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MODULATION OF HUMAN TELOMERASE ACTIVITY BY NUCLEOSIDE AND NUCLEOTIDE ANALOGUES

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Declaration

I hereby declare that this thesis summarizes my own work carried out since my registration for Ph.D. studies in 2000 and that it has not been previously included in a thesis submitted to Charles University, Prague, or to other intitutions for a degree.

Miroslav Hájek

Signed ……

TABLE OF CONTENTS

INTRODUCTION

1. Telomeres

Telomeres are the specialized nucleoprotein structures that form protective caps at the natural termini of linear eukaryotic chromosomes [1]. They allow the cell to distinguish the natural ends of linear chromosomes from intrachromosomal double-stranded breaks (DSBs) and thereby prevent end-to-end fusions or nucleolytic degradation [2-4]. Uncapped chromosome ends are prone to being recognized as DNA breaks, which could trigger inappropriate DNA repair processes such as homologous recombination (HR) and non-homologous DNA end-joining (NHEJ) [5,6]. These activities threaten the integrity of chromosome ends and result in extensive genomic instability. In healthy somatic cells, critically short (dysfunctional) telomeres activate a DNA damage checkpoint that induces either apoptosis or the proliferative arrest (replicative senescence), depending on the cell type and cell's genetic background [2,7,8].

In addition to this physical protection, eukaryotic telomeres have important roles in cellular processes including control of cell proliferation and chromatin organization [9]. Telomeres attach the chromosome ends to the nuclear matrix in all stages of interphase [5,10-13]. They also play an important role in the chromosomal pairing and segregation in both meiosis and mitosis [14-16]. Telomeres may also exert effects on subtelomeric gene transcription [17] and interact with cell cycle regulatory mechanisms [18,19].

To sum it up, these terminal caps are essential for maintaining genomic stability and conservation of genetic information. Without functional telomeres, genetic information can get lost, rearranged, or become unstable [20-23]. Changes in their structure and function occur during cancer development and aging [24,25].

1.1. Telomere composition and structure

1.1.1. Telomeric DNA

Almost 70 years ago the geneticists Hermann Joseph Müller and Barbara McClintock recognized the importance of telomeres (from the Greek, *telo* = end, and *mere* = part) for the protection of the genome in their studies in *Drosophilia melanogaster* and maize, respectively [26,27]. The telomeric DNA sequence, $(TTGGGG)_n$, was characterized for the first time in the ciliated protozoan *Tetrahymena thermophila* by Blackburn and Gall in 1978 [28]. Since that time it has been discovered that the telomere is a complex structure composed of both DNA and associated proteins [13,29]. Telomeric sequences are organism-specific [30,31]. In humans and other vertebrates, the telomeric DNA consists of tandem double-stranded repetitions of the hexameric sequence 5'-TTAGGG-3' [32]. The G-rich repeat strand is 50 - 400 nucleotides longer than the complementary 3'-AATCCC-5' strand, resulting in a single-stranded 3' overhang found at both chromosomal ends [33]. 3' overhangs result from both the "end replication problem" (the inability of DNA polymerase to replicate fully the very end of the telomeres) and postreplication processing [34,35]. They are a critical component of the telomere-end structure. Their importance exceeds simple telomere capping functions since they establish telomere shortening rates and influence replicative senescence [36,37]. The proximal 1-2 kilo base pairs (kb) of human telomeres contain complex patterns of TTAGGG repeats interspersed with telomere repeat variants such as TGAGGG and TCAGGG [38].

In vertebrates, telomeres do not contain genes and TTAGGG repeats are bound by a specialized multiprotein complex, known as "shelterin" or "telosome", which has fundamental roles in the regulation of telomere length and protection of telomeres [13]. Proper telomere functioning requires both a minimum length of TTAGGG repeats and the integrity of the shelterin complex [13,39,40]. The length and the structural organization of telomeres vary according to chromosomes and species, e.g. human telomeres are remarkably variable [41]. In normal human somatic cells, the mean telomere length at birth varies from 5 to 20 kb, but is as small as 1-2 kb in rapidly growing cancer cells. Moreover, telomere lengths show wide inter-individual variation. They also vary among cells in the same tissue and among chromosomes within the same cell [42,43] and, strikingly, between alleles at the same telomere [44]. Nevertheless, the

number of TTAGGG repeats on specific chromosome arms is very similar in different tissues from the same individual and varies only to some extent between individuals [45].

