al. (2000). "Telomerase-independent lengthening of yeast telomeres occurs by an abrupt Rad50p-dependent, Rif-inhibited recombinational process." Mol. Cell **6**: 947-952.

Teng, S. C. and V. A. Zakian (1999). "Telomere-telomere recombination is an efficient bypass pathway for telomere maintenance in Saccharomyces cerevisiae." Mol.Cell. Biol. **19**: 8083-8093.

Tentori, L., I. Portarena, et al. (2003). "Inhibition of telomerase increases resistance of melanoma cells to temozolomide, but not to temozolomide combined with poly (adp-ribose) polymerase inhibitor." Mol Pharmacol **63**(1): 192-202.

van Steensel, B. and T. de Lange (1997). "Control of telomere length by the human telomeric protein TRF1." Nature **385**(6618): 740-3.

Vaziri, H. and S. Benchimol (1998). "Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span." Curr Biol **8**: 279-282.

Vulliamy, T., A. Marrone, et al. (2004). "Disease anticipation is associated with progressive telomere shortening in families with dyskeratosis congenita due to mutations in TERC." Nat Genet **36**(5): 447-9.

Watson, J. D. (1972). "Origin of concatameric T4 DNA." Nature New Biol. **239**: 197-201.

Weinrich, S. L., R. Pruzan, et al. (1997). "Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT." Nat. Genet. **17**: 498-502.

Wellinger, R. J. and D. Sen (1997). "The DNA structures at the ends of eukaryotic chromosomes." Eur. J. Cancer **33**: 735-749.

Wenz, C., B. Enenkel, et al. (2001). "Human telomerase contains two cooperating telomerase RNA molecules." EMBO J. **20**: 3526-3534.

Wong, K.-K., S. Chang, et al. (2000). "Telomere dysfunction impairs DNA repair and enhances sensitivity to ionizing radiation." Nat. Genet. **26**: 85-88.

Wright, W. E., O. M. Pereira-Smith, et al. (1989). "Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts." Mol Cell Biol **9**(7): 3088-92.

Wright, W. E., V. M. Tesmer, et al. (1997). "Normal human chromosomes have long G-rich telomeric overhangs at one end." Genes Dev **11**(21): 2801-9.

Wu, G., W. H. Lee, et al. (2000). "NBS1and TRF1 colocalize at promyelocytic leukemia bodies during late S/G2 phases in immortalized telomerase-negative cells. Implications of NBS1 in alternative lengthening of telomeres." J. Biol. Chem. **275**: 30618-30622.

Yeager, T. R., A. A. Neumann, et al. (1999). "Telomerase-negative immortalized cells contain a novel type of promyelocytic (PML) body." Cancer Res. **59**: 4175-4179.

Yen, L., A. Woo, et al. (1995). "Enhanced host cell reactivation capacity and expression of DNA repair genes in human breast cancer cells resistant to bi-functional alkylating agents." Mutat Res **337**(3): 179-89.

Yoon, K. A., J. L. Ku, et al. (2003). "Telomerase activity, expression of Bcl-2 and cell cycle regulation in doxorubicin resistant gastric carcinoma cell lines." Int J Mol Med **11**(3): 343-8.

Yoshimura, S. H., H. Maruyama, et al. (2004). "Molecular mechanisms of DNA end-loop formation by TRF2." Genes Cells **9**(3): 205-18.

Yu, G.-L., J. D. Bradley, et al. (1990). "*In vivo* alteration of telomere sequences and senescence caused by mutated *Tetrahymena* telomerase RNAs." Nature **344**: 126-132.

