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STUDY OF THE EXTRA-TELOMERIC FUNCTIONS OF TELOMERASE IN *IN VITRO* AND *IN VIVO* MODELS

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Abstract

Maintenance of telomere length, required for the unlimited cell proliferation displayed by cancer cells, is provided by telomerase, a ribonucleoprotein complex containing a specialized reverse transcriptase, encoded by *TERT* gene, that uses an internal RNA template to maintain telomeres length, thus playing a critical role in tumor formation and progression. *TERT* is usually repressed in normal somatic cells, but is detectable in the vast majority of tumors.

Recent studies have suggested that TERT, besides maintaining telomere, is involved in other cellular functions, and it may contribute to carcinogenesis also *via* telomere length-independent mechanisms; therefore its inhibition could represent a promising strategy to improve cancer treatment, regardless of telomere length. The possible therapeutic effects of BIBR1532 (BIBR), a specific TERT inhibitor, have been evaluated in different cellular backgrounds, but no data are currently available regarding Epstein-Barr virus (EBV)-driven and virus-unrelated B-cell malignancies.

The aim of this study was to characterize the biological effects of short-term TERT inhibition by BIBR on EBV-immortalized lymphoblastoid cell lines (LCLs) and fully transformed Burkitt's lymphoma (BL) cell lines; in addition, we investigated the effects of short-term BIBR treatment *in vivo* in wild type zebrafish embryos.

We found that short-term inhibition of TERT by BIBR, in *in vitro* models of B-cell malignancies, led to decreased cell proliferation, accumulation of cells in the S-phase and ultimately increased apoptosis. The cell cycle arrest and apoptosis, consequent upon short-term TERT inhibition, were associated with and likely dependent on the activation of the DNA damage response (DDR), highlighted by the increased levels of γH2AX and activation of ATM and ATR pathways. Analyses of the mean and range of telomere lengths and telomere dysfunction-induced foci indicated that DDR after short-term TERT inhibition was not related to telomere dysfunction, thus suggesting that TERT, besides stabilizing telomere, may protect DNA via telomere-independent mechanisms. Notably, TERT-positive LCLs treated

with BIBR in combination with fludarabine or cyclophosphamide showed a significant increase in the number of apoptotic cells with respect to those treated with chemotherapeutic agents alone.

In agreement with *in vitro* results, short-term inhibition of Tert by BIBR in wild type zebrafish embryos reduced cell proliferation, induced an accumulation of cells in S-phase, increased apoptosis, and triggered the activation of DDR. These effects were telomere length-unrelated, since the range of telomere length was not affected by the short-term BIBR treatment and the DNA damage foci were distributed randomly, rather than specifically located at telomeres. All these effects were specifically related to Tert inhibition since BIBR treatment showed no effect in Tert-negative zebrafish embryos.

Taken together these data demonstrate that TERT inhibition impairs cell proliferation and induces pro-apoptotic effects unrelated to telomere dysfunction, enforcing the concept that TERT *per se* exerts telomere length-independent tumor-promoting effects, and thus supporting the introduction of TERT inhibitors to complement current anticancer treatment modalities.

Abstract

Il mantenimento dei telomeri, necessario per la proliferazione illimitata delle cellule tumorali, è esercitato dalla telomerasi, un complesso ribonucleoproteico contenente una trascrittasi inversa specializzata, codificata dal gene *TERT*, che utilizza un templato ad RNA per sintetizzare nuove sequenze telomeriche, svolgendo quindi un ruolo critico nella formazione e nella progressione dei tumori. TERT viene infatti solitamente represso in normali cellule somatiche, mentre è rilevabile nella maggior parte dei tumori.

Studi recenti hanno suggerito che TERT è coinvolto in altre funzioni cellulari e può contribuire alla carcinogenesi anche attraverso meccanismi indipendenti dal mantenimento dei telomeri, quindi la sua inibizione potrebbe rappresentare una strategia promettente per migliorare il trattamento antitumorale, al di là dell'effetto sui telomeri. I possibili effetti terapeutici di BIBR1532 (BIBR), un inibitore specifico del TERT, sono stati valutati in diversi contesti cellulari, ma non sono attualmente disponibili dati ottenuti su modelli di neoplasie delle cellule B sia associate al virus di Epstein-Barr (EBV) che virus-indipendenti.

Lo scopo di questo studio era di caratterizzare gli effetti biologici dell'inibizione di TERT a breve termine da parte del BIBR su linee cellulari linfoblastoidi immortalizzate da EBV (LCL) e su modelli in vitro di linfoma di Burkitt (BL); inoltre, sono stati studiati gli effetti del trattamento con BIBR a breve termine in vivo negli embrioni di zebrafish.

I risultati ottenuti hanno dimostrato che l'inibizione a breve termine di TERT da parte di BIBR, in modelli in vitro di tumori delle cellule B, ha portato a una diminuzione della proliferazione cellulare, all'accumulo di cellule nella fase S e infine all'aumento dell'apoptosi. L'arresto del ciclo cellulare e l'apoptosi, conseguenti all'inibizione di TERT a breve termine, erano associati e probabilmente dipendenti dall'attivazione della risposta al danno del DNA, come evidenziato dall'aumento dei livelli di γH2AX e dall'attivazione dei *pathway* di ATM e ATR. L'analisi della media e del *range* di lunghezza dei telomeri e dei foci di danno

al DNA ha indicato che la risposta al danno attivata in seguito all'inibizione TERT a breve termine non era legata a disfunzioni telomeriche, suggerendo quindi che TERT, oltre a stabilizzare il telomero, può proteggere il DNA tramite meccanismi telomero-indipendenti. In particolare, LCL-TERT positive trattate con BIBR in combinazione con fludarabina o ciclofosfamide hanno mostrato un aumento significativo del numero di cellule apoptotiche rispetto a quelle trattate con agenti chemioterapici da soli.

In accordo con i risultati in vitro, l'inibizione a breve termine di Tert da parte del BIBR in embrioni di zebrafish ha ridotto la proliferazione cellulare, indotto un accumulo di cellule nella fase S, aumentato il tasso di apoptosi e innescato l'attivazione della risposta al danno al DNA. Questi effetti non erano legati a disfunzioni telomeriche, poiché il *range* di lunghezza dei telomeri non era influenzato dal trattamento a breve termine con BIBR e i foci di danno al DNA erano distribuiti casualmente, piuttosto che localizzati in modo specifico sui telomeri. Tutti questi effetti erano specificamente associati all'inibizione di Tert poiché il trattamento con BIBR non mostrava alcun effetto negli embrioni di zebrafish Tert-negativi.

Nel complesso questi dati dimostrano che l'inibizione del TERT compromette la proliferazione cellulare e induce effetti pro-apoptotici non associati a disfunzioni telomeriche, rafforzando il concetto che TERT esercita di per sé funzioni pro-tumorali indipendenti dalla lunghezza del telomero e quindi supportando l'introduzione di inibitori di TERT per integrare le attuali modalità di trattamento antitumorale.

Chapter 1: General Introduction

1.1. Telomeres

1.1.1. Telomere Structure and Functions

The genome of eukaryotic cells is organized in linear structures called chromosomes, that contain all the information required by the cells for growth and metabolism. This genetic information is inevitably threatened by several endogenous and exogenous insults, such as normal metabolic processes, DNA replication, radiations and chemicals, altogether leading to the introduction of breaks or modification, referred to as DNA damages (1-3).

Damaged DNA leads to genomic instability that in turn can impair proper cellular functions, affecting organism's development, homeostasis and ageing (3). Thus, in order to preserve the integrity of the genetic information, a fine and complex DNA damage repair system evolved, named the DNA damage response (DDR) (4). DDR recognizes the DNA damage, adapts the cellular processes accordingly through the activation of several checkpoints (e.g. ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) checkpoint kinases (5)), and triggers DNA damage repair mechanisms.

A key point is that the DDR must distinguish between damaged DNA and the end of linear chromosomes (the end-protection problem), in order to avoid aberrant repair of the latter and preserve the spatial organization of the genome. For this purpose, the very ends of chromosomes assume characteristic protective structures called telomeres; they are composed of several tandem repeats, associated with specific protein complexes, with structural and regulative functions (1).

The length of telomeric repeats is a specie-specific trait, with high variability between individuals, different tissue and even across different chromosome arms (1). In particular, human telomeric DNA is 5-15 kilobases (kb) of double strand (ds) (TTAGGG)n repeats followed by a 50-300 nucleotides of G-rich single strand (ss) 3' overhang (6). This overhang folds back and invades the ds region in a short portion that assume a triple-stranded conformation, named displacement loop, D-loop. The resulting 3D loop structure of chromosome end, the telomeric loop (T-loop), hides the very end of the chromosome from the DDR machinery (7).

Telomere repeats are preceded by subtelomeric regions, composed of more variable repeats, that transcribe Telomere repeat-containing RNAs (TERRA), a G-rich long noncoding RNAs. TERRA transcripts have been proposed to be involved in telomere biology, including heterochromatin formation and telomere length homeostasis (8).

Thanks to the G-rich sequence, telomeric DNA can assume a particular conformation, called G-quadruplex, that was proposed to participate in the capping function; if these structures are stabilized and cannot unfold during DNA replication telomere homeostasis is impaired (9).

The three-dimensional structure of chromosome ends is sustained by the proteins of the shelterin complex, the chief telomere specific protein complex, the main functions of which are the recruitment of telomere maintenance machinery and the suppression of aberrant DNA break repair at chromosome ends, preserving the telomere structure and their protective capping function. Shelterin proteins, in turn, recruit a plethora of additional components, e.g. CST complex and chromatin modifiers, altogether involved in telomere homeostasis (6,10,11).

Shelterin consist of six telomere-specific proteins: Telomere Repeat Factor 1 and 2 (TRF1 and TRF2), Repressor/Activator Protein 1 (RAP1), Protection of Telomere 1 (POT1), TRF1- and TRF2-Interacting Nuclear Protein 2 (TIN2), and TPP1.

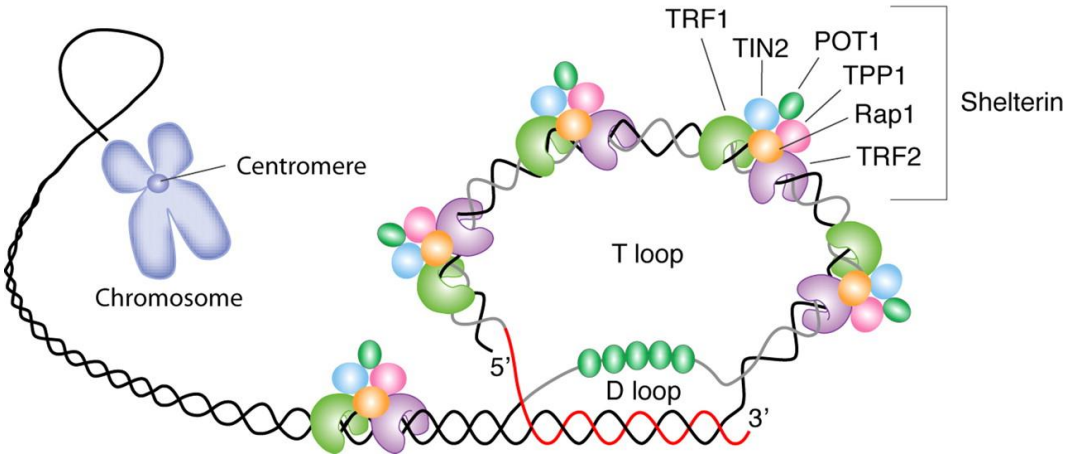


***Figure 1.1**. From Calado, Blood, 2008 (12)*

TRF1 and TRF2 specifically bind to the ds TTAGGG sequences, and are estimated to cover the telomeric DNA with thousands of dimers *per* telomere, contributing to 3-dimensional architecture (13). RAP1 lacks DNA-binding domain and is inserted in the shelterin complex through the association with TRF2. POT1 is the ss 3' overhang-binding protein, with specificity for ss TTAGGG repeats, while the POT1/TTP1 dimer is bridged to the ds-binding proteins by TIN2 (14).

Shelterin, besides stabilizing telomere structure, is mainly involved in the repression of DDR at telomeres; indeed, the lack of shelterin proteins at telomeres elicits a telomere-localized ATM/ATR-dependent DDR and, as a consequence, telomeres undergoes homologous recombination (HR) or non-homologous end joining (NHEJ) DNA damage repair mechanisms, becoming dysfunctional (15,16).

In particular, TRF2 inhibits ATM-dependent repair pathways by sustaining the T-loop configuration, hiding the end of chromosomes from the MRN (DNA binding) damage sensor complex, essential for ATM activation, and by directly inhibiting the ATM kinase as well as crucial downstream effectors. When telomeres are depleted of TRF2, they become substrate for NHEJ, leading to chromosome end-to-end fusion (6). On the other side, ssDNA bound POT1 prevents the recruitment of Replication protein A (RPA), crucial activator of ATR signaling for the repair of ss breaks by HR (6). Moreover, TRF1 and TRF2 suppress the nucleotide excision

repair (NER) mechanism that threatens telomeres; indeed NER is usually triggered by oxidative stress- and UV radiation-induced damage, to which telomeric DNA is particularly sensitive, because of the nature of the G-rich repeated sequence (17).

Another fundamental function of shelterin proteins concerns the telomere maintenance mechanism; indeed, shelterin participates in the regulation of telomere length through the recruitment of the telomerase, the ribonucleoprotein complex responsible for telomere maintenance (it will be detailed in 1.2.), and through the modulation of telomerase activity (18).

In addition, TRF1 has a major role in assisting the semi-conservative replication of telomeres by avoiding, with the help of helicases, the formation of fragile site at telomeres attributed to replication defects derived from impaired 3D structure unwinding (6)

Shelterin is also involved in the negative regulation of the physiological resection step at the end of telomere replication, the fundamental process that generates the 3' overhang functional to the T-loop structure; this process needs a fine regulation to avoid aberrant degradation/hyper-resection that would lead to telomere erosion and the consequent loss of these protective structures (6).

1.1.2. Telomere Erosion – The End Replication Problem

Besides solving the end-protection problem, telomeres are also fundamental to overcome the end-replication problem, because the canonical replication machinery is not sufficient to maintain the entire length of telomeres. As Wellinger summarized (19), the very last bit of the newly synthesized lagging strand cannot be turned into DNA, and this portion corresponds at least to the length of the RNA primer required to start the DNA synthesis. On the other side, the newly discontinuously synthesized leading strand must undergo 5' resection and fill-in synthesis steps (regulated by the CST complex), in order to form the 3' overhang,

unavoidably resulting in a shorter telomere compared to the parental one at each replication cycle.

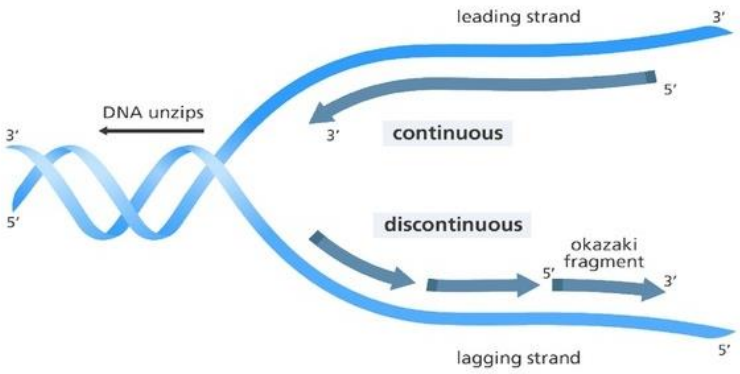


Figure 1.2. *From https://www.quora.com/What-is-the-difference-between-leading-and-lagging-strands*

Thus, the canonical replication machinery is not sufficient to replicate the very ends of chromosomes and, at each cell division, 50-200 base pairs (bp) of the telomeric repeats are lost (13). After continuous population doublings, telomere length reaches a critical threshold and telomeres become dysfunctional. The replicative life-span of cells with critically short telomeres is reduced; these cells are unable to undergo further division and morphological alteration and widespread genomic instability are induced (1).

In particular, critically short telomeres, which are no longer able to bind enough shelterins to repress the DDR, elicit the activation of ATM and ATR checkpoint kinases, that leads to the formation of DNA damage foci localized at telomeres (telomere dysfunction induced foci; TIF), followed by the upregulation of TP53 and the induction of cyclin-dependent kinase inhibitors p21 and p16. These cells usually stop their cell cycle in G1-phase and physiologically undergo senescence or apoptosis programs, depending on cell type (20). This telomere attrition is a physiological condition characterizing the aging of cells and the whole organism.

Critically short telomeres in cells with compromised checkpoints or unable to activate the TP53 and/or RB pathways, can bypass the senescence barrier and

continue to divide, thereby telomeres continue to shorten, increasing the number of dysfunctional chromosome ends (20).

In these cases, cells are in a stage referred to as crisis, characterized by telomeres that are not distinguishable from double strand break (DSB), and, then, continuously trigger DNA repair mechanisms; the consequence is the fusion of telomeres with other telomeric or non-telomeric damaged sequences (17). Fused chromosomes, after replication, form a bridge during anaphase, which eventually breaks when the two centromeres are pulled in opposite direction during cell division, creating novel chromosome free ends available for another fusion. This process is referred to as break-fusion-bridge (B-F-B) cycle (17).

Repeated B-F-B cycles lead to genomic instability that triggers gross genome reorganization by intra- or inter-chromosome events (e.g. resulting in dicentric, acentric or circular chromosomes, translocations, and karyotype alteration), and results in terminal deletion or end-to-end fusion (by NHEJ) and telomere length changes (by HR).

The escape from crisis requires the activation of a specific protein, called telomerase, in order to maintain telomere length, reconstitute the telomere capping function and restore the proliferative capacity (20,21). The result is a tumor promoting reorganization and stabilization of the genome; indeed, telomerase is expressed in the vast majority of tumors and is responsible for unlimited proliferative potential of tumor cells (see 1.2 section). In a very low percentage of cases, telomere healing is performed, instead of by telomerase, by the Alternative Lengthening of Telomere mechanism, ALT, which will be described later in section 1.3.2. (22).

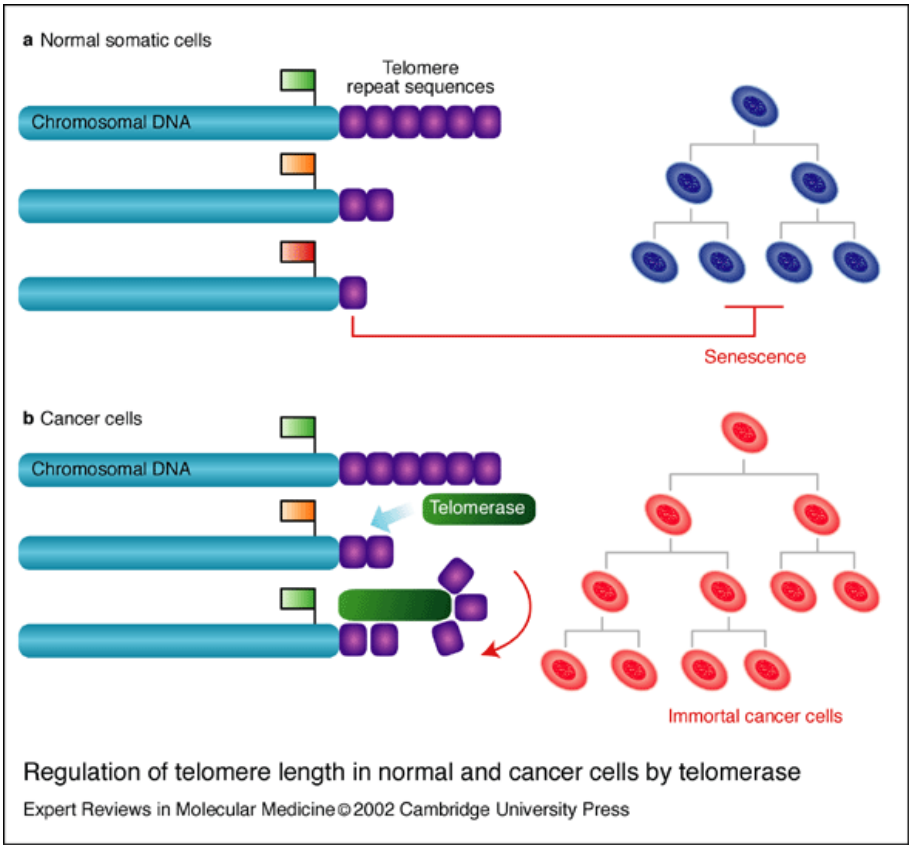


Figure 1.3. *From http://www.pharmstatus.com/telomerase.php*

1.2. Telomerase

1.2.1. Telomerase Structure and Activity

Telomerase is a ribonucleoprotein complex containing a catalytic protein with telomere-specific reverse transcriptase activity, TERT, which synthesizes telomeric sequences *de novo* utilizing an internal RNA as template, the TR. These two components are necessary and sufficient to exert catalytic activity in a cell free condition assay, while *in vivo* additional telomerase-associated proteins are needed for telomere maintenance (23), as they are involved in ribonucleoprotein assembly, post-translational modification, localization and activity regulation (18).

TR is constitutively transcribed from its own promoter (24) and has a diffuse nuclear localization, while TERT constitutes the rate limiting component of the complex, as it is expressed during embryogenesis, where it sustains the very high rate of proliferation, and then it is inhibited (18). Indeed, telomerase activity is undetectable in most normal somatic adult cells, with the exception of certain rapidly dividing tissues; e.g. telomerase activity is detectable at very low level in adult stem cell compartment, in proliferating/stimulated B and T cells, as well as in regenerating hepatocytes (22), intestinal crypts cells and male germ cells. Telomerase downregulation physiologically restricts the number of cellular divisions during organismal development, thanks to progressive telomere attrition, characterizing the aging process.

TERT, a 127 kDa protein, consists of four domains: (i) N-terminal (TEN) domain, involved in the telomerase recruitment to telomeres and in the catalysis of telomeric repeats synthesis; (ii) TR-binding domain (TRBD), which provides key interaction, together with RT domain, for the association with the RNA component; (iii) reverse transcriptase (RT) domain, that constitutes the catalytic domain for *de novo* telomeric repeats synthesis and displays clear homology with RT of transposons and retroviruses; (iv) C-terminal extension (CTE) domain (18).

TR sequence displays remarkable divergence between different species, but specific motifs are well conserved, as those for TR tridimensional folding, for the

high affinity interactions with TERT, and the template sequence for repeats synthesis, which is a well-defined and very short portion of TR (25).

Intramolecular interactions between domains induce a ring shaped-ribonucleoprotein complex with the catalytic active site located in the center of the ring (25).

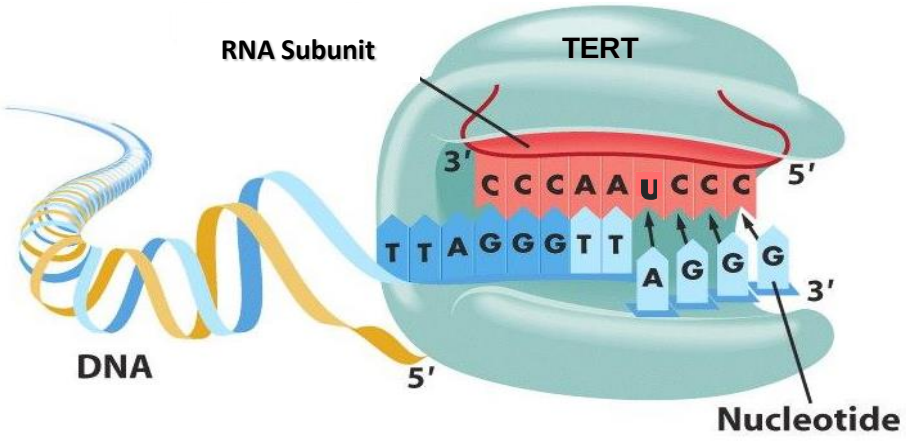


Figure 1.4. *From https://ronia.info/pages/t/telomerase/*

Telomerase maintains telomeres through catalytic cycles of repeats synthesis. Each cycle starts with the annealing of telomeric DNA primer to the template RNA within the telomerase complex; the entry of dNTP in the core of the enzyme triggers telomerase conformational changes that result in an elongation-competent closed conformation; at each dNTP addition, the active site switches between open and closed conformation. Repeat synthesis proceeds towards the 5’ boundary of the RNA template; then, the enzyme must undergo substantial protein remodeling to allow the DNA:RNA (telomeric DNA:template RNA) duplex separation and the translocation of the template for another cycle of repeat synthesis (25). The repeat addition processivity (RAP), i.e. is the ability of the telomerase enzyme to add more than one repeat at one telomere, usually allows

the addition of five-ten repeats per telomere elongation; then telomerase definitively dissociates from telomere (25).