1.1.2. Telomere-associated proteins

As mentioned above, a multiprotein complex called shelterin associates with telomeric DNA and fulfills the main functions of telomeres: the protection of chromosome end, the regulation of telomere length, and the recruitment and regulation of telomerase [13,46,47]. Pertubations in the shelterin complex and its components would result in the disruption of telomere maintenance and activation of the DNA damage response [22,48].

The mammalian shelterin contains the six core proteins TRF1, TRF2, POT1, RAP1, TIN2, and TPP1 (previously known as TINT1, PTOP, or PIP1). All shelterin components behave as negative regulators of telomere elongation by telomerase [13]. TRF1 (telomeric repeat binding factor 1), TRF2 and POT1 (protection of telomere) have been identified to bind directly and specifically to telomeric DNA (Fig. 1) [49-51]. TRF1 and TRF2 each form homodimers that bind to double-stranded telomeric DNA [49,50], whereas POT1 binds specifically to the single-stranded TTAGGG 3' overhang [52,53]. TRF1 (and its interacting partners) can limit telomere elongation in *cis* [54]. In contrast, TRF2 and POT1 appear to be particularly important for stabilizing the telomeric structure and protecting telomeres from degradation and end-to-end chromosomal fusions [52,55-59]. These two shelterin components act independently to repress two DNA damage response pathways specifically at telomeres. TRF2 represses the DNA damage sensing protein ATM, whereas POT1 prevents activation of ATR kinase signalling [60-66]. Both TRF1 and TRF2 recruit various proteins at telomeres which are involved in telomere length control and telomere protection, respectively (Table 1). An interacting partner of TRF1, PINX1, can inhibit telomerase and has been proposed to affect telomere length by altering the telomerase activity throughout the nucleus [67]. It is proposed that retaining hTERT in the nucleolus and inhibition of telomerase catalytic activity may be two independent processes of PINX1 on telomerase regulation in human cells [68,69]. The other member of shelterin complex TIN2 (TRF1-interacting nuclear factor 2) is an important scaffold molecule that links and coordinates

other telomeric proteins including TRF1 and TRF2 [48,70]. Additionally, TIN2 contributes to the regulation of telomere length by modulation of the poly(ADP-ribose) polymerase (PARP) activity of tankyrase 1 in the TRF1 complex [71]. When TIN2 is inhibited *in vivo*, TRF1 appears more sensitive to the endogenous tankyrase 1, and telomere elongation occurs. Due to its ability to bind both TRF1 and TRF2, TIN2 is a key player in telomere chromatin formation [70,72-74], and together with TPP1 mediates the shelterin assembly [48]. By means of its interaction with TIN2, TPP1 promotes the TIN2-TRF2 binding and stimulates the TRF1-TIN2-TRF2 complex formation [48]. Furthermore, TPP1 binds POT1 and is thought to bridge POT1 to TIN2 and ultimately to the TRF1 complex (Fig. 1) [75]. Thus, TIN2-TPP1 recruits POT1 as a terminal effector of TRF1 [76]. Interactions between TRF1 complex and POT1 affect the loading of POT1 on the single-stranded telomeric DNA and are crucial for telomere length control [75,76]. POT1, like the TRF1 complex, could function as a protein-counting device to measure telomere length. Longer telomeres contain more TRF1 and POT1. Thus, POT1 transduces information on the telomere length to the telomere terminus, where the regulation of telomerase takes place [76]. POT1 can either preclude telomerase from accessing the telomere ends by its physical presence at the 3' end or stabilize the t-loops by binding to the displaced TTAGGG repeats (D-loop; displacement loop) and thereby block telomerase from gaining access to the 3' telomere terminus [46,77]. Another shelterin member involved in telomere length regulation, RAP1, does not bind to telomeric DNA directly but is recruited to telomeres by TRF2 [78]. RAP1 affects telomere length heterogeneity and has been proposed to act as a negative regulator of telomere length [79].