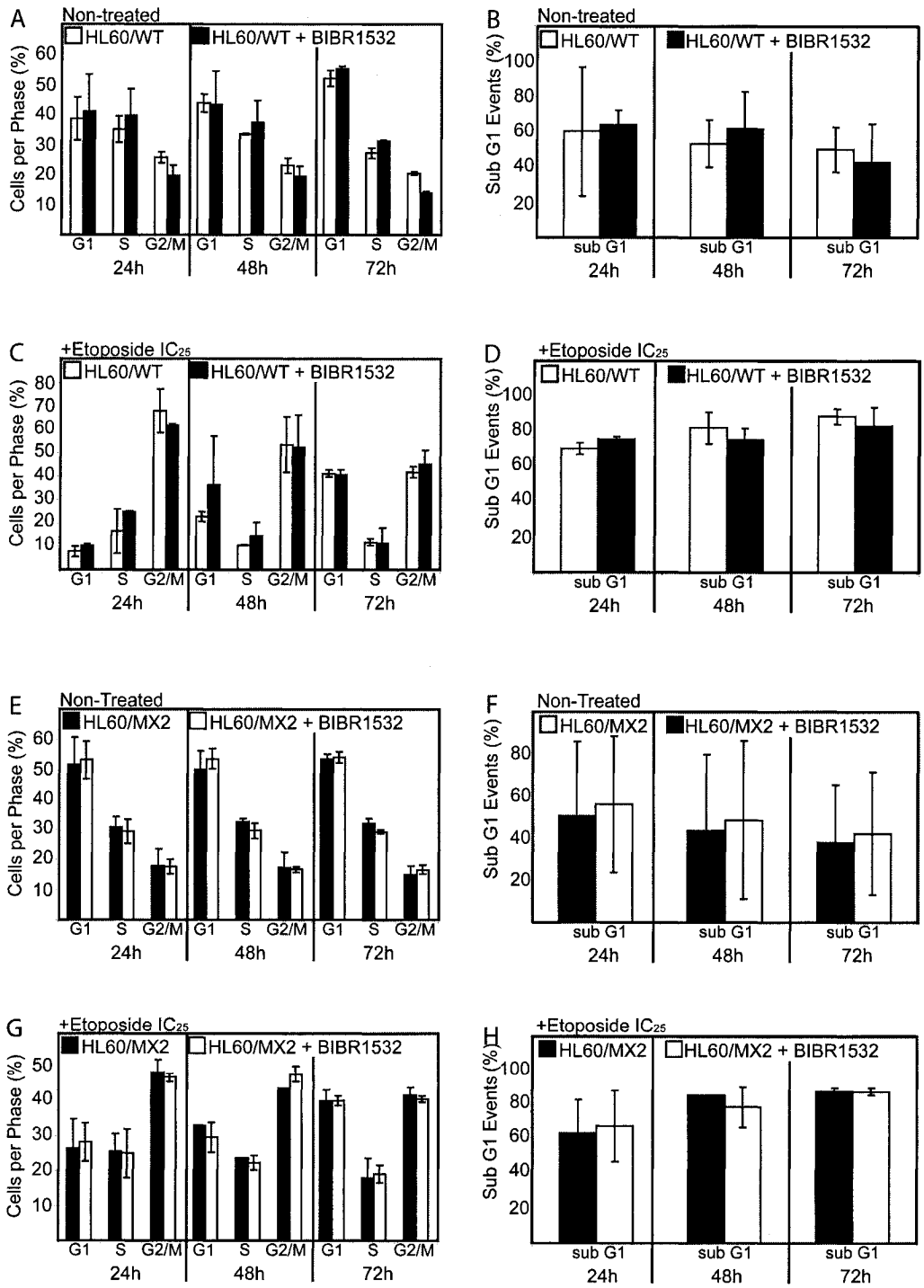
Zhang, P., S. L. Chan, et al. (2003). "TERT suppresses apoptotis at a premitochondrial step by a mechanism requiring reverse transcriptase activity and 14-3-3 protein-binding ability." Faseb J **17**(6): 767-9.

Zhang, X., V. Mar, et al. (1999). "Telomere shortening and apoptosis in telomerase-inhibited human tumor cells." Genes & Devel. **13**: 2388-2399.

Zhou, H. S., D. P. Liu, et al. (2004). "Challenges and strategies: the immune responses in gene therapy." Med Res Rev **24**(6): 748-61.

Zijlmans, J. M., U. M. Martens, et al. (1997). "Telomeres in the mouse have large inter-chromosomal variations in the number of T_2AG_3 repeats." Proc. Natl. Acad. USA **94**: 7423-7428.

Appendix A


A
Non-treated
HL60/WT
HL60/WT + BIBR1532
Cells per Phase (%)
G1 S G2/M G1 S G2/M G1 S G2/M
24h 48h 72h
B
Non-treated
HL60/WT
HL60/WT + BIBR1532
Sub G1 Events (%)
sub G1 sub G1 sub G1
24h 48h 72h
C
+Etoposide IC25
HL60/WT
HL60/WT + BIBR1532
Cells per Phase (%)
G1 S G2/M G1 S G2/M G1 S G2/M
24h 48h 72h
D
+Etoposide IC25
HL60/WT
HL60/WT + BIBR1532
Sub G1 Events (%)
sub G1 sub G1 sub G1
24h 48h 72h
E
Non-Treated
HL60/MX2
HL60/MX2 + BIBR1532
Cells per Phase (%)
G1 S G2/M G1 S G2/M G1 S G2/M
24h 48h 72h
F
Non-Treated
HL60/MX2
HL60/MX2 + BIBR1532
Sub G1 Events (%)
sub G1 sub G1 sub G1
24h 48h 72h
G
+Etoposide IC25
HL60/MX2
HL60/MX2 + BIBR1532
Cells per Phase (%)
G1 S G2/M G1 S G2/M G1 S G2/M
24h 48h 72h
H
+Etoposide IC25
HL60/MX2
HL60/MX2 + BIBR1532
Sub G1 Events (%)
sub G1 sub G1 sub G1
24h 48h 72h

Appendix A- Cell cycle FACS analysis of HL60/WT and HL60/MX2 cells. A-D, HL60/WT cells, untreated or treated with 2.5μM BIBR1532 for 65-75 population doublings (HL60/WT + BIBR) and co-treated with IC_{25} etoposide (C&D). Cell cycle analysis showing (A&C) the percentage of HL60/WT cells within each phase of the cell cycle, and (B&D) the percentage of sub-G1 events (apoptotic cells, mechanically damaged cells and cellular debris) within the HL60/WT cell populations. E-H, HL60/MX2 cells, untreated or treated with 2.5μM BIBR1532 for 95-100 population doublings (HL60/MX2 + BIBR) and co-treated with IC_{25} etoposide (G&H). Cell cycle analysis showing (E&G) the percentage of HL60/MX2 cells within each phase of the cell cycle, and (F&H) the percentage of sub-G1 events within the HL60/MX2 cell populations.

Appendix B