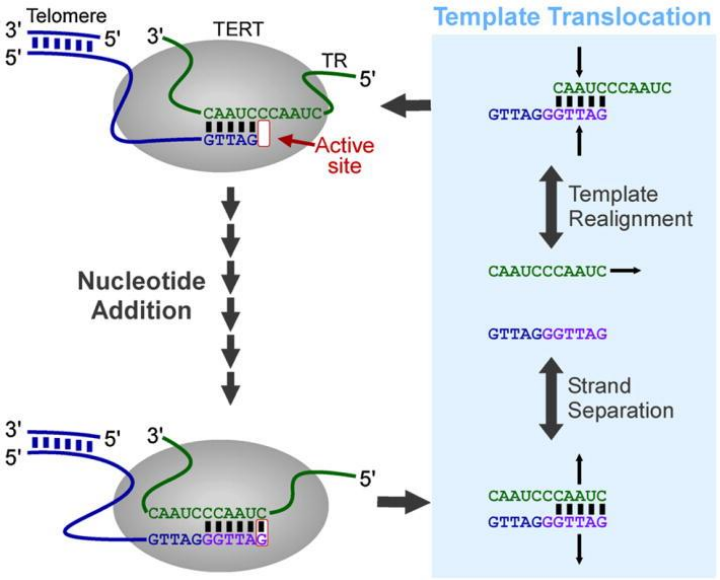


Figure 1.5. *From Podlevsky, Mutat Res, 2012 (26)*

1.2.2. Telomerase Regulation

TERT recruitment to telomeres is restricted to the S-phase of cell cycle, and its transcription and telomerase activity, which occurs after the completion of DNA replication, peak in S-phase (18).

Telomerase activity must be finely regulated in order to maintain functional telomeres, indeed, regulation of telomerase operates at several levels: transcription, mRNA splicing, subcellular location of each component, and assembly of TR and TERT in an active ribonucleoprotein. Transcription of the *TERT* gene is probably the key determinant in regulating telomerase activity, since *TERT* transcription is specifically upregulated in cancer cells but silent in most normal ones. The *TERT* promoter reveals complex regulation dynamics, whereby multiple

transcriptional regulatory elements play functional roles in different contexts, either individually or interactively. *TERT* promoter contains recognition sequences for many important transcription factors such as TP53, P21, SP1, ETS, E2F, AP-1, HIF1A and MYC (27). Regulation of *TERT* transcription may also involve DNA methylation, as the *TERT* promoter contains a cluster of CpG sites (27). Somatic mutations in the promoter of the *TERT* gene, which increase gene expression by creating de novo binding sites for the ETS/TCF transcription factors, have also recently been described (28).

At post-transcriptional level, more than 20 different TERT variants have been reported, some of which probably play critical roles in regulating telomerase activity (29). Telomerase activity is also controlled by post-translational modifications of the TERT protein. Phosphorylation of the protein at critical sites along the PI3K/AKT kinase pathway seems to be crucial for telomerase activity and nuclear localization. Active recruitment of telomerase to telomeres is a necessary regulatory step and involves shelterin proteins (18); for example, TTP1 is fundamental for telomerase recruitment to telomeres and, in complex with POT1, has been proposed to stimulate the RAP of telomerase (18). RAP is also regulated by the CST complex, which modulates processivity factors and binds to ssDNA to displace telomerase from replicated overhang in late S/G2-phase, thus inhibiting its activity; beyond this, CST complex affects telomere length by promoting the fill-in step during telomere replication (30). In addition, TERRA transcripts may inhibit telomerase activity by directly binding telomerase (23).

Human TERT contains putative nuclear localization signal and nuclear export signal, suggesting a nuclear-cytoplasmic shuttling possibly related to complex assembly or activity regulation (18); moreover, 10-20% of TERT is located in the mitochondria (mt) both in telomerase expressing normal cell and in cancer cells, where it shuttles thanks to its N-terminal mitochondrial targeting signal, and where it may perform telomere-unrelated functions (31).

The modulation of telomerase expression and/or activity is also affected by oncogenic viruses during the process of neoplastic transformation, as described in the next section.

On the other side, telomeric 3D structure must undergo unfolding to allow telomerase to reach telomeric sequences; topological interferences (17) due to telomeric chromatin state, G-quadruplex and T-loop unwinding, may prevent telomerase access to telomeres, thus impairing its activity.

1.3. Telomeres and Telomerase in Cancer

As anticipated before, after embryogenesis the expression of telomerase is suppressed. The progressive telomere attrition can be regarded as a tumor suppressive mechanism, since it restricts cell proliferative capacity.

The dysregulation of the telomerase suppressive mechanisms is responsible for the cell immortalization in the vast majority of tumors, which display a close correlation with high levels of *TERT* gene expression and telomerase activity (22).

It has been extensively described that the alteration of regulatory factors, the methylation status of regions proximal to *TERT* promoter, the gene amplification and/or genomic rearrangements involving *TERT* locus, and *TERT* promoter mutations strongly influence the cellular level of the telomerase complex, therefore the level of telomerase activity, which in turn affects telomere length homeostasis (22) providing unlimited proliferative potential to tumor cells.

As anticipated before, oncogenic viruses developed several strategies to engage telomerase during virus-driven tumorigenesis and, later, to sustain tumor progression, acting both at transcriptional and post-transcriptional levels. For instance, viral proteins, such as latent membrane protein 1 (LMP1) from Epstein-Barr virus (EBV) and E6 from human papilloma virus (HPV), activate TERT at transcriptional level (32,33); furthermore, E6 stabilizes TERT mRNA, participating in the post-transcriptional modulation of TERT (34).

1.3.1. Telomerase and Telomeres as Prognostic/Predictive Biomarkers

Given the ample participation of telomerase in the vast majority of tumors, and the requirement of telomere length maintenance for cell immortalization, TERT and telomere length have been proposed as prognostic biomarkers (32).

Indeed, high *TERT* mRNA and telomerase activity levels in cancer cells have been associated with aggressiveness of disease, advanced clinical stage, and poor overall survival (OS) and disease free survival (DFS) in several cancer types (e.g. bladder carcinoma, head and neck squamous cell carcinoma, medullary thyroid carcinoma, colorectal cancer, ampullary carcinoma) (32).

TERT mRNA is also detectable in plasma/serum (where it is released from cancer cells) and these levels are significantly correlated with the levels of *TERT* in tumors, thus it may be a useful non-invasive biomarker. It has been shown that plasma *TERT* mRNA level significantly correlates with clinical stage, metastasis and reduced (DFS) and (OS) in gastric cancer patients (35). Moreover, plasma *TERT* levels are independent markers of tumor response and are prognostic of disease progression in rectal cancer patients who undergo neoadjuvant therapy (36). In addition, *TERT* promoter mutations, which are associated with increased expression of TERT, resulted prognostic markers of aggressiveness and worsen course of disease in several cancer types (32,37).

It has been suggested that telomere length may also have a clinical significance, considering that, in many tumor types, neoplastic cells have shorter telomeres than their adjacent mucosa, suggesting that telomere shortening is a critical initial event in the oncogenetic process (32). There is no agreement concerning the role of telomere length in tumor cells as markers of disease progression of most investigated solid tumors; however, there is agreement that short telomere length in neoplastic cells is an independent predictor of disease progression and poor overall survival in chronic lymphocytic leukemia (CLL) (32,38).

1.3.2. Alternative Lengthening of Telomeres

As anticipated in 1.1.2, approximately 85% of all human cancers upregulate telomerase, while the residual 15% of tumors, mainly those of mesenchymal origin

(39), maintain their telomeres through a HR based mechanism, the Alternative Lengthening of Telomeres (ALT) (40).

ALT mechanism involves the synthesis of new telomeric repeats using as template the telomeric DNA from the same telomere, from the sister chromatid or from another chromosome, and it is usually repressed by the shelterin proteins (mainly POT1) that cover the telomeric DNA. The activation of ALT in cancer has been shown to correlate with the alteration of the chromatin remodeler ATRX and DAXX (as in pancreatic neuroendocrine tumors, liposarcomas and tumors of the central nervous system), and with high frequency of DDR foci at telomeres, attributable to decreased shelterin binding (41); moreover, ALT is in general associated with a more relaxed chromatin status (39).

As a consequence of this alternative mechanism, ALT cells display highly heterogeneous telomere length, high levels of telomere-sister chromatid exchange and extra-chromosomal telomeric repeats (linear and circular, e.g. C-circles), as well as the presence of ALT-associated PML bodies, APBs, that contain HR proteins, telomeric DNA and telomere binding proteins (39).

1.4. Telomere-Length Independent Functions of Telomerase

Telomerase reactivation in cancer cells is fundamental to provide unlimited proliferative potential, but a growing body of evidences indicates that there are additional, tumor-promoting functions of telomerase, that seem telomere-length independent.

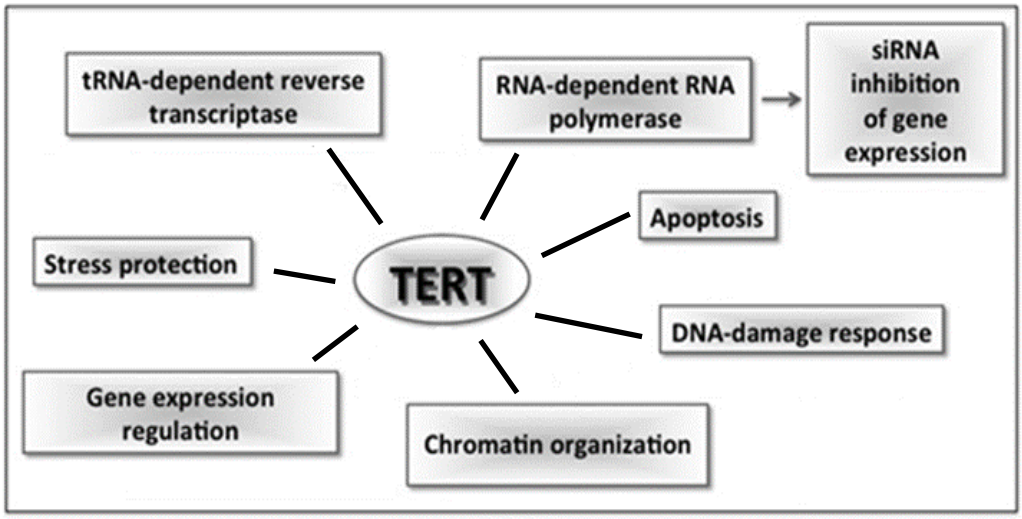


Figure 1.5. *Adapted from Chiodi, Front Oncol, 2012 (31)*

Extensive changes in gene expression have been observed after TERT overexpression (23); in particular, it has been reported the modulation of genes related to cell cycle progression, metabolism, differentiation, cell signaling, and survival among others (23). Of interest, the induction or inhibition of pro- or anti-proliferative genes respectively allows cells to proliferate even without mitogenic stimuli (31). Coherently, it has been demonstated that ectopic expression of TERT confers resistance to TGF-β growth inhibition (42) and reduces the requirement for exogenous mitogens, such as EGFR, whose alteration characterize many human malignancies (23).

Notably, as Chiodi and Mondello summarized (31), TERT was found to bind promoters responsive to WNT signaling. Indeed, TERT directly modulates the WNT signaling pathway by acting as a transcriptional cofactor, together with BRG1, in

the modulation of WNT target genes (43); in particular, it was found that TERT stimulates epithelial-to-mesenchymal transition (EMT) through the WNT/B-catenin pathway, thus promoting cancer invasiveness, and this effect was independent from TERT catalytic activity at telomeres (23). Interestingly, TERT-WNT relationship is bidirectional, as observed in both embryonic stem cells and in cancer cells; for example, β-catenin deficient human cancer cell lines had shorter telomeres, and TERT seems a target of the β-catenin/TCF4 mediated transcription. Therefore, WNT activation during transformation may promote cell immortalization through TERT induction (31), and, in turn, TERT modulation of WNT signaling clearly contributes to human tumor progression in a telomere-independent manner (44).

TERT also contributes to tumor-promoting processes by directly regulating the NF-kB-dependent transcription. For example, TERT promotes cells invasion and metastasizing capabilities by regulating the expression of matrix metalloproteases (MMPs) (that in turn degrade the basal membrane and extracellular matrix) *via* NF-kB signaling and independently from TERT catalytic activity (23). In addition, it has been demonstrated that in EBV-positive B lymphocytes TERT overexpression promotes NF-kB-dependent transcription of NOTCH2, as detailed below (45). On the other side, NF-kB can regulate TERT expression by binding next to its transcription start site (23).

In many cancers, the reactivation of *TERT* results concomitant with that of *MYC*; indeed, their interplay was shown to be multifaceted. The work of Kho and colleagues (46) demonstrated that TERT stabilize MYC protein level by affecting its ubiquitination and degradation by proteasome, independently from telomerase activity and telomere synthesis; thus MYC is stabilized on the promoter of its target genes, affecting their expression. Moreover, *Tert* depletion in murine model resulted in a delayed onset of *Myc*-driven lymphomas (46).

As in the case of WNT and NF-kB, TERT–MYC gf interplay is bidirectional; indeed, MYC directly binds TERT promoter and modulates its expression (47), as well as that of TR (48).

Intriguingly, TERT actively participates in the interplay between oncogenic viruses and host cells in the process of neoplastic transformation (32). In particular, our research group deeply investigated the relationship between telomerase and EBV (33,45,49-52). It has been demonstrated that LMP1, the major EBV oncoprotein, activates TERT at transcriptional level through NF-kB and by MAPK/ERK1/2 pathways (33). In turn, high TERT expression prevents the induction of EBV lytic cycle (49) and promotes the EBV latency tumorigenic program; indeed, TERT induced the transcriptional activation of NOTCH2 *via* NF-kB pathway (45). NOTCH2 activates the cellular transcription factor BATF, which, in turn, negatively affects the expression of viral BZLF, the master regulator of viral lytic cycle. Coherently, TERT inhibition by small interfering RNA (siRNA) or short hairpin RNA (shRNA) reduced the expression of NOTCH2, and consequently of BATF, resulting in the upregulation of BZLF and triggering a complete EBV lytic cycle with the death of infected cells (49,50).

Beside its role in modulation of gene expression, evidences suggest that TERT is also involved in mitochondrial processes, by which it modulates the resistance to apoptosis. Indeed, TERT contain a mitochondrial localization signal and it was shown to be active in mitochondria; it has been reported that, under oxidative stress, TERT (but not TR) is reversibly excluded from the nucleus and localizes in mitochondria, where it binds to several mtDNA regions, improves mitochondrial functions and stress resistance; of interest, these effects are linked to TERT catalytic activity but independent from TR (31). Indran and colleagues (53) demonstrated that TERT overexpression attenuated basal levels of cellular reactive oxygen species (ROS) and inhibited their production in response to ROS-inducing stimuli; this effects were associated with increased levels of cellular antioxidants as well as of cytochrome C oxidase. The resulting increase in mitochondrial transport chain activity, together with impaired translocation of BAX and decline in mitochondrial transmembrane potential among others, confers resistance to cell death induced by mitochondrial apoptotic pathway (53). Another possible explanation of the TERT-mediated modulation of apoptosis is the control

by TERT on the expression of apoptotic genes, through siRNA synthesis, but this mechanism remains to be uncovered (17).

Interestingly, Maida and colleagues demonstrated that TERT, beyond TR, can associate with other non coding RNA partners, like the mitochondrial RNA processing endoribonuclease (RMRP) (54,55). In this case, TERT acts as an RNA-dependent RNA polymerase (RdRP) that synthesizes RMRP dsRNA, subsequently processed in siRNA (by the RNA induced silencing complex, RISC) which in turn silences RMRP expression; this decrease in RMRP has been linked to TERT-dependent enhancement of cellular proliferation (54,56)). Thus, TERT was proposed as a modulator of cell proliferation *via* interfering RNA (31).

In addition, the work of Sharma N.K. and colleagues strongly indicated that TERT, in mitochondria, binds to several mt-tRNA and uses them as templates for TR-independent reverse transcriptase activity/cDNA synthesis; it has been suggested a role in mtDNA replication and repair (57).

On the other side, Sharma G.G. and colleagues proposed an involvement of TERT in genomic DNA repair. They found that TERT overexpression increased the DNA damage repair kinetics by increasing the levels of dNTPs and NTPs; they assumed that this may be due to the changes in gene expression consequent to TERT overexpression (58). By contrast, Shin and colleagues demonstrated that in human fibroblasts TERT overexpression improved the NER and the DNA-end-joining mechanisms; they proposed that TERT may attract DNA repair proteins to the site of DNA damage (59).

1.5. Strategies for Telomerase Inhibition

Different categories of telomerase inhibitors are currently available, comprising telomerase activity inhibitors, that targets TERT or TR, as well as inhibitors of telomerase interaction with telomeres or with telomerase binding proteins (60).

Among direct telomerase activity inhibitors, nucleoside analogues inhibit dNTPs incorporation in newly synthesized telomeric DNA and/or induce telomere dysfunctions; an example is Zidovudine (AZT), used in the first instance for the treatment of retroviral infections. Phytochemicals were shown to impair telomere maintenance through a mechanism that remain to be elucidated. Imetelstat (GRN163L) is a chemically modified oligonucleotide that binds to TR and impairs its association with TERT and with telomeric repeats, compromising telomere synthesis. Employing a different strategy, G-quadruplex stabilizers impair the unfolding of telomeric DNA during telomere replication and maintenance, thus telomerase is not able to reach telomeric repeats; they may also displace shelterin proteins, triggering telomere dysfunction (60).

In the growing list of promising anticancer drugs, BIBR1532 (BIBR), a synthetic non-nucleoside compound, can be regarded as one of the most potent specific inhibitors of TERT. BIBR directly inhibits telomerase catalytic activity by binding the TEN domain, in close proximity to the enzyme catalytic core, and blocks its conformation in a closed state; therefore, the switch to active open conformation is impaired, as well as the translocation step, thus the enzyme processivity are inhibited, reducing the number of repeats added (61,62). In this way the affinity for dNTPs is reduced, likely depending on reciprocal steric interference (63).

Objectives

A body of evidences indicates that TERT, beyond telomere maintenance, participates in the modulation of an ample spectrum of critical cellular processes, e.g. affecting tumor-promoting signaling pathways, as well as resistance to apoptosis. These findings enforce the interest in TERT inhibition as anticancer strategy, since it could be effective besides its effects on telomere length.

The aim of this study is to investigate the effects of the inhibition of telomerase activity in short-term experiments in *in vitro* models of B-cell malignancies, and in *in vivo* zebrafish model.

The ultimate aim is to provide a rationale supporting the inclusion of TERT inhibitors in treatment schedules for B-cell malignancies, taking in consideration that the potential therapeutic benefits may be extended theoretically to all TERT-positive tumors.

Chapter 2: *In Vitro* Study

2.1. Introduction

Telomerase is a ribonucleoprotein complex containing a catalytic protein with telomere-specific reverse transcriptase (TERT) activity, which synthesizes telomeric sequences *de novo* utilizing an internal RNA template. When the telomere reaches a critical length because of end-replication problems of DNA polymerase, cells cease to proliferate and undergo senescence. Maintenance of telomere length by telomerase is critical for overcoming replicative senescence and acquiring unlimited replicative potential (64,65). In humans, TERT is the rate-limiting component of the telomerase complex (66) and its expression, usually absent in normal somatic cells, is detectable in most cancer cells (67).

Recent studies have suggested that, besides maintaining telomere length, TERT is involved in other cellular functions of biological relevance (17). In fact, *in vitro* evidence indicates that TERT prevents cell cycle arrest and confers protection from apoptosis induced by adverse culture conditions (68) and DNA-damaging agents (69), prevents cell growth arrest induced by retinoic acid in promyelocytic leukemia-derived cell line (70), antagonizes p53-induced apoptosis in Burkitt's lymphoma (BL) cells (71) and inhibits apoptosis induced by tumor necrosis factor (TNF)-α (72).

TERT expression also affects the latent/lytic status of EBV in EBV-positive B lymphocytes (49,50). EBV is a ubiquitous human gamma herpesvirus causally linked to the development of several malignancies including BL, Hodgkin's lymphoma, post-transplant lymphoprolipherative disorders and AIDS-associated lymphomas (51). EBV has a potent transforming capacity, and efficiently *in vitro* induces uncontrolled proliferation of infected B lymphocytes and generates immortalized lymphoblastoid cell lines (LCLs), which are a suitable *in vitro* model

of EBV-driven B-cell lymphomas, mainly those arising in immunocompromised patients. Like many other tumors, EBV-associated malignancies maintain their ability to grow indefinitely through inappropriate activation of telomerase.

The latent membrane protein 1 (LMP1), the major EBV oncoprotein, activates the TERT promoter at the transcriptional level via NF-kB and MAPK/ERK1/2 pathways and increases telomerase activity in B lymphocytes (33). In addition, it has been reported that cells newly infected by EBV exhibit signs of telomere dysfunction and chromosomal rearrangements, mainly due to EBV-mediated displacement of shelterin proteins and uncapping problem at telomeres (73,74); however, established LCLs show minimal or no signal of telomere dysfunction and have a stable karyotype (73,74).

Given the ample spectrum of critical functions modulated by TERT, its inhibition could represent a promising strategy to improve cancer treatment, regardless of telomere length. In fact, TERT inhibition in different cellular backgrounds is associated with cell growth arrest, induction of apoptosis (69,75-77) and increased sensitivity to ionizing radiation (76). Our previous work has demonstrated that TERT inhibition by short hairpin RNA triggers the complete viral lytic cycle and cell death in EBV-positive cells (50).

In the growing list of promising anticancer drugs, BIBR1532 (BIBR), a synthetic non-nucleoside compound, can be regarded as one of the most potent specific inhibitors of TERT (61,78). This drug targets the catalytic activity of the telomerase enzyme by binding directly to the telomerase core component thereby reducing the affinity for deoxyribonucleotides (dNTPs). The drug's and TERT-binding sites for dNTPs are close or even overlap, thus creating reciprocal steric interference in binding efficiency (63,79). It has been demonstrated that in long-term cultures of human cancer cells of different histological origin, low doses of BIBR can induce a senescence phenotype associated with telomere shortening, which confirms the drug's ability to inhibit canonical TERT activity on telomere (79-83). It has also been demonstrated that short-term treatment with high doses of BIBR induces cytotoxicity in leukemia cells (83,84), most probably by directly inducing telomere

dysfunction (83). No data are as yet available concerning the effects of BIBR on EBV-immortalized LCLs and transformed BL cell lines.

On these grounds, we carried out this study aimed at characterizing the effects of BIBR in LCLs and BL cell lines. The impact of BIBR combined with fludarabine (FLU) or cyclophosphamide (CY) treatment on LCL viability, cell cycle profile and apoptosis was also evaluated. The study's ultimate aim was to provide a rationale supporting the inclusion of TERT inhibitors in treatment schedules for EBV-driven B-cell malignancies.

2.2. Materials and methods

2.2.1. Cell Lines

The 4134 LCL was obtained by infecting peripheral blood mononuclear cells from normal donor with the B95.8 EBV strain. Establishment and characterization of this cell line has already been described (49). 4134/TERT- and 4134/Late cells were derived from early and late passages after EBV infection and expressed very low and high level of endogenous TERT, respectively (45,49). The 4134/ TERT+ cell line, expressing ectopic TERT, was obtained by infecting 4134/TERT- cells with a retroviral vector (49). All three 4134 cell lines used in this study were negative for BZLF1 and viral lytic proteins EA-D and gp350. BL41 is an EBV-negative BL cell line with translocated *MYC* gene (kindly provided by Martin Rowe, Cancer Center, University of Birmingham, Birmingham, UK). BL41/B95.8 is the counterpart cell line infected *in vitro* with the B95.8 EBV strain (kindly provided by Martin Allday, Ludwig Institute for Cancer Research, London, UK). LCLs and BL41 were cultured in RPMI-1640 medium (Euroclone, Milano, Italy), supplemented with glutamine 4 mM, 50 mg/ml gentamycin (Sigma-Aldrich, St. Louis, MO, USA) and 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Milano, Italy; standard medium) at 37 °C and 5% CO_2. BL41/B95.8 cells were grown in standard medium supplemented with 1 mM sodium pyruvate, 1% nonessential amino acids (Sigma-Aldrich), and 50 mM β-mercaptoethanol. The human osteosarcoma cell line U2OS was used as TERT-negative control (85,86); cells were maintained in McCoy's5A medium modified (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (Gibco). Cell lines were checked and controlled by cytogenetic analyses. All cell lines were tested and resulted negative for mycoplasma contamination.

2.2.2. Compounds

A stock solution of BIBR (Selleck Chemicals LLC, Houston, TX, USA) at a concentration of 10 mM was prepared by dissolving the compound in sterile DMSO, divided into aliquots and stored at −80 °C until use. FLU (F9813; Sigma-Aldrich) was prepared by resuspending the compound in dimethylsulfoxide (DMSO) at a concentration of 10 mM, divided into aliquots and stored at −20 °C until use. CY (C0768; Sigma-Aldrich) was prepared by dissolving the compound in sodium chloride 0.9% solution at a concentration of 360 mM, divided into aliquots and stored at 4 °C. It was warmed to 37 °C for 30 s, immediately before use. LCLs were exposed to serial dilution of FLU and CY to identify the half-maximal inhibitory concentration (IC50) (Figure 2.1.). Optimal molarity was defined on the basis of the observed effects on the most sensitive cell line to each drug. FLU for 4134/TERT- had an IC50 concentration of 5 µM. CY on 4134/Late exhibited 50% of cell survival at a concentration of 4 mM. These concentrations were used for all drug experiments.

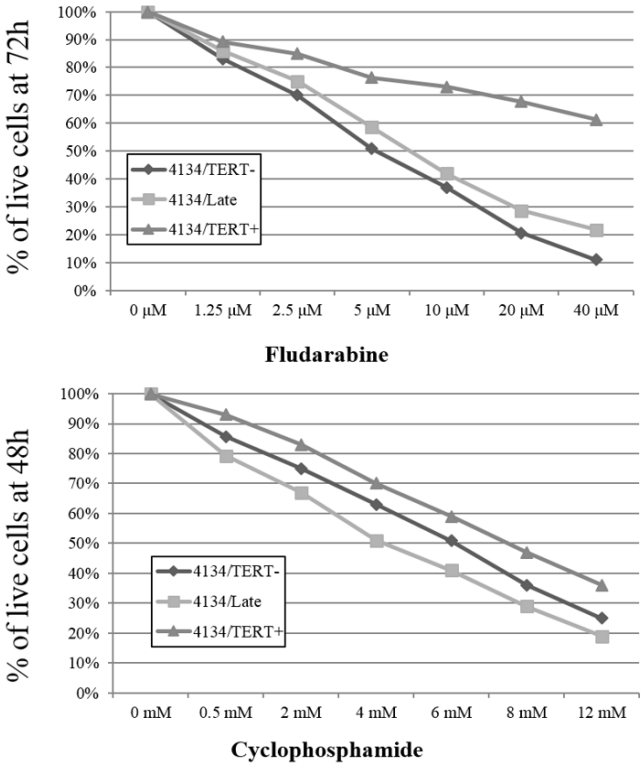


Figure 2.1. *Effects of serial concentrations of FLU and CY on percentage of cell viability in 4134/TERT-, 4134/Late and 4134/TERT+. IC50 values of FLU and CY were estimated as 5 µM at 72 h and 4 mM at 48 h, respectively.*

2.2.3. Real-time PCR for Quantification of TERT Transcripts

Cellular RNA was extracted and retrotranscribed into cDNA, as previously detailed (49). TERT transcripts were quantified by real-time PCR, with the AT1/AT2 primer pair, as previously described (49,87).

2.2.4. Analysis of Telomerase Activity

For each sample, three million cells were lysed in 50 µl of CHAPS buffer (0.5% CHAPS, 10 mM TrisHCl, pH 7.5, 1 mM MgCl2, 1 mM EGTA, 0.1 mM phenylmethyl-sulfonyl fluoride, 5 mM β-mercaptoethanol, 10% glycerol) and incubated at 4 °C for 30 min. The lysate was then centrifuged at 12 000 g for 30 min at 4 °C and the supernatant collected. Telomerase activity was assessed by the PCR-based TRAP, as previously reported (88). The TRAP assay was performed with 0.250 µg of total cell lysate.

2.2.5. Western Blotting

Western blot analyses from cell cultures were prepared as previously reported (89). The expression of TERT, RNR-R2, TRF2 and α-tubulin was evaluated by anti-TERT (ab94523, Abcam, Cambridge, UK), anti-RRM2/RNR-R2 (B-Bridge International, Cupertino, CA, USA), anti-TRF2 (Novus Biological, Littleton, CO, USA) and anti-α-tubulin (Sigma-Aldrich) antibodies (Ab), respectively. The ATM and ATR pathways were examined with specific Ab against ATM (ab32420, Abcam), ART (ab2905, Abcam), CHK1 (Ab47574, Abcam), CHK2 (Ab8108, Abcam), p53 (sc-6243, Santa Cruz Biotechnology, Dallas, TX, USA) and their respective phosphorylated/active form, p-ATM (ab81292, Abcam), p-ATR (ab178407, Abcam), p-CHK1 (ab195753, Abcam), p-CHK2 (ab195929, Abcam), p-p53 (9284, Cell Signaling, Danvers, MA, USA). Blots were incubated with an appropriate peroxidase-conjugated secondary antibody (Sigma-Aldrich) and stained with a chemiluminescence detection kit (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA). α-Tubulin was used as control for loading.

2.2.6. Viability, apoptosis and cell cycle analysis.

Cell viability was determined by Trypan blue exclusion in a Countess automated cell counter (Invitrogen, Carlsbad, CA, USA). To evaluate cell cycle distribution, cells were harvested and processed as previously described (50). Samples were analyzed by flow cytometry (FACS Calibur; Becton-Dickinson, Franklin Lakes, NJ, USA) and cell cycle profiles were analyzed with ModFit LT Cell Cycle Analysis software (version 2.0) (Verity Software House, Topsham, ME, USA). Apoptosis was evaluated by staining cells with annexin V and propidium iodide (PI; Sigma-Aldrich), as previously detailed (50), and analyzed by flow cytometry. At least 50 000 events were acquired; data were processed with CellQuestPro software (Becton-Dickinson), and analyzed by Kaluza Analyzing Software v1.2 (Beckman Coulter, Pasadena, CA, USA). Annexin V-positive/PI-negative and annexin V-positive/PI-positive samples were classified as early and late apoptotic cells, respectively; both fractions were considered apoptotic cells. The percentage of specific cell death was estimated with the following formula: % cell death = 100 x (percentage of dead cells in treated sample – percentage of dead cells in control)/(100% – percentage dead cells in control).

2.2.7. Analysis of DDR.

Approximately 1×10^6 cells were stained for 1 h in the dark with the labeled monoclonal antibody for γH2AX (Alexa Fluor 488 mouse anti-H2AX (pS139), clone N1-431, Becton-Dickinson). Samples were analyzed by flow cytometry (FACS Calibur, Becton-Dickinson). A total of 30 000 events were collected according to morphological parameters (forward- and side-scatter). Analysis was performed with Kalusa software (Beckman Coulter). The mean fluorescence intensity (MFI) was measured by BD FACSDiva software (Becton-Dickinson).

2.2.8. Combined FISH/Immunofluorescence

4134/Late cells were harvested following standard cytogenetic's procedure. Hypotonic treatment was carried out with 0.075 M KCl at 37 °C for 30 min and the resulting pellets were fixed with Carnoy's fixative (methanol/acetic acid 3:1). Slides

were prepared by dropping the fixative on to wet glass slides and were left to dry overnight at room temperature. The slides were treated with pepsin 0.5 mg/ml (Sigma-Aldrich) at 37 °C for 15 min. Telomeres were visualized with the Telomere PNA FISH Kit/Cy3 (DAKO, Glostrup, Denmark). After digestion, slides were dehydrated by consecutive 2 min in 80, 96 and 100% ethanol and air-dried. Ten microliters of probe was added and a denaturation step was performed at 80 °C for 5 min, followed by 2 h of hybridization at room temperature in the dark. Post-hybridization washes were done at 65 °C for 5 min and briefly at room temperature in PBS. Slides were then blocked with 0.2% fish gelatin and 0.5% BSA in PBS (PBG buffer) (90). To visualize TRF2 location slides were incubated for 1 h with a rabbit polyclonal anti-TRF2 antibody (1:1000, Novus Biological) in PBG buffer followed by Alexa Fluor 488 anti-rabbit (Thermo Fisher Scientific) in PBG buffer. To visualize DNA damage foci, slides were incubated for 1 h with a mouse monoclonal anti-γH2AX antibody (1:1000, Merck Millipore, Darmstadt, Germany) in PBG buffer, followed by Alexa Fluor 488 anti-mouse secondary antibody (Thermo Fisher Scientific) in PBG buffer. After washing, slides were air-dried and mounted with DAPI/antifade solution (250 ng DAPI/aml Antifade Solution, MetaSystems, Altlussheim, Germany). Microscope analysis were carried out on a fluorescence microscope (Zeiss Imager. Z2, Oberkochen, Germany) equipped with a single band filter for DAPI, Cy3 and FITC. Digital images were captured with CCD camera (iAi CV-M4+CL, Rohs, Yokohama, Japan) using ISIS software (MetaSystems, Heidelberg, Germany) and Z-stacking function with EC PLAN-NEUFLUAR × 100 magnification objective. At least 50 nuclei for each condition were scored in three independent experiments.

2.2.9. Telomere length measurement.

Telomere lengths were determined by quantitative multiplex PCR assay as previously described (91), and by the TeloTAGGG Telomere Length Assay Kit (Roche Diagnostic GmbH, Basel, Switzerland) according to the manufacturer's instructions.

2.2.10. Statistical analyses.

Statistical analyses were performed with SPSS software version 21 (IBM, Armonk, NY, USA). Results were analyzed with t-test, ANOVA and Mann–Whitney test and P-values = 0.05 were considered significant.

2.3. Results

2.3.1. TERT inhibition by BIBR

4134/Late and 4134/TERT+ LCLs were positive for TERT mRNA, protein expression and telomerase activity, whereas 4134/TERT- cells were not (Figures 2.2.a–c). The telomeric repeat amplification protocol (TRAP) assay, carried out by adding 2 µM BIBR to protein extracts of TERT-positive 4134/Late and 4134/TERT+ cells, demonstrated that BIBR efficiently inhibits telomerase activity in both TERT-positive cell lines (Figure 2d). Similar results were obtained in TERT-positive BL41 and BL41/B95.8 BL cells (data not shown).

The LCLs were then exposed to varying concentrations (from 10 to 60 µM) of BIBR and analyzed for cell viability at 16, 24, 36, 48 and 72 h (Figure 2.3.). Treatment with BIBR at 30 µM resulted in decreased proliferation rates of TERT-positive cells at all time points, whereas no effect was seen in TERT-negative 4134/TERT- and U2OS cells. Similar results were reached in the EBV-negative BL41 and its EBV-positive counterpart BL41/B95.8 BL cell lines (Figure 2.3.). At 60 µM, even the TERT-negative cell cultures (4134/TERT- and U2OS) showed reduced proliferation rates compared with untreated controls (Figure 2.3.). The concentration of 30 µM was then used for experiments in all cell lines.

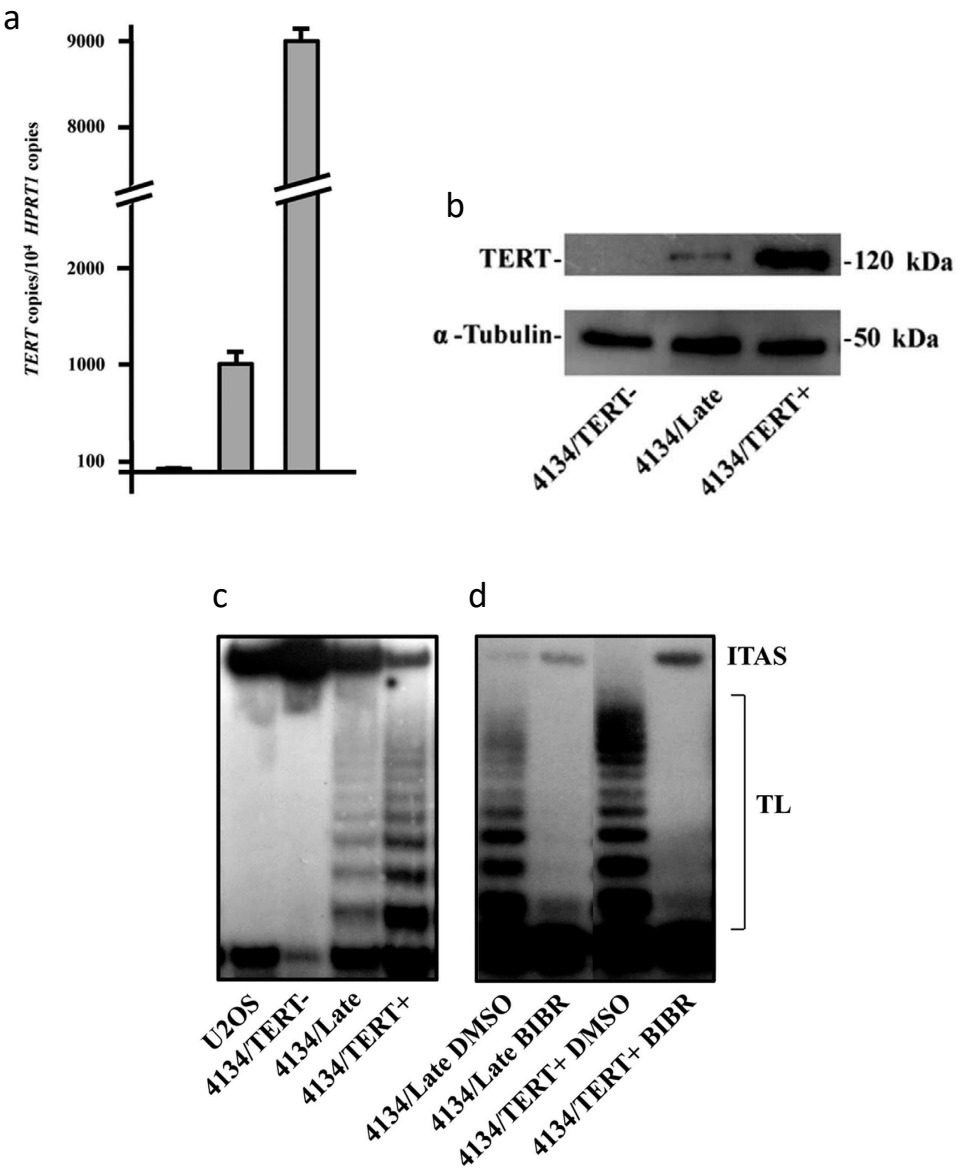


Figure 2.2. *TERT expression and activity in LCLs. (a) Levels of TERT transcripts in 4134/TERT-, 4134/Late and 4134/TERT+ LCLs. Means and S.D. (bar) of values from three independent experiments are shown. (b) Expression of TERT protein and housekeeping α-tubulin in LCLs assessed by western blotting. (c) Telomerase activity tested by TRAP assay in telomerase-negative U2OS and 4134/TERT- cells and in telomerase-positive 4134/Late and 4134/TERT+ cells. Panels from one representative experiment are shown. (d) In vitro efficiency of BIBR tested by TRAP assay in telomerase-positive 4134/Late and 4134/TERT+ by addition of BIBR (2 μM) or DMSO as control in protein extracts. Panels from one representative experiment are shown. TL, telomerase ladder; ITAS, internal telomerase assay standard*

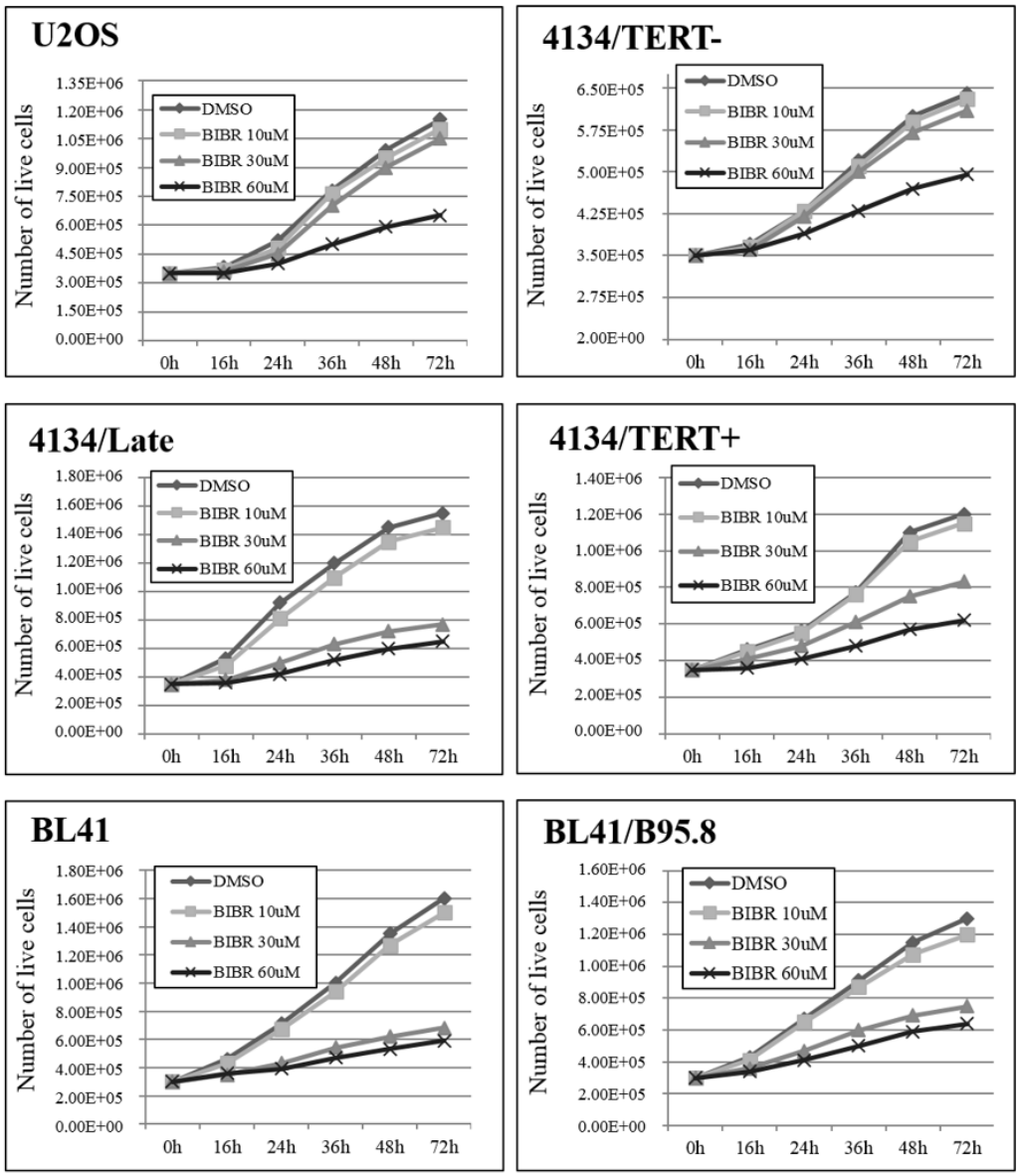


Figure 2.3. *LCLs, BL and U2OS cells, exposed to serial dilution of BIBR or DMSO as control, were analysed at 16, 24, 36, 48 and 72 h for cell viability by trypan blue exclusion. BIBR 30 µM led to decreased proliferation at 72 h of 57% ± 4% in 4134/Late cells, 30% ± 2% in 4134/TERT+ cells, 42% ± 3% in BL41 cells and 57% ± 2% in BL41/B95.8 cells. No effects were observed in TERT-negative 4134/TERT- or U2OS at this concentration.*

2.3.2. BIBR induces S-phase accumulation of TERT-positive LCLs and BL cells

We have previously demonstrated that TERT knockdown by shRNA induces cell cycle perturbations in both EBV-positive and EBV-negative lymphoma B cells (50). TERT-positive 4134/Late and 4134/TERT+ cells treated with BIBR also showed alterations in cell cycle profile, with decreased cells in the G1-phase, disappearance of the G2/M-phase and a significant accumulation of cells in the S-phase (Figure 2.4.). In particular, the S-phase was significantly increased compared with DMSO-treated control cells in both cell cultures, particularly at 16 and 24 h of exposure. Similar findings were observed in both EBV-negative BL41 and EBV-positive BL41/B95.8 BL cells; at 24 h of exposure, both cell lines showed a significant increase of cells in S-phase compared with DMSO-treated control cells (Figure 2.4.). Instead, BIBR treatment did not affect the cell cycle profile of 4134/TERT- (Figure 2.4.) and U2OS cells (Figure 2.5).

Consistently, the expression of the protein ribonucleotide reductase RNR-R2, a molecular marker of the S-phase, was higher in BIBR-treated TERT-positive LCLs than in untreated controls, whereas 4134/TERT- BIBR-treated cells showed no RNR-R2 upregulation (Figure 2.6.). These findings, taken together, support the hypothesis that BIBR can affect cell cycle progression by promoting selective accumulation of cells in the S-phase in TERT-positive B cells.

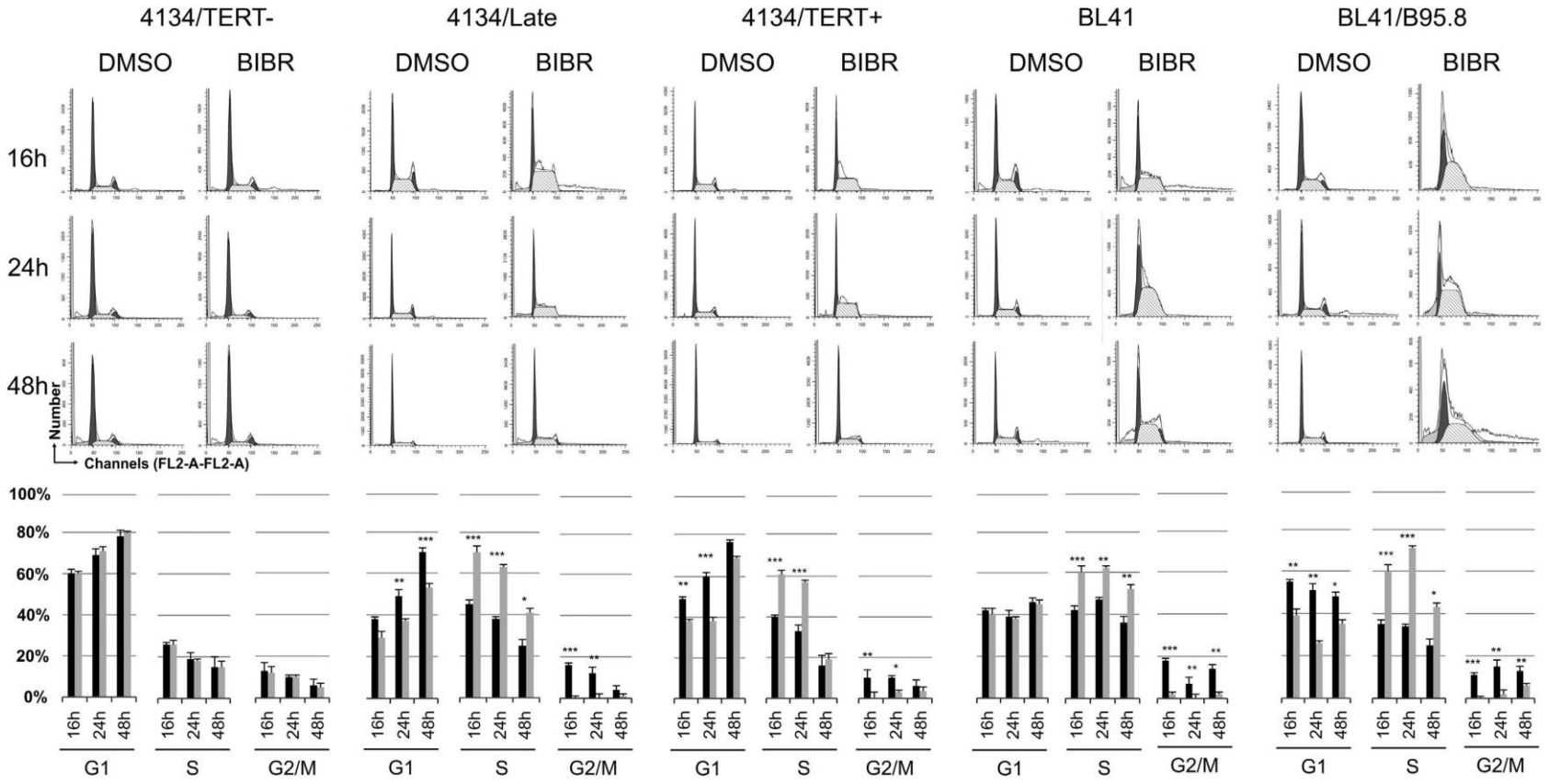


Figure 2.4. *Effect of TERT inhibition by BIBR on cell cycle profiles in LCLs and BL cells. Cells, treated with BIBR (30 µM) and DMSO as control at 16, 24 and 48 h, were labeled with PI and analyzed by flow cytometry for cell cycle distribution. Panels from one representative experiment are shown. Percentages of cells in G1-, S- and G2/M-phase are shown in graphs below. Black bars: DMSO-treated cells; gray bars: BIBR-treated control cells. Values are means and S.D. (bar) of three separate experiments. Significant differences between values in BIBR-treated versus DMSO-treated cells are shown: *P=0.05, **P=0.01 and ***P=0.001.*

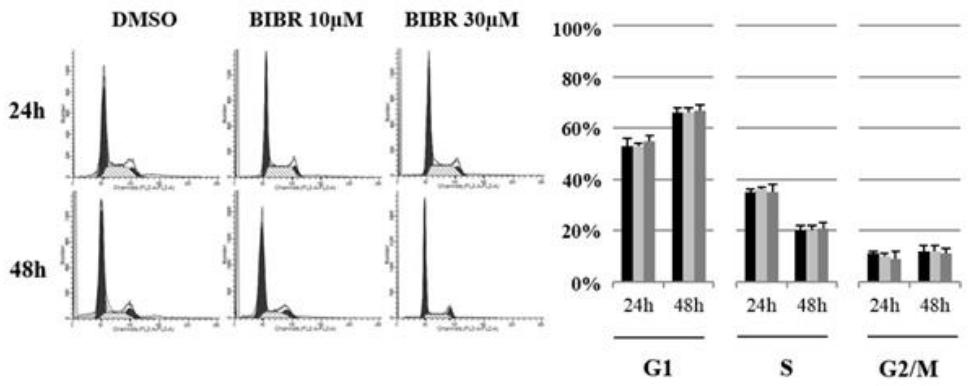


Figure 2.5. *U2OS cells were treated with BIBR (10 and 30 µM) and DMSO as control and analyzed at 24 and 48 h. U2OS cells were labeled with PI and cell cycles were analyzed by flow cytometry. Panels from one representative experiment are shown. Graphs on the right: percentages of cells in G1-, S-, and G2/M-phase. Values are means and SD (bar) of 3 separate experiments.*

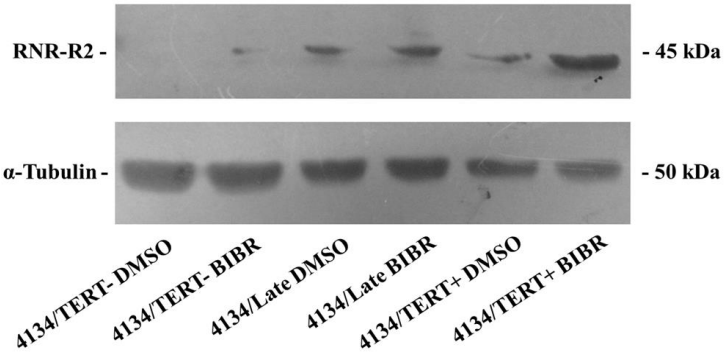


Figure 2.6. *LCLs were treated with BIBR (30 µM) and DMSO as control for 24 h. Expression of RNR-R2 and housekeeping α-Tubulin were assessed by western blot.*

2.3.3. TERT inhibition leads to apoptosis in TERT-positive LCLs and BL cells

As previous data had indicated that BIBR can promote apoptosis (83,92), we analyzed the pro-apoptotic effects of this drug in both LCL and BL models. TERT-positive LCLs treated with 30 μM BIBR showed a progressive increase in the number of apoptotic cells compared with controls at all the time points considered (Figure 2.7.). Similar results were observed in BL41 and BL41/B95.8 cells treated with BIBR; a significant increase in the number of apoptotic cells compared with controls was observed at 48 h of exposure, and the rate of apoptotic cells was higher in BL41/B95.8 than BL41 cells (Figure 2.7.). Conversely, 4134/TERT- (Figure 2.7.) and U2OS cells (Figure 2.8) exposed to BIBR showed no increase in the number of apoptotic cells.

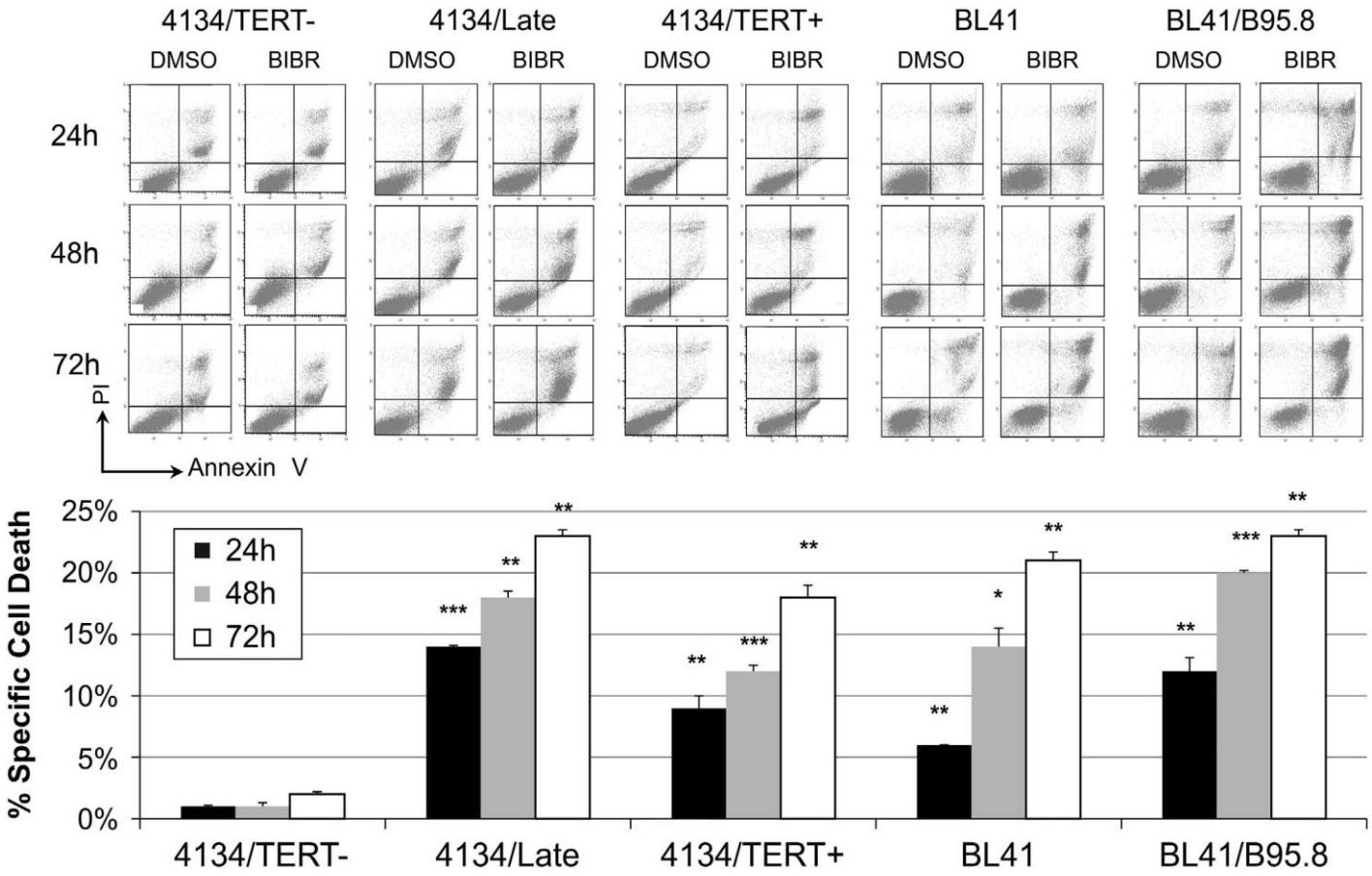


Figure 2.7. *Effect of TERT inhibition by BIBR on cell viability in LCLs and BL cells. Cells treated with BIBR (30 µM) and DMSO at 24, 48 and 72 h, were labeled with annexin V/PI and analyzed by flow cytometry for cell viability. Panels from one representative experiment are shown. Percentages of specific cell death were calculated as described in Materials and Methods section, with DMSO-treated samples as controls. Values are means and S.D. (bar) of three separate experiments. Significant differences between values in BIBR-treated versus DMSO-treated cells are shown. *P=0.05, **P=0.01 and*

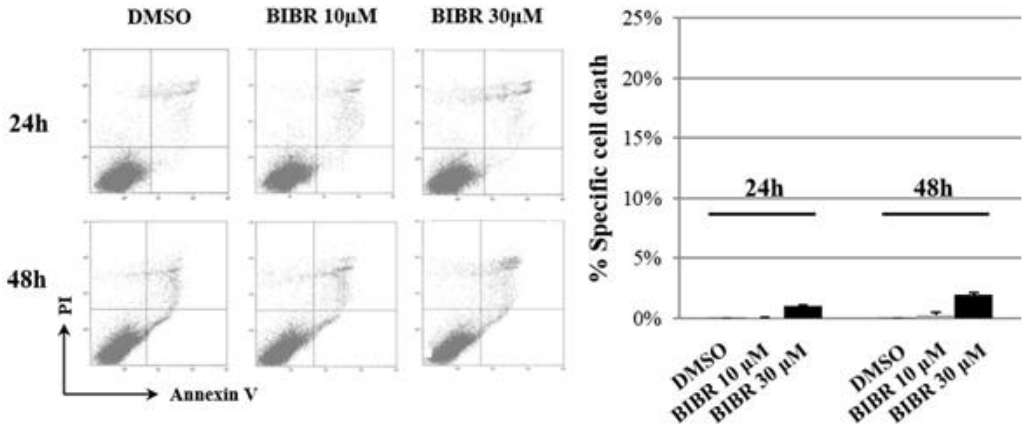


Figure 2.8. *U2OS cells were treated with BIBR (10 and 30 µM) and DMSO as control and analysed at 24 and 48 h. Cells were labeled with annexin V/PI and analysed by flow cytometry. Panels from one representative experiment are shown. Graphs on the right: percentages of specific cell death. Values are means and SD (bar) of 3 separate experiments.*

2.3.4. TERT inhibition activates the ATM/ATR cascade

To shed light on the possible mechanism underlying the cell cycle arrest and apoptosis consequent upon TERT inhibition by BIBR, we studied the involvement of the ATM and ATR pathways, which are critical regulators of cell cycle progression and apoptosis. BIBR treatment resulted in increased levels of the phosphorylated active form of ATM and ATR and their downstream substrates CHK1, CHK2 and pro-apoptotic p53 protein in 4134/Late and 4134/TERT+ cells, as well as in both EBV-negative BL41 and EBV-positive BL41/B95.8 cell lines (Figure 2.9.). This effect was not due to the modulation of their respective total forms, as shown in the panel below, indicating the substantial activation of the ATM/ATR DNA damage response pathway.

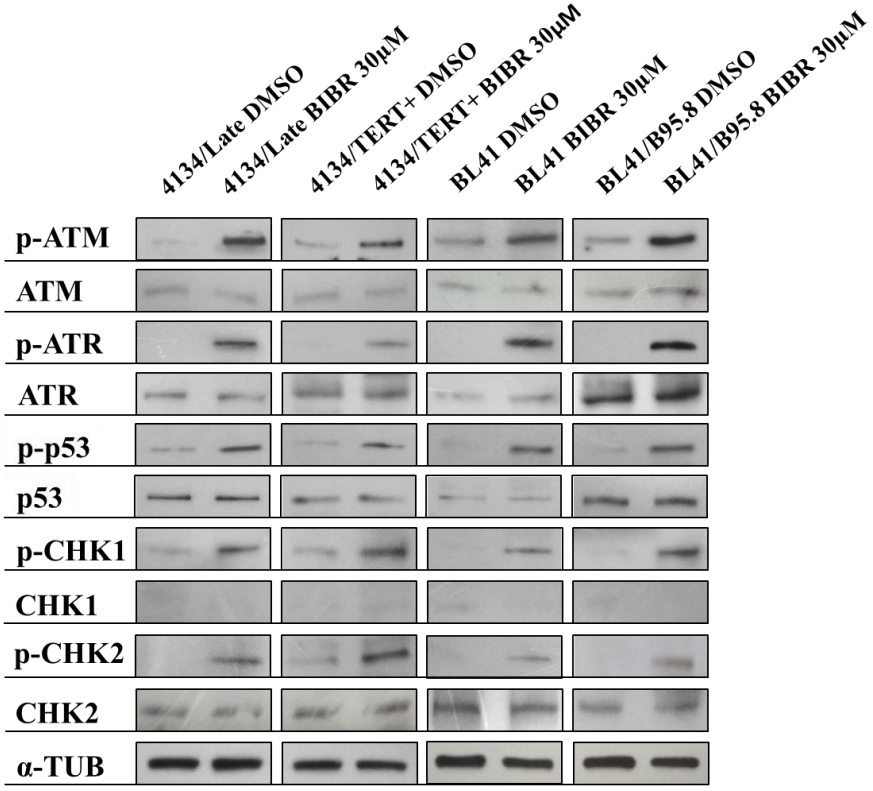
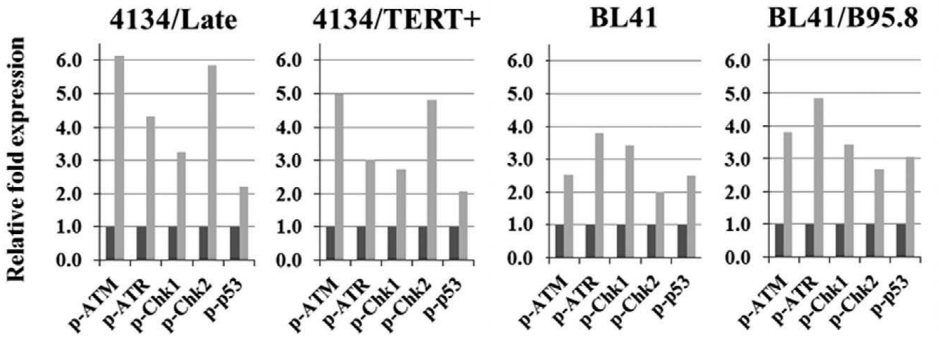


Figure 2.9. *TERT inhibition activates the ATM and ATR cascades. TERT inhibition by BIBR results in activation of ATM/ATR pathways in 4134/Late, 4134/TERT+, BL41 and BL41/B95.8 cell lines. Cells were treated with BIBR (30 μM) and analyzed after 36 h of exposure by western blot. Phospho-ATM (p-ATM), ATM, phospho-ATR (p-ATR), ATR, phospho-CHK1 (p-CHK1), CHK1, phospho-CHK2 (p-CHK2), CHK2, phospho-p53 (p-p53) and p53 (p53) protein expression, detected by specific antibodies, are shown.* ***Continue…***



Continue… (Figure 2.9.) *Graphs shows the densitometry analysis in arbitrary units performed with ImageJ software (NIH, Bethesda, MD, USA), with value of 1 assigned to DMSO-treated control samples. Gray bars: BIBR-treated cells; black bars: DMSO-treated control cells.*

Conversely, no changes in the phosphorylation level of these proteins were noted in 4134/TERT- and U2OS cells (Figure 2.10.). Thus, TERT inhibition activates ATM and ATR cascades in TERT-positive LCLs and BL cells.

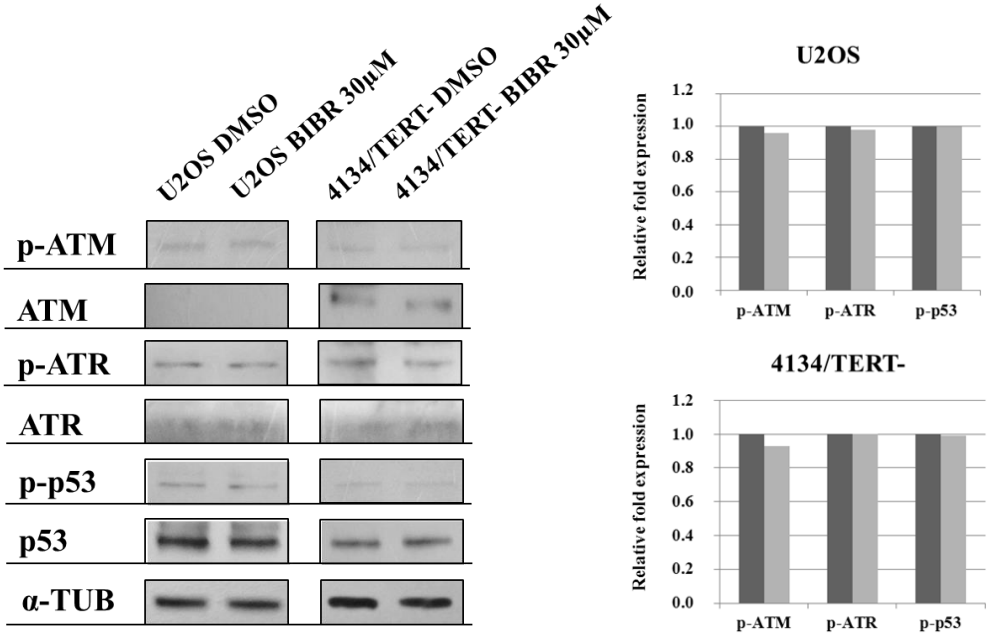


Figure 2.10. *BIBR treatment did not induce the activation of ATM/ATR pathway in TERT-negative cells. Cells were treated with BIBR (30 μM) and analyzed after 36 h. 4134/TERT- and U2OS cells expressed same level of total and* ***Continue…***

Continue… (Figure 2.10.) *phosphorylated/active form of ATM, ATR and p53 with and without BIBR treatment. Western blotting shows total and phosphorylated/active forms of ATM, ATR and phosphorylated/unphosphorylated forms of p53 with specific antibodies. Graphs on right: densitometry analysis in arbitrary units performed with ImageJ software (NIH, Bethesda, MD, USA), with value of 1 assigned to DMSO-treated samples. Grey bars: BIBR-treated cells; black bars: DMSO-treated control cells.*

2.3.5. TERT inhibition leads to H2AX activation in TERT-positive LCLs and BL cells

ATM and ATR are the key sensors of DNA damage (93). Findings that both these proteins are activated in BIBR-treated TERT-positive cells suggested that TERT inhibition could induce DNA damage and activate the DNA damage response (DDR). To assess this possibility, cells were stained for γH2AX, a marker of DNA damage (94). As shown in Figure 2.11.a, TERT-positive 4134/Late, 4134/TERT+, BL41 and BL41/B95.8 cells showed a significant increase in γH2AX-positive cells, even after 24 h of exposure (Figure 2.11.a). Conversely, 4134/TERT- and U2OS cells exposed to BIBR showed no evidence of increased DNA damage. The γH2AX MFI also increased significantly in TERT-positive BIBR-treated cell lines compared with DMSO-treated control ones, whereas in TERT-negative cells no differences in MFI levels were observed between cells treated with BIBR or DMSO (Figure 2.11.b).

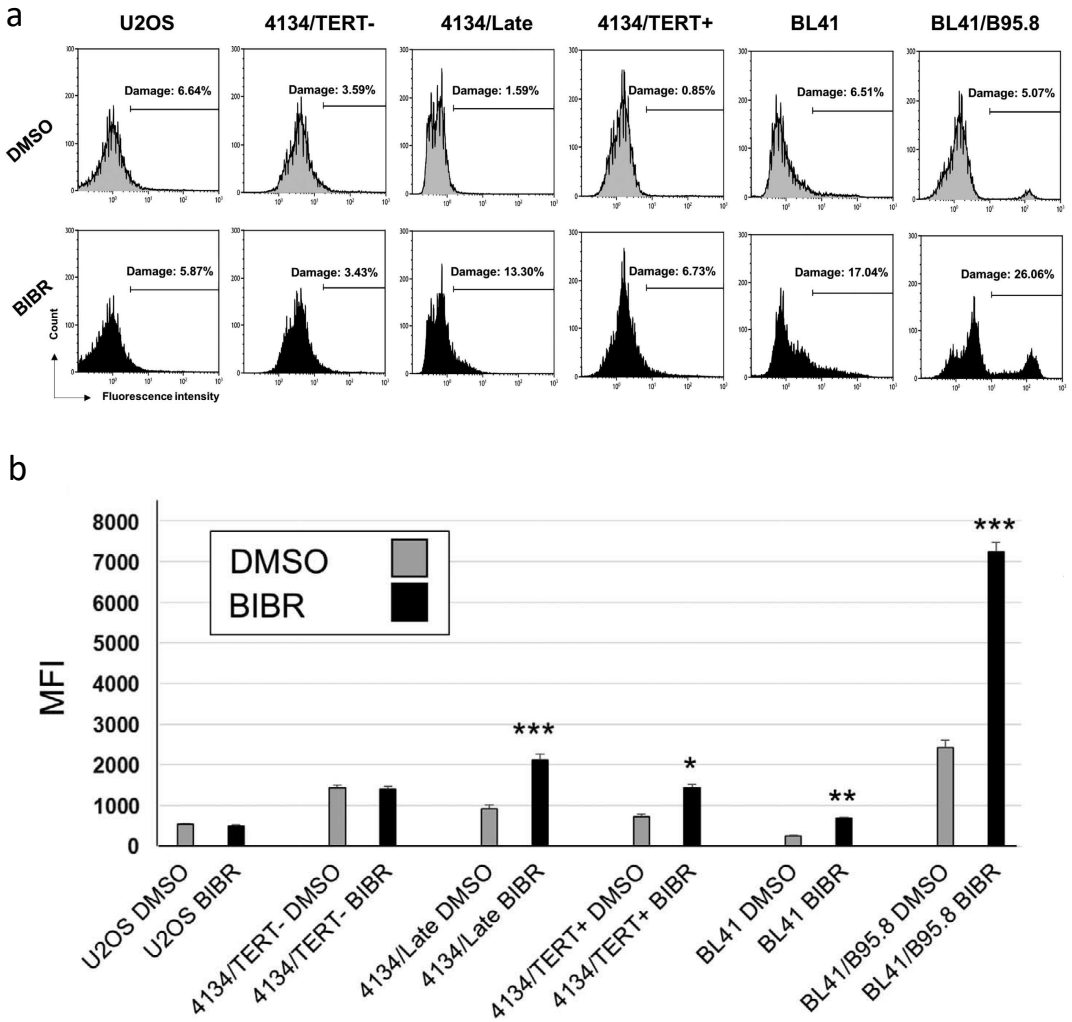


Figure 2.11. *TERT inhibition by BIBR increases DNA damage in TERT-positive cells. (a) TERT-positive 4134/Late, 4134/TERT+, BL41 and BL41/B95.8 cells and TERT-negative 4134/TERT- and U2OS cells exposed for 24 h to BIBR or to DMSO as control, were stained with γH2AX to evaluate DNA damage and analyzed by flow cytometry. Panels from one representative experiment are shown. (b) Levels of γH2AX MFI in BIBR- and DMSO-treated cells. Significant differences between values in BIBR-treated versus DMSO-treated cells are shown: *P=0.05, **P=0.01 and ***P=0.001*

2.3.6. Short-term inhibition of TERT does not affect telomeres

Replicative telomere attrition leads to activation of ATM and ATR. To elucidate whether the DDR in BIBR-treated cells can be activated by telomere erosion, we assessed the effects of the drug on telomere length. BIBR treatment did not affect the telomere length of LCLs or BL cells, as measured by quantitative multiplex PCR at 72 h of exposure (Figure 2.12.a). This finding was confirmed by terminal restriction fragment (TRF) analysis (Figure 2.12.b). Unlike the PCR-based assay, which gives a mean estimate of telomere length of the cellular population, TRF makes it possible to visualize the range of telomere length. The results showed that the TRF ranges are the same in cells treated with BIBR or DMSO, thus excluding the possibility that BIBR selectively targets cells with short telomeres within one cellular population.

Figure 2.12.*a*

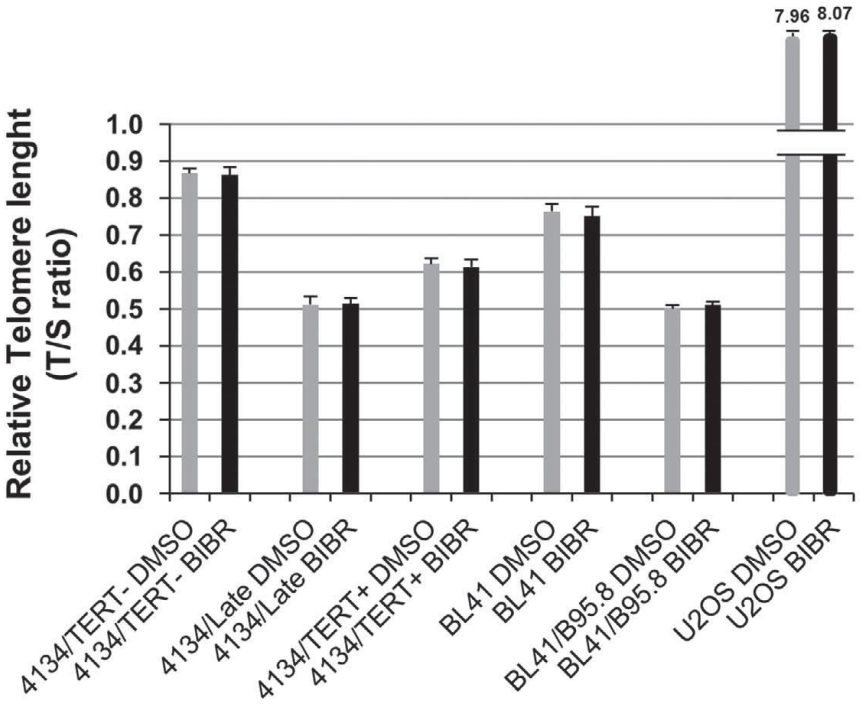
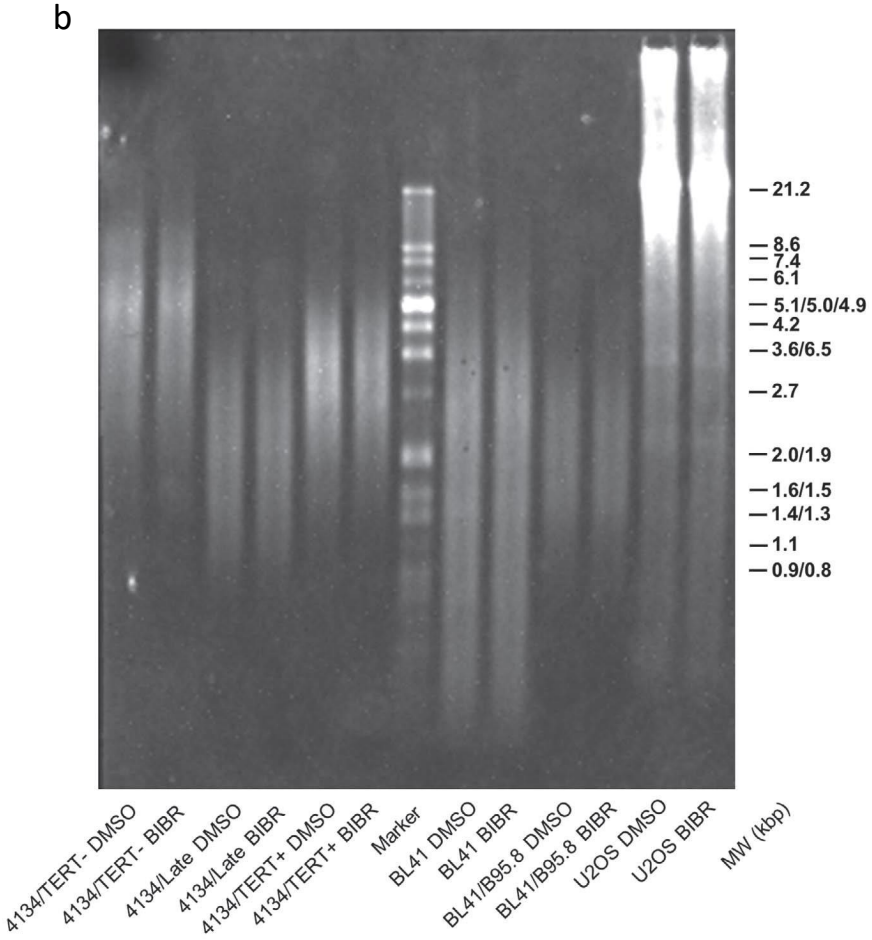


Figure 2.12.

b



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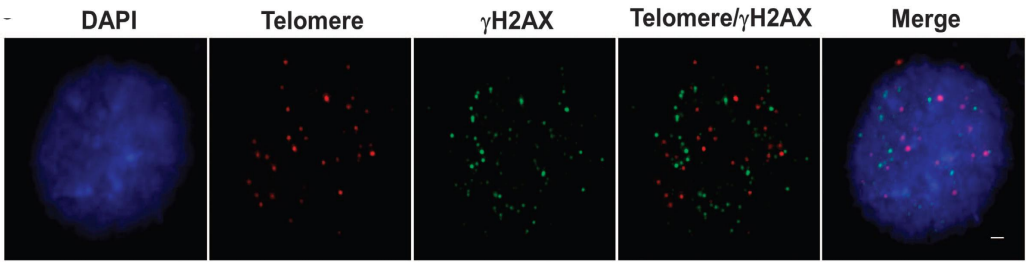


Figure 2.12. *Short-term TERT inhibition by BIBR did not affect telomere. TERT-positive 4134/Late, 4134/TERT+, BL41 and BL41/B95.8 cells and TERT-negative 4134/TERT- and U2OS cells exposed for 72 h to BIBR or to DMSO as control were analyzed for telomere length. (a) Telomere length measured by quantitative multiplex PCR assay. T/S values are e means and S.D. (bar) of three separate experiments. (b) Telomere lengths analyzed by TRF by the TeloTAGGG telomere length assay. Panel from one representative experiment is shown.* ***Continue…***

Continue… (Figure 2.12.) *(c) TIF analysis. Representative micrographs showing combined telomere FISH/γH2AX immunofluorescence of 4134/Late cells treated with BIBR at 24 h. From the left: DAPI (nuclear marker, blue), telomere probe (red), γH2AX (DNA damage marker, green), combined Telomere/γH2AX and the merged image. Scale bar: 2 µm.*

It has been demonstrated that EBV infection may cause telomere dysfunction, mainly due to reduction and displacement of TRF2 shelterin protein from telomeres; however, this effect was greatly reduced in LCLs kept in culture for an extended period of time (73,74). In agreement with these observations in established LCLs, in our 4134/Late cells, combined telomere FISH/TRF2 immunofluorescence showed that TRF2 was expressed and localized on telomeres (Figure 2.13.b). In addition, the treatment of 4134/Late cells with 30 µM BIBR at 24 h did not modify the expression of TRF2 protein compared with DMSO control cells (Figures 2.13.a). These results suggest that DDR is not driven by TRF2 displacement and uncapping problems at telomeres.

To elucidate in greater detail whether DNA damage is associated with telomeres, we examined the presence of telomere dysfunction-induced foci (TIF) in cells exposed to BIBR. Most of the γH2AX foci, markers of DNA damage, did not colocalize with telomere probe signals, and the number of TIF per nucleus was always lower than 3 (Figure 2.12.c.). All together, these findings indicate that inhibition of TERT by BIBR may lead to DNA damage randomly rather than specifically on telomeres.

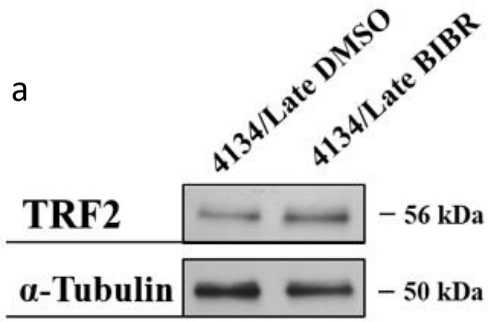
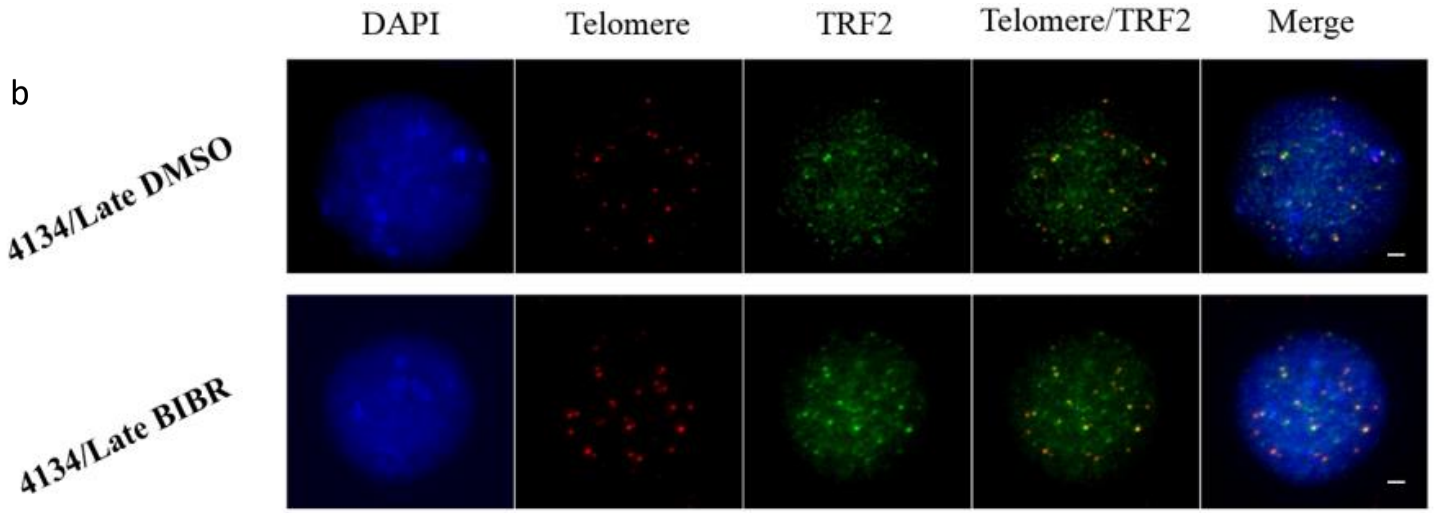


Figure 2.13. *(a) Western blotting shows TRF2 expression in 4134/Late treated with BIBR and DMSO as controls. α-Tubulin was used as control for loading. (b) Representative micrographs showing combined telomere FISH/TRF2 immunofluorescence of 4134/Late cells treated with BIBR or DMSO at 24h. From the left: DAPI (nuclear marker, blu), telomere probe (red), TRF2 (green), combined Telomere/TRF2 and the merged image. Scale bar: 2 µm.*



2.3.7. Effects of combined treatment with BIBR and FLU or CY

The observation that TERT inhibition by BIBR leads to cell cycle arrest and apoptosis prompted us to investigate whether TERT inhibition increases susceptibility to antineoplastic drugs. We therefore examined the effects of BIBR in combination with FLU or CY, two of the agents most frequently used to treat B-cell malignancies, in the LCL model.

Each drug was used alone or in combination with BIBR. Cells exposed to FLU were analyzed at 48 and 72 h (Figure 2.14.a). In both 4134/Late and 4134/TERT+, treatment with FLU alone did not modify the cell cycle profile, whereas cells treated with BIBR+FLU showed a significant increase of cells in the S-phase and a decrease in the G1-phase, at both 48 and 72 h. Conversely, in 4134/TERT- cells, neither treatments with FLU alone or FLU+BIBR induced significant cell cycle changes (Figure 2.14.a).

Cells treated with FLU and FLU+BIBR were also analyzed for apoptosis. 4134/TERT- cells were more sensitive to FLU alone (apoptosis of 29±2% at 72 h) than 4134/Late cells (14±2% at 72 h) and 4134/TERT+ cells (3 ±1% at 72 h) (Figure 2.14.b). In 4134/Late and 4134/TERT+ cells, BIBR+FLU treatment significantly increased the percentage of apoptotic cells compared with that obtained with FLU alone. In contrast, BIBR+FLU treatment of TERT-negative cells did not increase the number of apoptotic cells compared with that obtained with FLU alone (Figure 2.14.b).

Figure 2.14.a

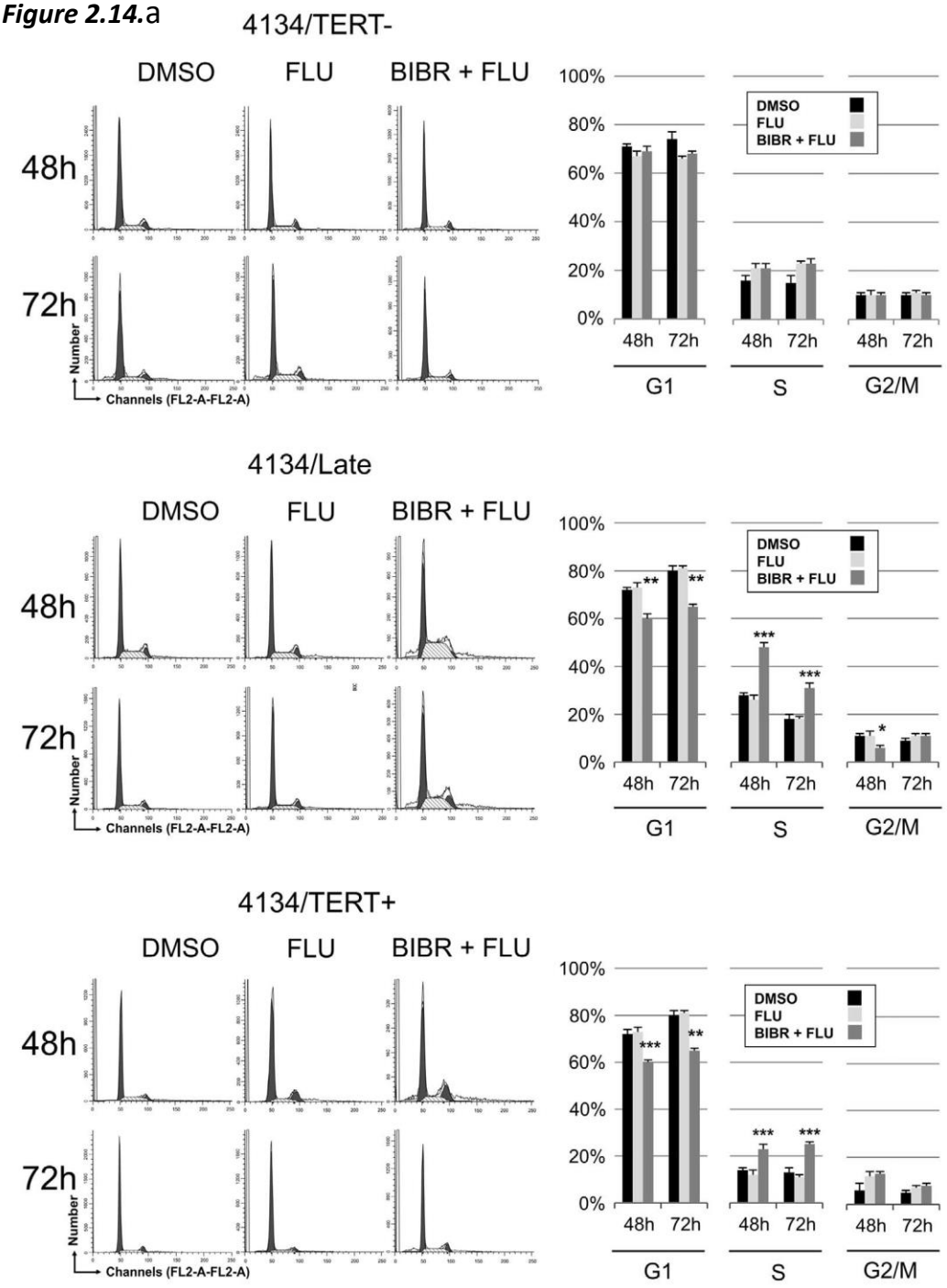
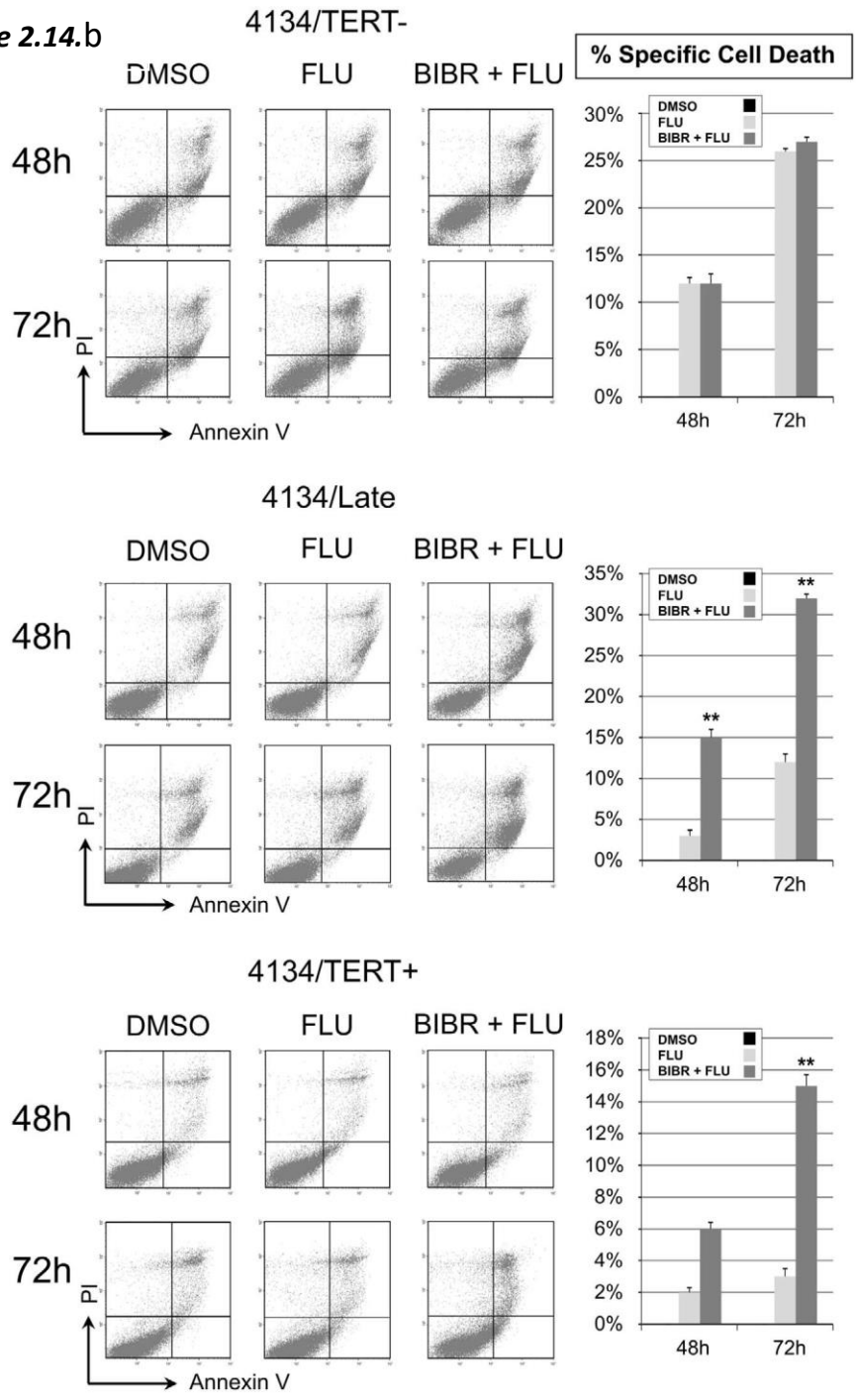


Figure 2.14. *Effects of FLU and BIBR+FLU treatments on cell cycle profiles and cell viability in LCLs. Cells were treated with FLU (5 µM) and BIBR (30 µM) plus FLU (5 µM) (BIBR+FLU) and analyzed at 48 and 72 h. DMSO was used as control. (a) Cells were labeled with PI and analyzed by flow cytometry. Panels from one representative experiment are shown. Graphs on right: percentages of cells in G1-, S- and G2/M-phase. Values are means and S.D. (bar) of three independent experiments.* ***Continue…***

Figure 2.14.b



Continue … (Figure 2.14) *(b) Cells were labeled with annexin V/PI and analyzed by flow cytometry. Panels from one representative experiment are shown. Graphs on right: percentages of specific cell death. Values are means and S. D. (bar) of three separate experiments. Significant differences between values in BIBR+FLU-treated versus FLU-treated cells are shown: *P=0.05, **P=0.01 and ***P=0.001*

Parallel experiments were performed with CY alone or in combination with BIBR. Cell cycle profiles were analyzed at 24 and 48 h in all LCLs (Figure 2.15.a). In 4134/Late and 4134/TERT+, CY induced a decrease of cell number in G1-phase at 48 h, whereas in 4134/TERT- cells CY treatment slightly increased cell number in the S-phase. Treatment with BIBR+CY in TERT-positive cells induced complete arrest of the cell cycle, whereas in 4134/TERT- the pattern observed with BIBR+CY had no effect compared with cell cultures treated with CY alone (Figure 2.15.a). The number of apoptotic cells after exposure to CY alone was higher in 4134/Late (47±4% at 48 h) than in 4134/TERT- cells (21±2%) (Figure 2.15.b). In 4134/Late and 4134/TERT+ cells treatment with BIBR+CY significantly increased the apoptotic effect compared with those obtained with CY alone, whereas BIBR+CY did not change the number of apoptotic cells compared with that obtained with CY alone in 4134/TERT- cell culture (Figure 2.15.b).

Figure 2.15.a

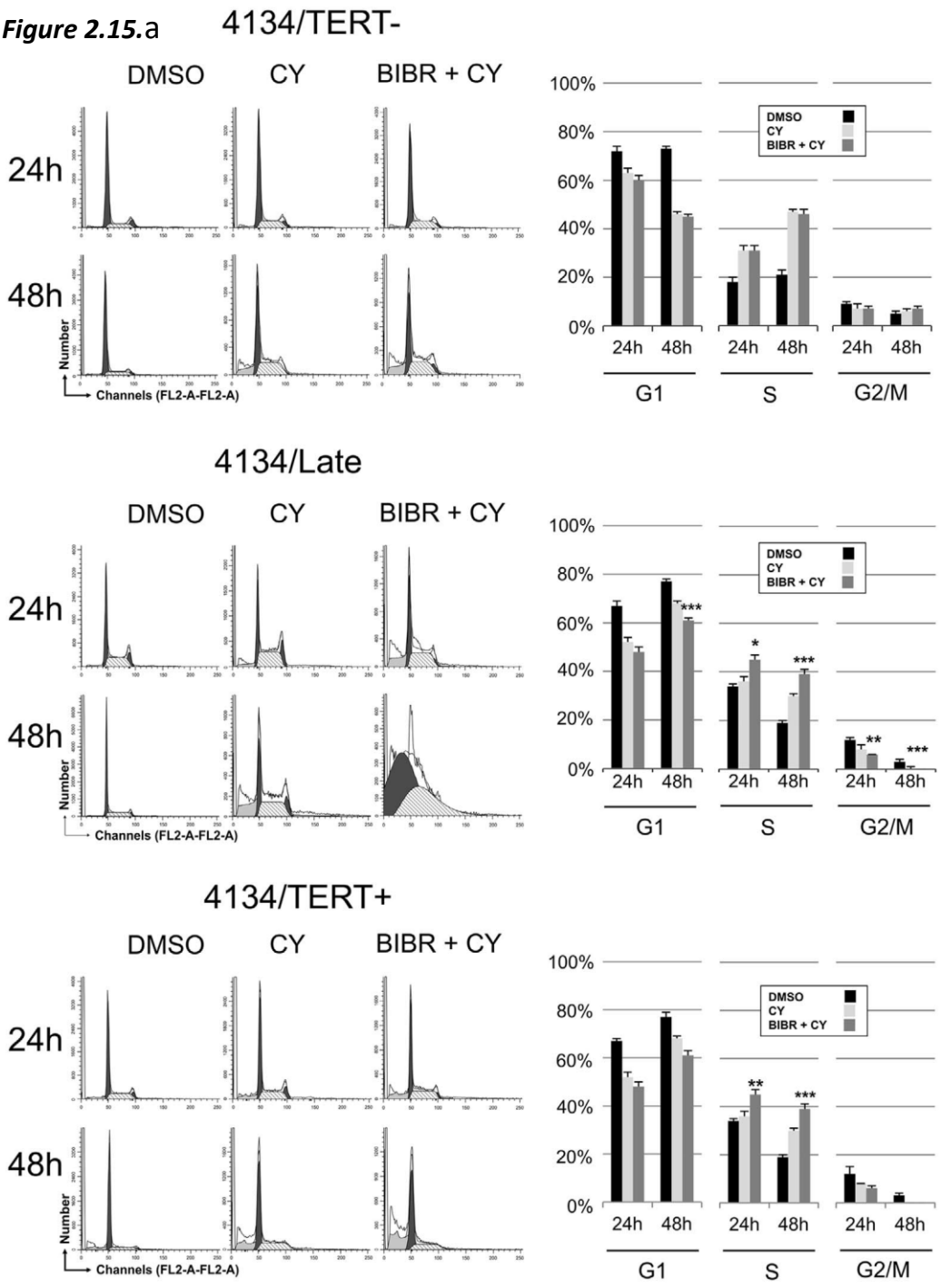
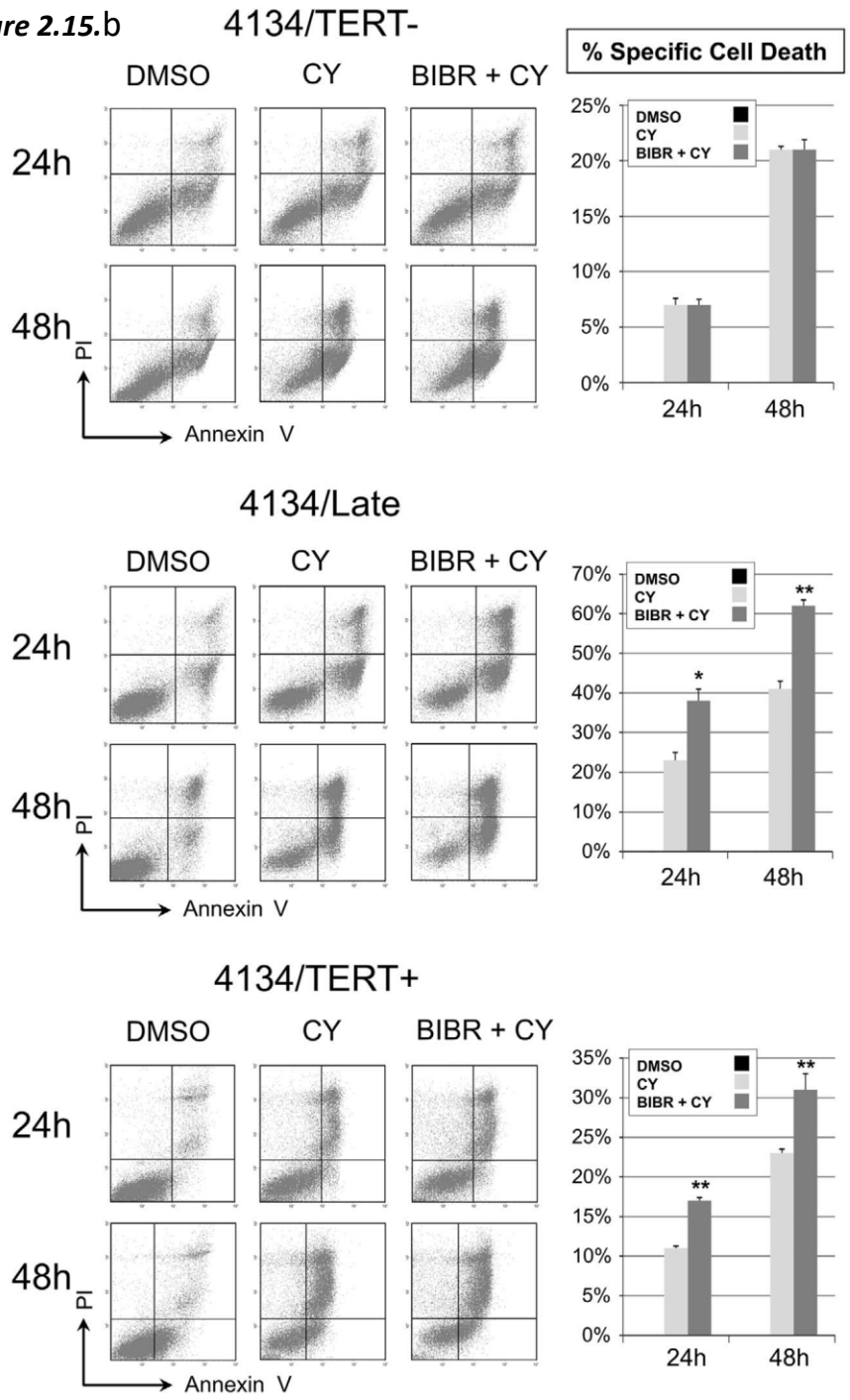


Figure 2.15. *Effects of CYand BIBR+CY treatments on cell cycle profiles and cell viability in LCLs. Cells were treated with CY (4 mM) and BIBR (30 µM) plus CY (4 mM) (BIBR +CY) and analyzed at 24 and 48 h. DMSO was used as control. (a) Cells were labeled with PI and analyzed by flow cytometry. Panels from one representative experiment are shown. Graphs on right: percentages of cells in G1-, S- and G2/M-phase. Values are means and S.D. (bar) of three separate experiments.* ***Continue…***

Figure 2.15.b



Continue … (Figure 2.15) *(b) Cells were labeled with annexin V/PI and analyzed by flow cytometry. Panels from one representative experiment are shown. Graphs on right: percentages of specific cell death. Values are means and S.D. (bar) of three independent experiments. Significant differences between values in BIBR+CY-treated versus CY-treated cells are shown: *P=0.05, **P=0.01 and ***P=0.001.*

2.4. Discussion

In this study, we demonstrate that in TERT-positive LCLs short-term TERT inhibition by BIBR causes cell cycle arrest, accumulation of cells in the S-phase and apoptosis. Similar results were obtained in the BL41 and its EBV convertant BL41/B95.8 BL cell lines. These effects driven by BIBR were telomerase-specific, as they were not observed in telomerase-negative LCL 4134/TERT- and U2OS cells. This study provides evidence indicating that cell cycle arrest and apoptosis induced by BIBR-mediated TERT inhibition are related and probably dependent on the activation of the DDR pathway. In particular, TERT inhibition induces DNA damage, highlighted by increased levels of γH2AX, resulting in the activation of DDR and phosphorylation of the ATM and ATR kinases, which in turn activate the mitotic checkpoints CHK1, CHK2 and the pro-apoptotic p53 protein to induce cell cycle arrest with accumulation of cells in the S-phase and apoptosis. Notably, in the EBV-positive BL41/B95.8 cells, the inhibition of TERT by BIBR leads to an earlier and greater accumulation of cells in the S-phase, as well as a higher number of apoptotic cells, than in the EBV-negative counterpart BL41 cells. This effect may be due to the underlying EBV infection and in particular to the effects consequent upon TERT inhibition in this cellular background. In fact, it has been demonstrated that the EBV protein BGLF4 can directly promote elongation of the S-phase (95). Intriguingly, this protein is expressed during the EBV lytic cycle and we have previously demonstrated that TERT inhibition in EBV-infected cells triggers a complete viral lytic replication (50). From a therapeutic perspective, these findings suggest that TERT inhibition may induce more pronounced effects of potential relevance in EBV-associated lymphoproliferations as compared with EBV-unrelated B-cell malignancies.

It is well-known that shelterin proteins binding to telomeres enable cells to distinguish their chromosome ends from DNA breaks and to repress DNA repair reactions (13,96). Replicative telomere attrition with depletion of TRF2 and POT1 shelterin proteins leads to activation of both ATM- and ATR-mediated DDR (97).

Notably, it has been demonstrated that EBV in newly infected cells may cause telomere dysfunction, mainly due to decreased expression of shelterin proteins and displacement of TRF2 from telomeres (73,74); in addition, the EBV-encoded LMP1 transfected in EBV-negative BL cells promotes downregulation of shelterin proteins (98). However, in agreement with previous observations on established LCLs (73,74), we did not find any TRF2 displacement from telomeres in our LCL cells. Nakashima et al. (80) have reported that long-term BIBR treatment of HeLa-EM2-11ht cells is associated with telomere shortening and activation of DDR at telomeres. Telomere shortening after long-term BIBR treatment has also been reported in chronic myeloid leukemia cells (81) and in human promyelocytic leukemia cells (82). Besides these results supporting the ability of BIBR to inhibit the canonical TERT activity on telomeres during long-term treatment, it has been reported that high doses of BIBR induced growth arrest and apoptosis in short-term culture assays in both leukemia cell lines and primary cells from patients with acute myeloid leukemia and chronic lymphocytic leukemia (83). Notably, similar findings were also observed in cells without detectable telomerase activity, and the authors suggested that they were due to direct damage by high doses of BIBR on telomere structures, being thus independent of telomerase (83).

In our *in vitro* models, the DDR pathway activated after short-term exposure to low doses of BIBR seemed to be substantially unrelated to telomere dysfunction, being instead dependent on TERT inhibition *per se*, as none of above effects were observed in TERT-negative cells. BIBR-treated cells have exactly the same mean telomere length, estimated by multiplex PCR, and range, estimated by TRF analysis, as control DMSO-treated cells. In addition, BIBR treatment did not modify the expression and telomere localization of TRF2, which is compatible with the persistence of its capping function on telomeres. The diffuse localization of γH2AX foci and the limited number of TIF in BIBR-treated cells clearly demonstrated that the DNA damage induced by TERT inhibition in short-term experiments was randomly rather than specifically localized on telomeres.

Thus, the findings that TERT inhibition determines DNA damage, unrelated to telomere dysfunction, reinforces the concept that TERT may have additional roles

other than maintaining telomere length, and are in line with the growing body of data describing the extra-telomeric functions of telomerase in many biological processes, including cellular proliferation, gene expression regulation, DNA repair process and mitochondrial functionality (17,31). In particular, several lines of evidence demonstrate that TERT is partially targeted to mitochondria, in which it may influence the production of reactive oxygen species (ROS), and thus DNA damage and apoptosis (23,99,100). TERT is also involved in DNA repair processes (58,101), and TERT inhibition may lead to perturbation of chromatin structure with diminished capacity for DNA repair and thus accumulation of DNA damage (101). On these grounds, the DNA damage we observed after short-term TERT inhibition may be due to increased ROS levels and/or perturbation of the chromatin structure. Further studies are warranted to define the mechanisms underlying the short-term consequences of TERT inhibition unrelated to telomere dysfunction.

In the light of the possible integration of TERT inhibitors in chemotherapeutic regimens, we treated the LCLs with two drugs used to manage lymphoproliferative disorders (FLU and CY), both alone and in combination with BIBR. Notably, treatment with FLU alone did not alter the cell cycle profile and induced more pronounced apoptotic effects in TERT-negative than in TERT-positive cells. These observations support the finding that high TERT levels confer protection against apoptosis (69,82). Indeed, TERT inhibition does sensitize cells to the drug-induced apoptotic effect, as demonstrated by the high number of apoptotic cells induced by BIBR+FLU in TERT-positive cell cultures. Consistently, the percentage of apoptotic cells in 4134/Late culture treated with BIBR+FLU at 72 h was similar to that observed in 4134/TERT- cell culture treated with FLU alone.

CY alone induced stronger apoptotic effects in TERT-positive than in TERT-negative cells; this is consistent with its effect in proliferating cells, taking into account the fact that TERT-positive cells proliferated more rapidly than TERT-negative cells. Nonetheless, the addition of BIBR caused cell cycle arrest and an increased apoptotic effect in TERT-positive cells.

Our findings support the concept that inhibition of the extra-telomeric functions of TERT could be exploited as an effective therapeutic strategy for a variety of

tumors, including B-cell malignancies, regardless of telomere length. The inclusion of telomerase inhibitors in chemotherapy protocols for cancer patients may have strong effects on cell proliferation and survival and thus may represent a valid strategy to complement current treatment modalities, as also suggested by others (102,103). Confirmation of these findings in primary tumors cells from patients with EBV-driven and unrelated B-cell malignancies and in suitable animal models will pave the way for a solidly based pre-clinical rationale for including TERT inhibitors in chemotherapy protocols for the treatment of these malignancies.

Chapter 3: *In vivo* study

3.1. Introduction

3.1.1. *In vivo* zebrafish model

The employment of animal model is unavoidable to study the pathogenesis of human diseases and to develop and test new therapies (104).

In the last few decades zebrafish (*Danio rerio*) emerged as an excellent model of early vertebrate development (105,106,107) and of an ample spectrum of human diseases, including cancer (108).

Zebrafish, originally coming from the river Ganges in India, belongs to the genus of teleost. As a model, it combines the advantages of large clutch size (up to 200 eggs *per* week *per* female) and transparency (characteristics of invertebrate models) with high similarity of histology of both normal and cancer tissue with those of humans (109). In particular, the major oncogenes and tumor suppressor genes, as well as signalling pathways regulating proliferation, apoptosis, differentiation and cell migration, are highly conserved between zebrafish and human (109). Moreover, the very fast and *ex vivo* embryonic development strongly facilitates the employment of this model (108), allowing an *in vivo* and in real time examination of the pathological processes (104).

Of interest, zebrafish spontaneously develops tumors preferentially in advancing age, as mammals, and, with the introduction of techniques to enhance tumorigenesis, zebrafish became a largely employed useful tool to model human cancers (108) and a powerful system to perform large scale *in vivo* anticancer drug screenings (109). Moreover, many mutant zebrafish lines are available nowadays, e.g. the transparent *casper* mutant and several fluorescent reporter lines (109).

3.1.2. Telomeres and telomerase in zebrafish

In the field of telomere and telomerase research, zebrafish results an appropriate model thanks to the very high conservation of telomere and telomerase biology between zebrafish and human.

Contrary to the laboratory mouse, that have 20-150 kb telomeres (110), zebrafish has heterogeneous telomeres of human-like length, 5-15 kb (110). A similar trend of accentuated telomere shortening during puberty followed by stabilization in length at later ages has been described in human as well as in zebrafish (110); in both species, telomeres shortening occurs both in high-turnover (e.g. gut) and low-turnover (e.g. muscle) organs, regardless of differences in proliferation rates, (111). Moreover, zebrafish telomerase promoter is activated by MYC and NF-κB, as in humans (110,112,113).

Zebrafish TERT is a 126 kD protein composed by 1091 amino acids; it display 36% whole sequence similarity with human TERT (32% with mouse TERT), but functional domains of zebrafish telomerase are highly similar to their human counterparts (N-terminal domain, TR binding site, RT motif) (113,114). Zebrafish TR is also quite conserved, both in structure and in function, between zebrafish and human, thus supporting the employment of zebrafish in telomere/telomerase research (115).

It has been established a direct correlation between the levels of telomerase expression, telomerase activity and telomere length in zebrafish (112). Of interest, low levels of telomerase, insufficient to maintain telomeres, can be detected in different zebrafish adult tissue, like in humans (116), even in those that do not further divide; this found suggests that zebrafish telomerase may be involved in functions other than the elongation of telomere (113).

Thus, our *in vivo* study of the extra-telomeric functions of telomerase is performed in the zebrafish model.

3.2. Materials and methods

3.2.1. Zebrafish Lines

The embryos of wild type (wt) zebrafish present high tert mRNA levels from 9 to 24 hours post-fertilization (hpf) and Tert protein expression is maintained at high level up to 72 hpf, after which it decreases (112); we selected 24 hpf as the timing for our short-term experiments. The telomerase mutant line $tert^{hu3430}$, generated by N-Ethyl-Nitrosourea (ENU) mutagenesis, has a T-A point mutation in the *tert* gene, that introduces an early stop codon (117); this line was employed as negative control.

Wt zebrafish embryos were treated with serial dilutions of BIBR (S1186, Selleckchem) (0.5, 2 and 4 µM) or with DMSO (in which BIBR is dissolved in) as control treatment. At a concentration of 0.5 µM, BIBR did not show any significant effect on wt embryos, whereas at 4 µM almost 50% of them died; 2 µM BIBR induced an embryos death rate of 15% in wt, but showed no effects on $tert^{hu3430/hu3430}$ ($tert^{-/-}$) zebrafish. 2 µM BIBR was therefore employed for the subsequent experiments.

BIBR or DMSO were administered to wt and $tert^{-/-}$ zebrafish embryos at the stage of 12 hpf and samples were analysed at 24 hpf, i.e. after 12 h of treatment, to investigate the effects of short-term Tert inhibition.

3.2.2. Immunohistochemistry and Immunofluorescence

Cell cycle alterations after Tert inhibition were analysed in wt and $tert^{-/-}$ embryos. Samples were manually dechorionated, fixed with 4% paraformaldehyde (PFA) and stored in 100% methanol. Embryos were then permeabilized in cold acetone and saturated in 0.5% Triton X-100, 1% DMSO, 1% BSA, 2% Normal Goat Serum. The mitotic foci were highlighted by anti-phospho Histone H3 (pHH3) antibody (s10) (05-570, Millipore) followed by appropriate secondary antibody conjugated with alkaline phosphatase (12-448, Millipore) and visualized with optic

microscope. S-phases were stained employing the anti-PCNA antibody (M0879, DAKO) followed by appropriate secondary antibody and visualized with fluorescence microscope.

3.2.3. TUNEL assay

Apoptosis rate was evaluated in BIBR-treated and DMSO-treated wt and tert$^{-/-}$ embryos, employing the DeadEnd™ Fluorometric TUNEL System (G3250, Promega). Shortly, after manual dechorionation, embryos were fixed with 4% PFA and stored in 100% methanol. Samples were treated with proteinase k, incubated in 2:1 ethanol:acetic acid and then subjected to TUNEL assay, according to manufacturer's instruction. Apoptotic foci were visualized with fluorescence microscope.

3.2.4. Western Blotting

Protein lysates were prepared from BIBR-treated and control-treated embryos at 24 hpf. Briefly, embryos were manually dechorionated, deyolked with appropriate salt solution, flash frozen and incubated in RIPA lysis buffer. The expression of RNR-R2, a marker of S-phase, γH2AX, a marker of DNA damage, and α-tubulin (as loading control) were evaluated by anti-RNR-R2 (70-050, B-Bridge), anti-γH2AX (pS139) (GTX127342, GeneTex) and anti-α-tubulin (T5168, Sigma) antibodies, respectively. Blots were incubated with appropriate peroxidase-conjugated secondary antibody and stained with chemiluminescent detection kit.

3.2.5. Telomere Length Measurement by TRF Analysis

The telomere length was determined using the TeloTAGGG telomere length assay kit (12209136001, Roche); it measures the range of length of the terminal restriction fragments, obtained after enzymatic digestion of genomic DNA. Shortly, DNA was extracted with the phenol-chloroform method from dechorionated embryos; after digestion, the fragments were separated by gel

electrophoresis and transferred to a nylon membrane by Southern blotting. Telomeric sequences were labelled by a specific probe, then recognized by an alkaline phosphatase conjugated antibody and visualized with a chemiluminescent substrate.

3.2.6. Combined FISH/Immunofluorescence

The rate of dysfunctional telomeres was evaluated by the Telomere Dysfunction-induced Foci (TIF) analysis, which measures the co-localization of telomere signals (FISH) and DNA damage foci (IF). Cellular suspensions were obtained from dechorionated and deyolked treated and mock-treated embryos by incubation with 0.25% Tripsin-EDTA for 12 minutes at 28°C; these cells were cytospinned onto glass slides. Telomeres were stained with the Telomere PNA FISH Kit/Cy3 (5326, DAKO) and DNA damage foci were highlighted by anti-γH2AX antibody (GTX127342, GeneTex), followed by appropriate secondary antibody. Slides were mounted with DAPI/antifade solution and analyzed with fluorescence microscope, as detailed in 2.2.8.

3.3. Results

3.3.1. Short-term Tert inhibition by BIBR reduced cell proliferation and induced an accumulation of cells in S-phase.

In agreement with previous *in vitro* studies on LCLs and BL cell lines, short-term Tert inhibition by BIBR reduced the proliferation rate in wt treated zebrafish embryos. This was demonstrated by a 30% decrease in the number of mitotic foci, evaluated by IHC for pHH3, a molecular marker of mitosis. Conversely, no effect was observed in tert$^{-/-}$ treated embryos (Figure 3.1.).

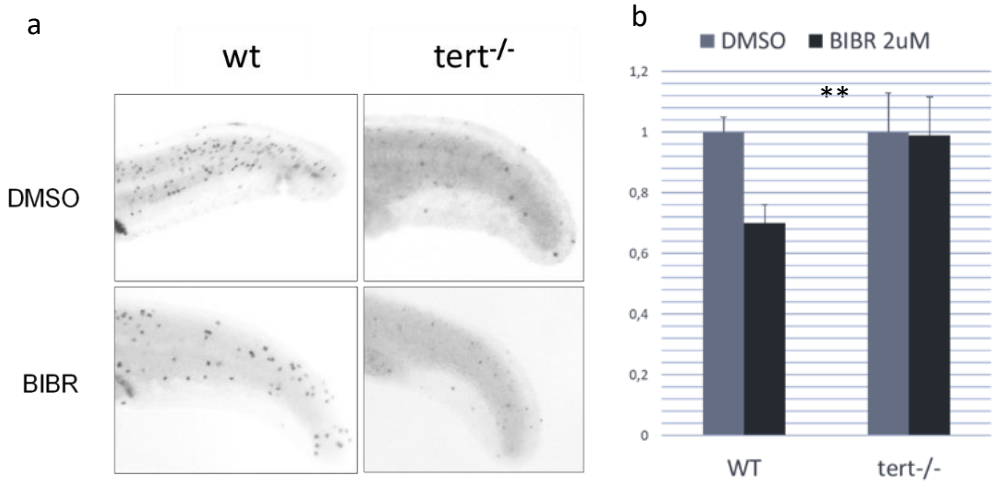


Figure 3.1. *(a) Representative wt and tert$^{-/-}$ embryos treated with BIBR or DMSO are shown. The quantification analysis of pHH3 foci was made by ImageJ software. (b) Graph shows the means and S.D. (bar) of pHH3 foci in at least 20 embryos, with value of 1 assigned to DMSO-treated control specimens. Significant difference between values in BIBR-treated embryos versus DMSO-treated ones is shown: ** p<0.01.*

Furthermore, to shed light on cell cycle profile alterations induced by BIBR treatment, we measured the level of two S-phase specific markers. PCNA, revealed by IF techniques, highlighted a 1.8-fold increase of mitotic foci in wt but not in tert$^{-/-}$ embryos (Figure 3.2.); the level of RNR-R2, measured by Western Blot, confirmed the accumulation of cells in S-phase by a 1.81-fold increase in wt embryos (Figure 3.3.).

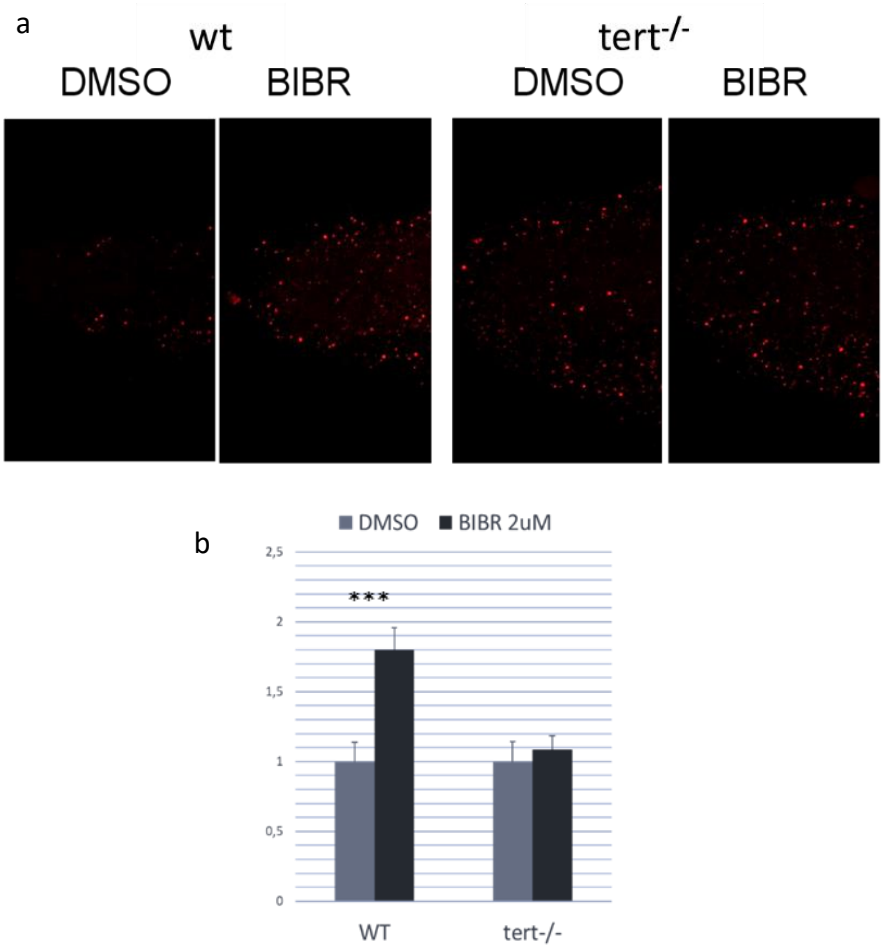


Figure 3.2. *(a) Representative wt and tert$^{-/-}$ embryos treated with BIBR or DMSO are shown. The quantification analysis of PCNA expression was made by ImageJ software. (b) Graph shows the relative means and S.D. (bar) of PCNA expression in at least 20 embryos, with value of 1 assigned to DMSO-treated control specimens. Significant difference between values in BIBR-treated embryos versus DMSO-treated embryos is shown: *** p<0.001.*

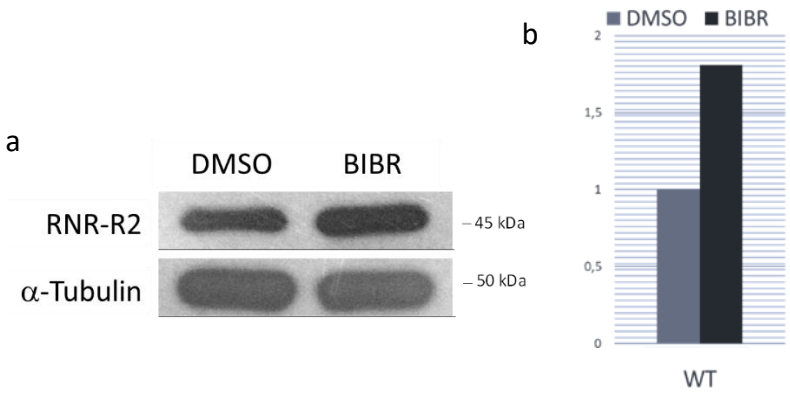


Figure 3.3. *(a) RNR-R2 protein (45 kDa) levels, detected by specific antibody, and normalized on tubulin content, are shown. (b) Graphs shows densitometry analysis in arbitrary units performed with ImageJ software, with value of 1 assigned to DMSO-treated control samples.*

3.3.2. Short-term Tert inhibition by BIBR increased apoptosis.

We investigated if zebrafish embryos short-term treated with BIBR responded, in term of cell death, coherently to the previously studied *in vitro* models. We observed a 1.97-fold increase in the number of apoptotic foci, measured by the TUNEL assay, in wt BIBR-treated embryos, compared to control-treated ones, while the treatment did not induce apoptosis in tert$^{-/-}$ embryos (Figure 3.4.).

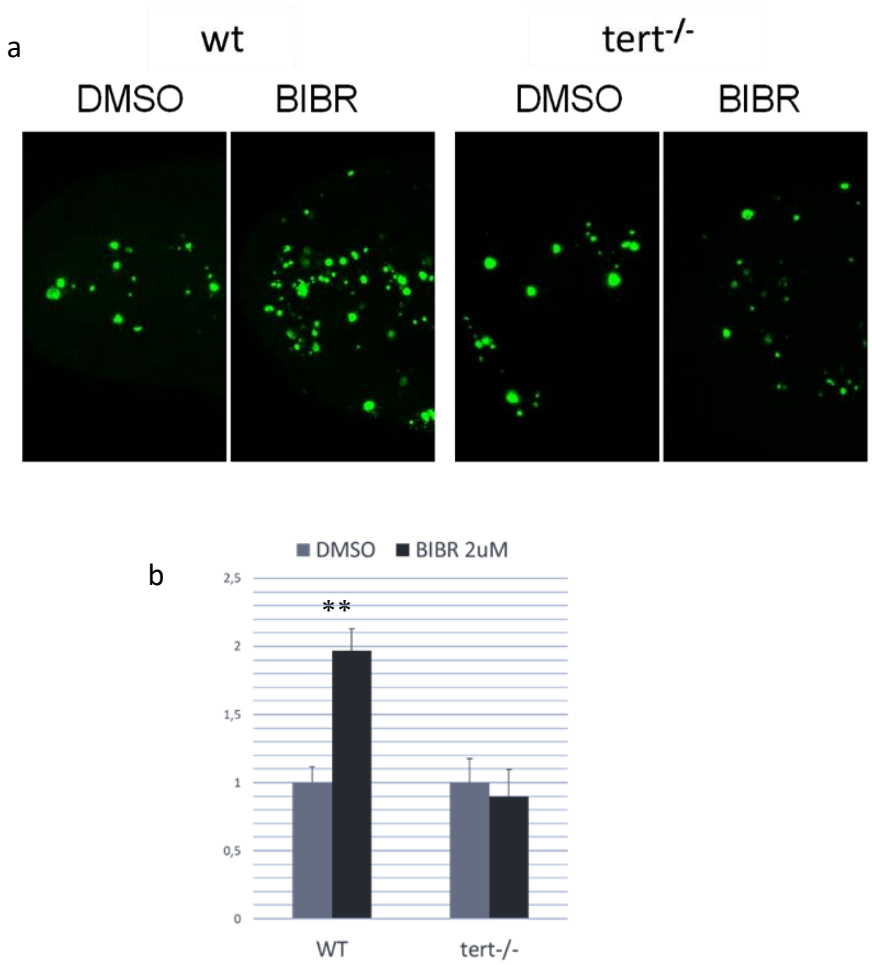


Figure 3.4. *(a) Representative wt and tert$^{-/-}$ embryos treated with BIBR or DMSO are shown. The quantification analysis of apoptotic foci was made by ImageJ software. (b) Graph shows the relative means and S.D. (bar) of expression of apoptotic foci in at least 20 embryos, with value of 1 assigned to DMSO-treated control specimens. Significant difference between values in BIBR-treated embryos versus DMSO-treated embryos is shown: ** p<0.01.*

3.3.3. Short-term Tert inhibition by BIBR induced DDR activation.

The cell cycle alteration and apoptosis induced by BIBR treatment may result from the activation of DDR, as mentioned above. To elucidate whether the same mechanism is activated after short-term Tert inhibition in wt zebrafish embryos, we measured the level of a histonic variant which is phosphorylated at sites of DNA damage, the γH2AX. It is evident that BIBR treatment induced a 3.78-fold increase in the level of γH2AX, compared to control treated samples, indicating induction of DNA damage and consequent activation of DDR (Figure 3.5.).

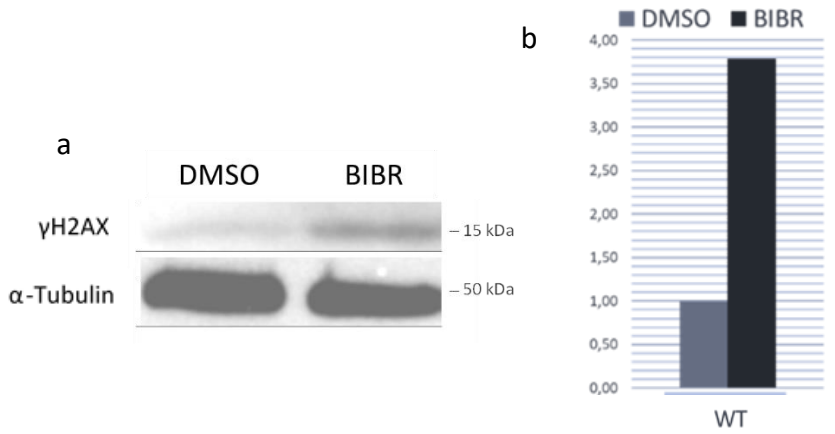


Figure 3.5. *(a) γH2AX protein (15 kDa) levels, detected by specific antibody, and normalized on tubulin content, are shown. (b) Graph shows densitometry analysis in arbitrary units performed with ImageJ software, with value of 1 assigned to DMSO-treated control samples.*

3.3.4. Short-term Tert inhibition did not affect telomeres.

Given that excessive telomere erosion or telomere dysfunction lead to the activation of DDR, we evaluated the involvement of telomeres in the effects observed after the short-term inhibition of TERT. On this regard, we analyzed the telomere length of treated and control-treated wt embryos by Southern Blot, and we demonstrated that the range of telomere length was not affected by 36 h BIBR treatment (Figure 3.6.).

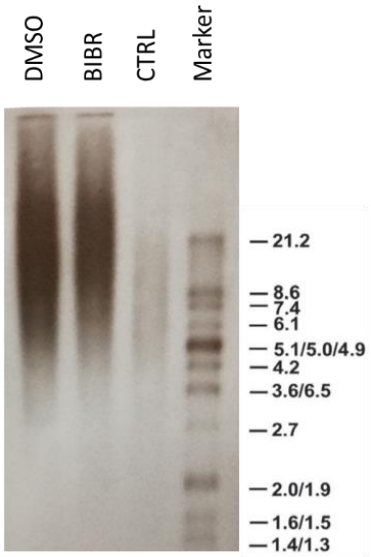


Figure 3.6. *TRF analyses of telomere lengths by TeloTAGGG telomere length assay. A panel from one representative experiment is shown.*

Moreover, we investigated, through the TIFs analysis, if the DNA damage induced by the treatment was specifically located at telomeres, thus indicating the participation of telomere dysfunction in the effects observed after short-term TERT inhibition. TIF assay showed that the DNA damage foci did not specifically co-localize with the telomere signals, rather they are randomly distributed throughout the genome (Figure 3.7.).

These results suggest that the effects observed after short-term Tert inhibition in wt zebrafish embryos were not to be attributable to telomere alteration, thus they are likely linked to extra-telomeric function of Tert.

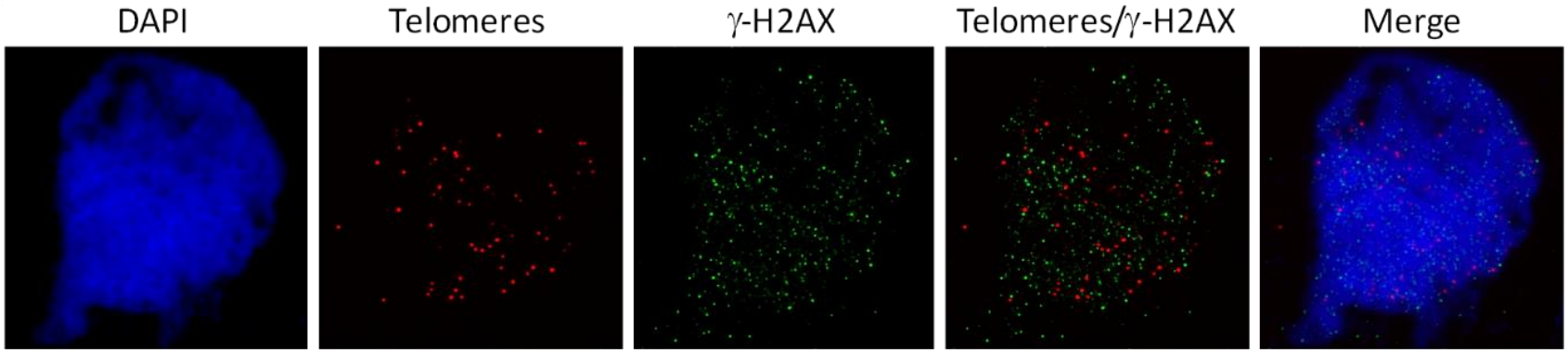


Figure 3.7. *Representative micrographs showing combined telomere FISH/γH2AX IF on cells from wt zebrafish embryos treated with BIBR. From the left: DAPI (nuclear marker, blue), telomere probe (red), γH2AX antibody (green), combined Telomere/γH2AX, merged image.*

3.4. Discussion

This study demonstrates that short-term inhibition of Tert in 24 hpf wt zebrafish embryos reduces cell proliferation, induces an accumulation of cells in S-phase, and ultimately leads to apoptosis; these effects are associated with the activation of DDR. Notably, they are telomere length unrelated, since the DNA damage foci are distributed randomly in the genome, rather than specifically located at telomeres, and the range of telomere length is not affected by the short-term treatment. All these effects are specifically related to Tert inhibition since BIBR treatment shows no effect in $tert^{-/-}$ embryos.

These *in vivo* results confirm our previous work in *in vitro* models, enforcing the concept that telomerase has telomere length-independent effects on cell proliferation and survival and that these effects involve the induction of telomere unrelated DNA damage. Thus, Tert *per se* seems to exert tumor-promoting activities that are independent from its canonical role of telomere length maintenance. Accordingly, telomerase inhibition could be a useful therapeutic strategy to counteract tumor growth, and could be effective besides its effects on telomere length.

In support of these findings, it will be important to continue this line of research confirming the effects of short-term inhibition of Tert in *in vivo* zebrafish model, employing a different inhibitory strategy, morpholino (MO). MO is an antisense oligonucleotide that, injected in zebrafish embryos, directly impairs gene transcription or mRNA translation in a sequence specific manner (104).

4. General Conclusions

This research program aimed at evaluating the effects of short-term treatment with specific TERT inhibitor in *in vitro* models of B-cell malignancies and in *in vivo* zebrafish model, in order to support the introduction of telomerase inhibitors as anticancer therapeutic strategy.

Telomerase inhibition has already been exploited as anticancer strategy, since it reduces the proliferative potential of cancer cells after continuous cell divisions within the tumor; indeed, several classes of telomerase inhibitors have been developed, most of which specifically affects telomere maintenance.

Considering that, in theory, the time to antineoplastic effectiveness of telomerase inhibitors depends on the original length of the telomeres, cancer cells with short telomere are the preferential targets of telomerase inhibition; indeed, telomere length reaches the critical threshold in a reasonable period of treatment, exhausting the proliferative potential of cancer cells and, therefore compromising the tumor growth. By contrast, in tumor cells with long telomeres, a very long period of treatment with telomerase inhibitors would be required to obtain telomere-related therapeutic benefits. In both cases, a lag period between the beginning of treatment and the therapeutic benefit is inherent in this approach.

However, a growing list of evidences shows the involvement of TERT in several tumor-promoting processes, such as enhancement of cell proliferation and resistance to apoptosis, independently to its role in telomere maintenance (118). Therefore, targeting the functions of TERT may lead to broader efficacy in cancer treatment.

The results presented in this thesis demonstrate that short-term inhibition of TERT by the specific inhibitor BIBR impairs cell proliferation, and induces a pro-apoptotic effect associated with the activation of DDR, in *in vitro* B-cell malignancies models and *in vivo* in zebrafish embryos; these effects are unrelated to telomere dysfunction, since the telomere length is not affected by the short-term TERT inhibition and the DNA damage is randomly distributed, rather than specifically

located at telomeres. These findings enforce the concept that TERT inhibition may be taken into account as a valid approach to counteract tumor cell proliferation and viability, regardless of tumor telomere length.

The mechanism(s) underlying these effects require further investigation and several hypotheses regarding the telomere length-independent functions of TERT may be explored.

For instance, the ability of TERT to modulate gene expression by affecting signalling pathways such as WNT and NF-kB, producing siRNA (by RdRP activity of TERT), and regulating miRNA expression, may in turn be responsible for the effects observed after TERT inhibition by BIBR treatment (23,31,45,54-56). It can also be speculated the involvement of chromatin alterations as a result of TERT activity loss; this may be due to a reduced DNA damage repair capacity or the impairment of DNA replication mechanisms, with the consequent accumulation of damages (58,59). Moreover, the effects induced by TERT activity inhibition may be mediated, at least partially, by the telomerase role in mitochondria (31,53,57).

In any case, our *in vitro* experiments indicate that the therapeutic approach based on the inhibition of TERT enhances the pro-apoptotic and anti-proliferative effects of chemotherapeutic agents currently employed in the treatment of lymphoproliferative disorders. This observation supports the clinical application of TERT inhibitors in combination with standard chemotherapeutic protocols to treat B-cell malignancies, and suggests that the effectiveness of the combined therapeutic strategy may be potentially extended to all TERT-positive tumors.

Classically, a limitation of the approach based on telomerase inhibition may results from the activation of the ALT mechanism, which has been described as a compensatory mechanism triggered by telomere erosion after long-term telomerase inhibition (39). Nevertheless, the efficacy soon after the beginning of the treatment, and the independence of the effects observed after BIBR treatment from telomere shortening, prompt to re-evaluate telomerase inhibition as an effective anticancer therapeutic strategy.

Therefore, it will be important to validate the effects of TERT activity inhibition on cancer cells in an *in vivo* context to further support the efficacy of TERT inhibitors in anticancer therapy.

4.1 Future Perspective

Relevant insights have been obtained by xenotransplantation, that is the transfer of human cancer cells or tissue (cell lines or primary tumors) in zebrafish; this tool allows the direct observation of drug response by human tumor material in *in vivo* platform (109,119-121).

On this ground, we plan to xenotransplant fluorescent-labelled TERT-positive and TERT-negative (as control) cell lines in zebrafish embryos, that do not require immunosuppression since they have not yet developed an adaptive immune response. We will employ the *casper* zebrafish line, which does not express pigments, and thus allows direct fluorescence imaging of labelled transplanted cells. Xenotransplanted zebrafish will be treated with the TERT inhibitor BIBR, and its short-term effects on human tumor cells proliferation and viability will be evaluated in an *in vivo* context, to confirm the validity of TERT inhibition as anticancer strategy. It will be also of great interest to investigate the consequences of combined treatment with TERT inhibitor and currently employed antineoplastic drugs and study the possible cumulative effects on tumor cells, to further support the introduction of TERT inhibitors in anticancer therapy.

5. References

1. Cleal K, Norris K, Baird D. Telomere Length Dynamics and the Evolution of Cancer Genome Architecture. Int J Mol Sci **2018**;19(2) doi 10.3390/ijms19020482.
2. Seifert M, Scholtysik R, Kuppers R. Origin and pathogenesis of B cell lymphomas. Methods Mol Biol **2013**;971:1-25 doi 10.1007/978-1-62703-269-8_1.
3. Hakem R. DNA-damage repair; the good, the bad, and the ugly. Embo j **2008**;27(4):589-605 doi 10.1038/emboj.2008.15.
4. Sirbu BM, Cortez D. DNA damage response: three levels of DNA repair regulation. Cold Spring Harb Perspect Biol **2013**;5(8):a012724 doi 10.1101/cshperspect.a012724.
5. Gilson E, Geli V. How telomeres are replicated. Nat Rev Mol Cell Biol **2007**;8(10):825-38 doi 10.1038/nrm2259.
6. Lazzerini-Denchi E, Sfeir A. Stop pulling my strings - what telomeres taught us about the DNA damage response. Nat Rev Mol Cell Biol **2016**;17(6):364-78 doi 10.1038/nrm.2016.43.
7. Lu W, Zhang Y, Liu D, Songyang Z, Wan M. Telomeres-structure, function, and regulation. Exp Cell Res **2013**;319(2):133-41 doi 10.1016/j.yexcr.2012.09.005.
8. Cusanelli E, Chartrand P. Telomeric noncoding RNA: telomeric repeat-containing RNA in telomere biology. Wiley Interdiscip Rev RNA **2014**;5(3):407-19 doi 10.1002/wrna.1220.
9. Rhodes D, Lipps HJ. G-quadruplexes and their regulatory roles in biology. Nucleic Acids Res **2015**;43(18):8627-37 doi 10.1093/nar/gkv862.
10. Rog O, Cooper JP. Telomeres in drag: Dressing as DNA damage to engage telomerase. Curr Opin Genet Dev **2008**;18(2):212-20 doi 10.1016/j.gde.2008.01.011.
11. Martinez P, Blasco MA. Replicating through telomeres: a means to an end. Trends Biochem Sci **2015**;40(9):504-15 doi 10.1016/j.tibs.2015.06.003.
12. Calado RT, Young NS. Telomere maintenance and human bone marrow failure. Blood **2008**;111(9):4446-55 doi 10.1182/blood-2007-08-019729.
13. Palm W, de Lange T. How shelterin protects mammalian telomeres. Annu Rev Genet **2008**;42:301-34 doi 10.1146/annurev.genet.41.110306.130350.
14. de Lange T. How telomeres solve the end-protection problem. Science **2009**;326(5955):948-52 doi 10.1126/science.1170633.
15. Longhese MP. DNA damage response at functional and dysfunctional telomeres. Genes Dev **2008**;22(2):125-40 doi 10.1101/gad.1626908.

16. Cesare AJ, Karlseder J. A three-state model of telomere control over human proliferative boundaries. Curr Opin Cell Biol **2012**;24(6):731-8 doi 10.1016/j.ceb.2012.08.007.

17. Martinez P, Blasco MA. Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. Nat Rev Cancer **2011**;11(3):161-76 doi 10.1038/nrc3025.

18. Schmidt JC, Cech TR. Human telomerase: biogenesis, trafficking, recruitment, and activation. Genes Dev **2015**;29(11):1095-105 doi 10.1101/gad.263863.115.

19. Wellinger RJ. In the end, what's the problem? Mol Cell **2014**;53(6):855-6 doi 10.1016/j.molcel.2014.03.008.

20. Maciejowski J, de Lange T. Telomeres in cancer: tumour suppression and genome instability. Nat Rev Mol Cell Biol **2017**;18(3):175-86 doi 10.1038/nrm.2016.171.

21. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell **1985**;43(2 Pt 1):405-13.

22. Kumar M, Lechel A, Gunes C. Telomerase: The Devil Inside. Genes (Basel) **2016**;7(8) doi 10.3390/genes7080043.

23. Saretzki G. Extra-telomeric functions of human telomerase: cancer, mitochondria and oxidative stress. Curr Pharm Des **2014**;20(41):6386-403.

24. Nachajova M, Brany D, Dvorska D. Telomerase and the process of cervical carcinogenesis. Tumour Biol **2015**;36(10):7335-8 doi 10.1007/s13277-015-3976-z.

25. Wu RA, Upton HE, Vogan JM, Collins K. Telomerase Mechanism of Telomere Synthesis. Annu Rev Biochem **2017**;86:439-60 doi 10.1146/annurev-biochem-061516-045019.

26. Podlevsky JD, Chen JJ. It all comes together at the ends: telomerase structure, function, and biogenesis. Mutat Res **2012**;730(1-2):3-11 doi 10.1016/j.mrfmmm.2011.11.002.

27. Akincilar SC, Unal B, Tergaonkar V. Reactivation of telomerase in cancer. Cell Mol Life Sci **2016**;73(8):1659-70 doi 10.1007/s00018-016-2146-9.

28. Heidenreich B, Kumar R. TERT promoter mutations in telomere biology. Mutat Res **2017**;771:15-31 doi 10.1016/j.mrrev.2016.11.002.

29. Liu X, Wang Y, Chang G, Wang F, Geng X. Alternative Splicing of hTERT Pre-mRNA: A Potential Strategy for the Regulation of Telomerase Activity. Int J Mol Sci **2017**;18(3) doi 10.3390/ijms18030567.

30. Pfeiffer V, Lingner J. Replication of telomeres and the regulation of telomerase. Cold Spring Harb Perspect Biol **2013**;5(5):a010405 doi 10.1101/cshperspect.a010405.

31. Chiodi I, Mondello C. Telomere-independent functions of telomerase in nuclei, cytoplasm, and mitochondria. Front Oncol **2012**;2:133 doi 10.3389/fonc.2012.00133.

32. Giunco S, Rampazzo E, Celeghin A, Petrara MR, De Rossi A. Telomere and Telomerase in Carcinogenesis: Their Role as Prognostic Biomarkers. Curr Pathobiol Rep **2015;**3:315–28 doi 10.1007/s40139-015-0087-x.

33. Terrin L, Dal Col J, Rampazzo E, Zancai P, Pedrotti M, Ammirabile G*, et al.* Latent membrane protein 1 of Epstein-Barr virus activates the hTERT promoter and enhances telomerase activity in B lymphocytes. J Virol **2008**;82(20):10175-87 doi 10.1128/JVI.00321-08.

34. Chen X, Kamranvar SA, Masucci MG. Tumor viruses and replicative immortality--avoiding the telomere hurdle. Semin Cancer Biol **2014**;26:43-51 doi 10.1016/j.semcancer.2014.01.006.

35. Kang Y, Zhang J, Sun P, Shang J. Circulating cell-free human telomerase reverse transcriptase mRNA in plasma and its potential diagnostic and prognostic value for gastric cancer. Int J Clin Oncol **2013**;18(3):478-86 doi 10.1007/s10147-012-0405-9.

36. Rampazzo E, Del Bianco P, Bertorelle R, Boso C, Perin A, Spiro G*, et al.* The predictive and prognostic potential of plasma telomerase reverse transcriptase (TERT) RNA in rectal cancer patients. Br J Cancer **2018**;118(6):878-86 doi 10.1038/bjc.2017.492.

37. Yuan P, Cao JL, Abuduwufuer A, Wang LM, Yuan XS, Lv W*, et al.* Clinical Characteristics and Prognostic Significance of TERT Promoter Mutations in Cancer: A Cohort Study and a Meta-Analysis. PLoS One **2016**;11(1):e0146803 doi 10.1371/journal.pone.0146803.

38. Rampazzo E, Bonaldi L, Trentin L, Visco C, Keppel S, Giunco S*, et al.* Telomere length and telomerase levels delineate subgroups of B-cell chronic lymphocytic leukemia with different biological characteristics and clinical outcomes. Haematologica **2012**;97(1):56-63 doi 10.3324/haematol.2011.049874.

39. De Vitis M, Berardinelli F, Sgura A. Telomere Length Maintenance in Cancer: At the Crossroad between Telomerase and Alternative Lengthening of Telomeres (ALT). Int J Mol Sci **2018**;19(2) doi 10.3390/ijms19020606.

40. Maicher A, Lockhart A, Luke B. Breaking new ground: digging into TERRA function. Biochim Biophys Acta **2014**;1839(5):387-94 doi 10.1016/j.bbagrm.2014.03.012.

41. Pickett HA, Reddel RR. Molecular mechanisms of activity and derepression of alternative lengthening of telomeres. Nat Struct Mol Biol **2015**;22(11):875-80 doi 10.1038/nsmb.3106.

42. Stampfer MR, Garbe J, Levine G, Lichtsteiner S, Vasserot AP, Yaswen P. Expression of the telomerase catalytic subunit, hTERT, induces resistance

to transforming growth factor beta growth inhibition in p16INK4A(-) human mammary epithelial cells. Proc Natl Acad Sci U S A **2001**;98(8):4498-503 doi 10.1073/pnas.071483998.

43. Park JI, Venteicher AS, Hong JY, Choi J, Jun S, Shkreli M*, et al.* Telomerase modulates Wnt signalling by association with target gene chromatin. Nature **2009**;460(7251):66-72 doi 10.1038/nature08137.

44. Polakis P. Wnt signaling in cancer. Cold Spring Harb Perspect Biol **2012**;4(5) doi 10.1101/cshperspect.a008052.

45. Giunco S, Celeghin A, Gianesin K, Dolcetti R, Indraccolo S, De Rossi A. Cross talk between EBV and telomerase: the role of TERT and NOTCH2 in the switch of latent/lytic cycle of the virus. Cell Death Dis **2015**;6:e1774 doi 10.1038/cddis.2015.145.

46. Koh CM, Khattar E, Leow SC, Liu CY, Muller J, Ang WX*, et al.* Telomerase regulates MYC-driven oncogenesis independent of its reverse transcriptase activity. J Clin Invest **2015**;125(5):2109-22 doi 10.1172/jci79134.

47. Khattar E, Tergaonkar V. Transcriptional Regulation of Telomerase Reverse Transcriptase (TERT) by MYC. Front Cell Dev Biol **2017**;5:1 doi 10.3389/fcell.2017.00001.

48. Baena-Del Valle JA, Zheng Q, Esopi DM, Rubenstein M, Hubbard GK, Moncaliano MC*, et al.* MYC drives overexpression of telomerase RNA (hTR/TERC) in prostate cancer. J Pathol **2018**;244(1):11-24 doi 10.1002/path.4980.

49. Terrin L, Dolcetti R, Corradini I, Indraccolo S, Dal Col J, Bertorelle R*, et al.* hTERT inhibits the Epstein-Barr virus lytic cycle and promotes the proliferation of primary B lymphocytes: implications for EBV-driven lymphomagenesis. Int J Cancer **2007**;121(3):576-87 doi 10.1002/ijc.22661.

50. Giunco S, Dolcetti R, Keppel S, Celeghin A, Indraccolo S, Dal Col J*, et al.* hTERT inhibition triggers Epstein-Barr virus lytic cycle and apoptosis in immortalized and transformed B cells: a basis for new therapies. Clin Cancer Res **2013**;19(8):2036-47 doi 10.1158/1078-0432.ccr-12-2537.

51. Dolcetti R, Dal Col J, Martorelli D, Carbone A, Klein E. Interplay among viral antigens, cellular pathways and tumor microenvironment in the pathogenesis of EBV-driven lymphomas. Semin Cancer Biol **2013**;23(6):441-56 doi 10.1016/j.semcancer.2013.07.005.

52. Giunco S, Petrara MR, Zangrossi M, Celeghin A, De Rossi A. Extra-telomeric functions of telomerase in the pathogenesis of Epstein-Barr virus-driven B-cell malignancies and potential therapeutic implications. Infect Agent Cancer **2018**;13:14 doi 10.1186/s13027-018-0186-5.

53. Indran IR, Hande MP, Pervaiz S. hTERT overexpression alleviates intracellular ROS production, improves mitochondrial function, and inhibits ROS-mediated apoptosis in cancer cells. Cancer Res **2011**;71(1):266-76 doi 10.1158/0008-5472.can-10-1588.

54. Maida Y, Yasukawa M, Furuuchi M, Lassmann T, Possemato R, Okamoto N*, et al.* An RNA-dependent RNA polymerase formed by TERT and the RMRP RNA. Nature **2009**;461(7261):230-5 doi 10.1038/nature08283.

55. Lassmann T, Maida Y, Tomaru Y, Yasukawa M, Ando Y, Kojima M*, et al.* Telomerase reverse transcriptase regulates microRNAs. Int J Mol Sci **2015**;16(1):1192-208 doi 10.3390/ijms16011192.

56. Mukherjee S, Firpo EJ, Wang Y, Roberts JM. Separation of telomerase functions by reverse genetics. Proc Natl Acad Sci U S A **2011**;108(50):E1363-71 doi 10.1073/pnas.1112414108.

57. Sharma NK, Reyes A, Green P, Caron MJ, Bonini MG, Gordon DM*, et al.* Human telomerase acts as a hTR-independent reverse transcriptase in mitochondria. Nucleic Acids Res **2012**;40(2):712-25 doi 10.1093/nar/gkr758.

58. Sharma GG, Gupta A, Wang H, Scherthan H, Dhar S, Gandhi V*, et al.* hTERT associates with human telomeres and enhances genomic stability and DNA repair. Oncogene **2003**;22(1):131-46 doi 10.1038/sj.onc.1206063.

59. Shin KH, Kang MK, Dicterow E, Kameta A, Baluda MA, Park NH. Introduction of human telomerase reverse transcriptase to normal human fibroblasts enhances DNA repair capacity. Clin Cancer Res **2004**;10(7):2551-60.

60. Arndt GM, MacKenzie KL. New prospects for targeting telomerase beyond the telomere. Nat Rev Cancer **2016**;16(8):508-24 doi 10.1038/nrc.2016.55.

61. Shirgahi Talari F, Bagherzadeh K, Golestanian S, Jarstfer M, Amanlou M. Potent Human Telomerase Inhibitors: Molecular Dynamic Simulations, Multiple Pharmacophore-Based Virtual Screening, and Biochemical Assays. J Chem Inf Model **2015**;55(12):2596-610 doi 10.1021/acs.jcim.5b00336.

62. Ruden M, Puri N. Novel anticancer therapeutics targeting telomerase. Cancer Treat Rev **2013**;39(5):444-56 doi 10.1016/j.ctrv.2012.06.007.

63. Pascolo E, Wenz C, Lingner J, Hauel N, Priepke H, Kauffmann I*, et al.* Mechanism of human telomerase inhibition by BIBR1532, a synthetic, non-nucleosidic drug candidate. J Biol Chem **2002**;277(18):15566-72 doi 10.1074/jbc.M201266200.

64. Greider CW. Telomere length regulation. Annu Rev Biochem **1996**;65:337-65 doi 10.1146/annurev.bi.65.070196.002005.

65. Blackburn EH, Greider CW, Szostak JW. Telomeres and telomerase: the path from maize, Tetrahymena and yeast to human cancer and aging. Nat Med **2006**;12(10):1133-8 doi 10.1038/nm1006-1133.

66. Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J*, et al.* Telomerase catalytic subunit homologs from fission yeast and human. Science **1997**;277(5328):955-9.

67. Kelland L. Targeting the limitless replicative potential of cancer: the telomerase/telomere pathway. Clin Cancer Res **2007**;13(17):4960-3 doi 10.1158/1078-0432.ccr-07-0422.

68. Liang W, Ye D, Dai L, Shen Y, Xu J. Overexpression of hTERT extends replicative capacity of human nucleus pulposus cells, and protects against serum starvation-induced apoptosis and cell cycle arrest. J Cell Biochem **2012**;113(6):2112-21 doi 10.1002/jcb.24082.

69. Cerone MA, Londono-Vallejo JA, Autexier C. Telomerase inhibition enhances the response to anticancer drug treatment in human breast cancer cells. Mol Cancer Ther **2006**;5(7):1669-75 doi 10.1158/1535-7163.mct-06-0033.

70. Pendino F, Dudognon C, Delhommeau F, Sahraoui T, Flexor M, Bennaceur-Griscelli A*, et al.* Retinoic acid receptor alpha and retinoid-X receptor-specific agonists synergistically target telomerase expression and induce tumor cell death. Oncogene **2003**;22(57):9142-50 doi 10.1038/sj.onc.1207093.

71. Rahman R, Latonen L, Wiman KG. hTERT antagonizes p53-induced apoptosis independently of telomerase activity. Oncogene **2005**;24(8):1320-7 doi 10.1038/sj.onc.1208232.

72. Dudognon C, Pendino F, Hillion J, Saumet A, Lanotte M, Segal-Bendirdjian E. Death receptor signaling regulatory function for telomerase: hTERT abolishes TRAIL-induced apoptosis, independently of telomere maintenance. Oncogene **2004**;23(45):7469-74 doi 10.1038/sj.onc.1208029.

73. Lacoste S, Wiechec E, Dos Santos Silva AG, Guffei A, Williams G, Lowbeer M*, et al.* Chromosomal rearrangements after ex vivo Epstein-Barr virus (EBV) infection of human B cells. Oncogene **2010**;29(4):503-15 doi 10.1038/onc.2009.359.

74. Kamranvar SA, Chen X, Masucci MG. Telomere dysfunction and activation of alternative lengthening of telomeres in B-lymphocytes infected by Epstein-Barr virus. Oncogene **2013**;32(49):5522-30 doi 10.1038/onc.2013.189.

75. Hahn WC, Stewart SA, Brooks MW, York SG, Eaton E, Kurachi A*, et al.* Inhibition of telomerase limits the growth of human cancer cells. Nat Med **1999**;5(10):1164-70 doi 10.1038/13495.

76. Nakamura M, Masutomi K, Kyo S, Hashimoto M, Maida Y, Kanaya T*, et al.* Efficient inhibition of human telomerase reverse transcriptase expression by RNA interference sensitizes cancer cells to ionizing radiation and chemotherapy. Hum Gene Ther **2005**;16(7):859-68 doi 10.1089/hum.2005.16.859.

77. Noureini SK, Wink M. Dose-dependent cytotoxic effects of boldine in HepG-2 cells-telomerase inhibition and apoptosis induction. Molecules **2015**;20(3):3730-43 doi 10.3390/molecules20033730.

78. Bashash D, Ghaffari SH, Zaker F, Hezave K, Kazerani M, Ghavamzadeh A*, et al.* Direct short-term cytotoxic effects of BIBR 1532 on acute promyelocytic leukemia cells through induction of p21 coupled with downregulation of c-Myc and hTERT transcription. Cancer Invest **2012**;30(1):57-64 doi 10.3109/07357907.2011.629378.

79. Damm K, Hemmann U, Garin-Chesa P, Hauel N, Kauffmann I, Priepke H*, et al.* A highly selective telomerase inhibitor limiting human cancer cell proliferation. Embo j **2001**;20(24):6958-68 doi 10.1093/emboj/20.24.6958.

80. Nakashima M, Nandakumar J, Sullivan KD, Espinosa JM, Cech TR. Inhibition of telomerase recruitment and cancer cell death. J Biol Chem **2013**;288(46):33171-80 doi 10.1074/jbc.M113.518175.

81. Brassat U, Balabanov S, Bali D, Dierlamm J, Braig M, Hartmann U*, et al.* Functional p53 is required for effective execution of telomerase inhibition in BCR-ABL-positive CML cells. Exp Hematol **2011**;39(1):66-76.e1-2 doi 10.1016/j.exphem.2010.10.001.

82. Ward RJ, Autexier C. Pharmacological telomerase inhibition can sensitize drug-resistant and drug-sensitive cells to chemotherapeutic treatment. Mol Pharmacol **2005**;68(3):779-86 doi 10.1124/mol.105.011494.

83. El-Daly H, Kull M, Zimmermann S, Pantic M, Waller CF, Martens UM. Selective cytotoxicity and telomere damage in leukemia cells using the telomerase inhibitor BIBR1532. Blood **2005**;105(4):1742-9 doi 10.1182/blood-2003-12-4322.

84. Bashash D, Ghaffari SH, Mirzaee R, Alimoghaddam K, Ghavamzadeh A. Telomerase inhibition by non-nucleosidic compound BIBR1532 causes rapid cell death in pre-B acute lymphoblastic leukemia cells. Leuk Lymphoma **2013**;54(3):561-8 doi 10.3109/10428194.2012.704034.

85. Nabetani A, Yokoyama O, Ishikawa F. Localization of hRad9, hHus1, hRad1, and hRad17 and caffeine-sensitive DNA replication at the alternative lengthening of telomeres-associated promyelocytic leukemia body. J Biol Chem **2004**;279(24):25849-57 doi 10.1074/jbc.M312652200.

86. Dejardin J, Kingston RE. Purification of proteins associated with specific genomic Loci. Cell **2009**;136(1):175-86 doi 10.1016/j.cell.2008.11.045.

87. Terrin L, Rampazzo E, Pucciarelli S, Agostini M, Bertorelle R, Esposito G*, et al.* Relationship between tumor and plasma levels of hTERT mRNA in patients with colorectal cancer: implications for monitoring of neoplastic disease. Clin Cancer Res **2008**;14(22):7444-51 doi 10.1158/1078-0432.ccr-08-0478.

88. Ballon G, Ometto L, Righetti E, Cattelan AM, Masiero S, Zanchetta M*, et al.* Human immunodeficiency virus type 1 modulates telomerase activity in peripheral blood lymphocytes. J Infect Dis **2001**;183(3):417-24 doi 10.1086/318072.

89. Colombrino E, Rossi E, Ballon G, Terrin L, Indraccolo S, Chieco-Bianchi L*, et al.* Human immunodeficiency virus type 1 Tat protein modulates cell cycle and apoptosis in Epstein-Barr virus-immortalized B cells. Exp Cell Res **2004**;295(2):539-48 doi 10.1016/j.yexcr.2004.01.018.

90. McKerlie M, Walker JR, Mitchell TR, Wilson FR, Zhu XD. Phosphorylated (pT371)TRF1 is recruited to sites of DNA damage to facilitate homologous recombination and checkpoint activation. Nucleic Acids Res **2013**;41(22):10268-82 doi 10.1093/nar/gkt775.

91. Gianesin K, Noguera-Julian A, Zanchetta M, Del Bianco P, Petrara MR, Freguja R*, et al.* Premature aging and immune senescence in HIV-infected children. Aids **2016**;30(9):1363-73 doi 10.1097/qad.0000000000001093.

92. Bashash D, Zareii M, Safaroghli-Azar A, Omrani MD, Ghaffari SH. Inhibition of telomerase using BIBR1532 enhances doxorubicin-induced apoptosis in pre-B acute lymphoblastic leukemia cells. Hematology **2017**;22(6):330-40 doi 10.1080/10245332.2016.1275426.

93. Liang Y, Lin SY, Brunicardi FC, Goss J, Li K. DNA damage response pathways in tumor suppression and cancer treatment. World J Surg **2009**;33(4):661-6 doi 10.1007/s00268-008-9840-1.

94. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T*, et al.* A DNA damage checkpoint response in telomere-initiated senescence. Nature **2003**;426(6963):194-8 doi 10.1038/nature02118.

95. Chang YH, Lee CP, Su MT, Wang JT, Chen JY, Lin SF*, et al.* Epstein-Barr virus BGLF4 kinase retards cellular S-phase progression and induces chromosomal abnormality. PLoS One **2012**;7(6):e39217 doi 10.1371/journal.pone.0039217.

96. Xu L, Li S, Stohr BA. The role of telomere biology in cancer. Annu Rev Pathol **2013**;8:49-78 doi 10.1146/annurev-pathol-020712-164030.

97. Denchi EL, de Lange T. Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. Nature **2007**;448(7157):1068-71 doi 10.1038/nature06065.

98. Lajoie V, Lemieux B, Sawan B, Lichtensztejn D, Lichtensztejn Z, Wellinger R*, et al.* LMP1 mediates multinuclearity through downregulation of shelterin proteins and formation of telomeric aggregates. Blood **2015**;125(13):2101-10 doi 10.1182/blood-2014-08-594176.

99. Barzilai A, Yamamoto K. DNA damage responses to oxidative stress. DNA Repair (Amst) **2004**;3(8-9):1109-15 doi 10.1016/j.dnarep.2004.03.002.

100. Tanaka T, Halicka HD, Huang X, Traganos F, Darzynkiewicz Z. Constitutive histone H2AX phosphorylation and ATM activation, the reporters of DNA damage by endogenous oxidants. Cell Cycle **2006**;5(17):1940-5 doi 10.4161/cc.5.17.3191.

101. Masutomi K, Possemato R, Wong JM, Currier JL, Tothova Z, Manola JB*, et al.* The telomerase reverse transcriptase regulates chromatin state and

DNA damage responses. Proc Natl Acad Sci U S A **2005**;102(23):8222-7 doi 10.1073/pnas.0503095102.

102. Shawi M, Chu TW, Martinez-Marignac V, Yu Y, Gryaznov SM, Johnston JB*, et al.* Telomerase contributes to fludarabine resistance in primary human leukemic lymphocytes. PLoS One **2013**;8(7):e70428 doi 10.1371/journal.pone.0070428.

103. Tahtouh R, Azzi AS, Alaaeddine N, Chamat S, Bouharoun-Tayoun H, Wardi L*, et al.* Telomerase inhibition decreases alpha-fetoprotein expression and secretion by hepatocellular carcinoma cell lines: in vitro and in vivo study. PLoS One **2015**;10(3):e0119512 doi 10.1371/journal.pone.0119512.

104. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. Nat Rev Genet **2007**;8(5):353-67 doi 10.1038/nrg2091.

105. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn **1995**;203(3):253-310 doi 10.1002/aja.1002030302.

106. Parichy DM, Elizondo MR, Mills MG, Gordon TN, Engeszer RE. Normal Table of Post-Embryonic Zebrafish Development: Staging by Externally Visible Anatomy of the Living Fish. Dev Dyn **2009**;238(12):2975-3015 doi 10.1002/dvdy.22113.

107. Ackermann GE, Paw BH. Zebrafish: a genetic model for vertebrate organogenesis and human disorders. Front Biosci **2003**;8:d1227-53.

108. Goessling W, North TE, Zon LI. New waves of discovery: modeling cancer in zebrafish. J Clin Oncol **2007**;25(17):2473-9 doi 10.1200/jco.2006.08.9821.

109. Veinotte CJ, Dellaire G, Berman JN. Hooking the big one: the potential of zebrafish xenotransplantation to reform cancer drug screening in the genomic era. Dis Model Mech **2014**;7(7):745-54 doi 10.1242/dmm.015784.

110. Carneiro MC, de Castro IP, Ferreira MG. Telomeres in aging and disease: lessons from zebrafish. Dis Model Mech **2016**;9(7):737-48 doi 10.1242/dmm.025130.

111. Carneiro MC, Henriques CM, Nabais J, Ferreira T, Carvalho T, Ferreira MG. Short Telomeres in Key Tissues Initiate Local and Systemic Aging in Zebrafish. PLoS Genet **2016**;12(1):e1005798 doi 10.1371/journal.pgen.1005798.

112. Anchelin M, Murcia L, Alcaraz-Perez F, Garcia-Navarro EM, Cayuela ML. Behaviour of telomere and telomerase during aging and regeneration in zebrafish. PLoS One **2011**;6(2):e16955 doi 10.1371/journal.pone.0016955.

113. Lau BW, Wong AO, Tsao GS, So KF, Yip HK. Molecular cloning and characterization of the zebrafish (Danio rerio) telomerase catalytic subunit (telomerase reverse transcriptase, TERT). J Mol Neurosci **2008**;34(1):63-75 doi 10.1007/s12031-007-0072-x.

114. Imamura S, Uchiyama J, Koshimizu E, Hanai J, Raftopoulou C, Murphey RD*, et al.* A non-canonical function of zebrafish telomerase reverse transcriptase is required for developmental hematopoiesis. PLoS One **2008**;3(10):e3364 doi 10.1371/journal.pone.0003364.

115. Xie M, Mosig A, Qi X, Li Y, Stadler PF, Chen JJ. Structure and function of the smallest vertebrate telomerase RNA from teleost fish. J Biol Chem **2008**;283(4):2049-59 doi 10.1074/jbc.M708032200.

116. Henriques CM, Carneiro MC, Tenente IM, Jacinto A, Ferreira MG. Telomerase is required for zebrafish lifespan. PLoS Genet **2013**;9(1):e1003214 doi 10.1371/journal.pgen.1003214.

117. Anchelin M, Alcaraz-Perez F, Martinez CM, Bernabe-Garcia M, Mulero V, Cayuela ML. Premature aging in telomerase-deficient zebrafish. Dis Model Mech **2013**;6(5):1101-12 doi 10.1242/dmm.011635.

118. Li Y, Tergaonkar V. Noncanonical functions of telomerase: implications in telomerase-targeted cancer therapies. Cancer Res **2014**;74(6):1639-44 doi 10.1158/0008-5472.can-13-3568.

119. Pruvot B, Jacquel A, Droin N, Auberger P, Bouscary D, Tamburini J*, et al.* Leukemic cell xenograft in zebrafish embryo for investigating drug efficacy. Haematologica **2011**;96(4):612-6 doi 10.3324/haematol.2010.031401.

120. Corkery DP, Dellaire G, Berman JN. Leukaemia xenotransplantation in zebrafish--chemotherapy response assay in vivo. Br J Haematol **2011**;153(6):786-9 doi 10.1111/j.1365-2141.2011.08661.x.

121. Haldi M, Ton C, Seng WL, McGrath P. Human melanoma cells transplanted into zebrafish proliferate, migrate, produce melanin, form masses and stimulate angiogenesis in zebrafish. Angiogenesis **2006**;9(3):139-51 doi 10.1007/s10456-006-9040-2.

6. Abbreviations

Ab	antibodies
ALT	alternative lengthening of telomere
APB	ALT-associated PML bodies
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related
BL	Burkitt lymphoma
bp	base pair
BSA	bovine serum albumin
CHK1	checkpoint kinase 1
CHK2	checkpoint kinase 2
CLL	chronic lymphocytic leukemia
CTE	C-terminal extension domain
CY	cyclophosphamide
DDR	DNA damage response
DFS	disease free survival
DMSO	dimethylsulfoxide
DSB	double strand break
EBV	Epstein-Barr virus
EMT	epithelial-to-mesenchymal transition
ENU	N-Ethyl-Nitrosourea
FBS	fetal bovine serum
FISH	fluorescence in situ hybridization
FLU	fludarabine
h	hours
hpf	hours post-fertilization
HPV	human papilloma virus
HR	homologous recombination
IC50	half-maximal inhibitory concentration
IF	immunofluorescence
IHC	immunohistochemistry
LCL	lymphoblastoid cell lines
LMP1	latent membrane protein 1

MFI mean fluorescence intensity

Min minutes

MMPs matrix metalloproteases

MO morpholino

Mt mitochondria

NER nucleotide excision repair

NF-kB nuclear factor kappa b

NHEJ non-homologous end joining

nt nucleotides

TEN N-terminal domain

OS overall survival

PBS phosphate-buffered saline

PFA paraformaldehyde

PI propidium iodide

POT1 protection of Telomere 1

RAP repeat addition processivity

RAP1 repressor/activator protein 1

RdRP RNA-dependent RNA polymerase

RT reverse transcriptase domain

RISC RNA induced silencing complex

RMRP mitochondrial RNA processing endoribonuclease

RNR-R2 ribonucleotide reductase

ROS reactive oxygen species

RPA replication protein A

s seconds

S.D. Standard Deviation

shRNA short hairpin RNA

siRNA small interfering RNA

ss single strand

ds double strand

TERRA telomere repeat-containing RNAs

TERT telomere reverse transcriptase

TIF telomere dysfunction-induced foci

TIN2	TRF1- and TRF2-Interacting Nuclear Protein 2
TNF-α	tumor necrosis factor-α
TRAP	telomeric repeat amplification protocol
TRBD	TR-binding domain
TRF	terminal restriction fragment
TRF1	Telomere Repeat Factor 1
TRF2	Telomere Repeat Factor 2
wt	wild type

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8. Publications

Celeghin A, Giunco S, Freguja R, **Zangrossi M**, Nalio S, Dolcetti R, De Rossi A. Short-term inhibition of TERT induces telomere length-independent cell cycle arrest and apoptotic response in EBV-immortalized and transformed B cells. *Cell Death Dis*. 2016;7:e2562.

Giunco S, Petrara MR, **Zangrossi M**, Celeghin A, De Rossi A. Extra-telomeric functions of telomerase in the pathogenesis of Epstein-Barr virus-driven B-cell malignancies and potential therapeutic implications. *Infect Agent Cancer*. 2018;13:14.