Other telomere-associated proteins that bind to telomeric DNA indirectly through TRF1 and TRF2 have also different functions, in addition to their presumed roles at the telomeres (Fig. 1). Tankyrase 1 and 2 (TANK1/2), two non-classical, highly related telomeric poly(ADP-ribose) polymerases (PARPs), enzymes commonly associated with DNA repair and maintenance of chromosome stability [80,81], also interact with TRF1 [82,83]. Poly(ADP-ribosyl)ation of TRF1 by tankyrase 1 leads to the loss of its DNA binding activity [76,83] and subsequent degradation of TRF1 [84]. This suggests that tankyrase 1 may attenuate the effect of telomerase inhibitors by accelerating access of residual telomerase activity to telomeres [85]. Conversely,

tankyrase inhibitors have been shown to enhance telomere shortening caused by telomerase inhibitors in telomerase-positive cells [86]. Thus, targeting of tankyrases has emerged as a potential target for cancer therapy in conjunction with inhibitors of telomerase [87]. Tankyrases might also participate in repairing and/or signaling the occurrence of dysfunctional telomeres. Several other DNA repair proteins were recently found at telomeres. Both TRF1 and TRF2 interact with Ku (the 70 kDa Ku subunit) [88,89], the DNA end-binding component of DNA-dependent protein kinase (DNA-PK), which is essential for DSB repair by nonhomologous end-joining (NHEJ) [90,91]. Thus, DNA-PK, in addition to its role in DNA repair, may also play a role in telomere maintenance. This idea is supported by the data showing that DNA-PK, together with TRF2, is required for strand-specific processing of the telomeres after they are replicated in S-phase [57]. Likewise, RAD50, MRE11 and NBS1 (RMN), another important DNA repair complex, which is crucial for homologous recombinational repair (HR) and may also participate in NHEJ [92], associates with mammalian telomeres, at least during S-phase. It is probably due to an interaction between NBS1 and TRF1 [93] and TRF2 [66]. TRF2 also interacts with WRN helicase [94], the protein that appears to participate in both the NHEJ and the HR DNA repair pathways [95-98].

1.1.3. Telomere structure

Using electron microscopy it was revealed that telomeres are not linear but were shown to end in a large tailed loop resembling a lasso, known as the t-loop [99,100]. It has been proposed that the 3' overhang can fold back and invade the double-stranded region of telomeres forming this protective t-loop [99,100] while the displacement is called D-loop (Fig. 1) [101]. Telomeric t-loops have been isolated from mice, humans and protozoa, and may be an evolutionarily conserved structure [99,102].

The formation of telomeric t-loops is critically dependent on TRF2 [99,103]. In addition, the formation and/or maintenance of this protective structure may be facilitated or stabilized by other telomere-associated proteins, particularly TRF1 and TIN2 [70,99,104]. Based on its biochemical features, POT1 should have the ability to bind to the displaced TTAGGG repeats at the base of the t-loop (the D-loop) [77]. The binding of POT1 to the D-loop could potentially stabilize t-loops (by preventing branch-

migration) [105]. Disruption of the t-loop is thought to signal a cellular response that, in at least some regards, resembles the cellular response to DSB in the genome. It has been proposed that t-loops represent the basic mechanism by which the telomeric nucleoprotein complex sequesters the natural ends of chromosomes from DNA damage checkpoints, DNA repair enzymes, and telomerase [22,29,100].

Mammalian telomeres have epigenetic marks of constitutive heterochromatin; however, the telomeric chromatin is functionally and structurally distinct from bulk nuclear chromatin. Most of the telomeric DNA is organized into nucleosomes with dense spacing typical for heterochromatin, whereas the shelterin structure is present at the very ends of the chromosomes [106,107]. DNA linker length is usually ~40 bp shorter than that in bulk chromatin [108]. Interestingly, the telomeric DNA is excluded from nucleosomal cleavage during apoptosis [109]. It was shown that telomeric nucleosomes are intrinsically mobile and are less stable than non-telomeric nucleosomes [110]. This seems to be a feature of the telomeric repeat sequence TTAGGG. Furthermore, the binding of TRF1 and, to a lesser extent, TRF2 is able to remodel telomeric nucleosomes. The binding of TRF1 was suggested to induce the movement of nucleosomes and nucleosome-nucleosome interactions [110]. Thus, nucleosomes contribute to the establishment of a telomeric capping complex, whose structure and dynamics can be modulated by the binding of telomeric factors [111]. Additionally, loss of telomeric repeats leads to a change in the architecture of telomeric and subtelomeric chromatin consisting of loss of heterochromatic features leading to a more “open“ chromatin state [112].

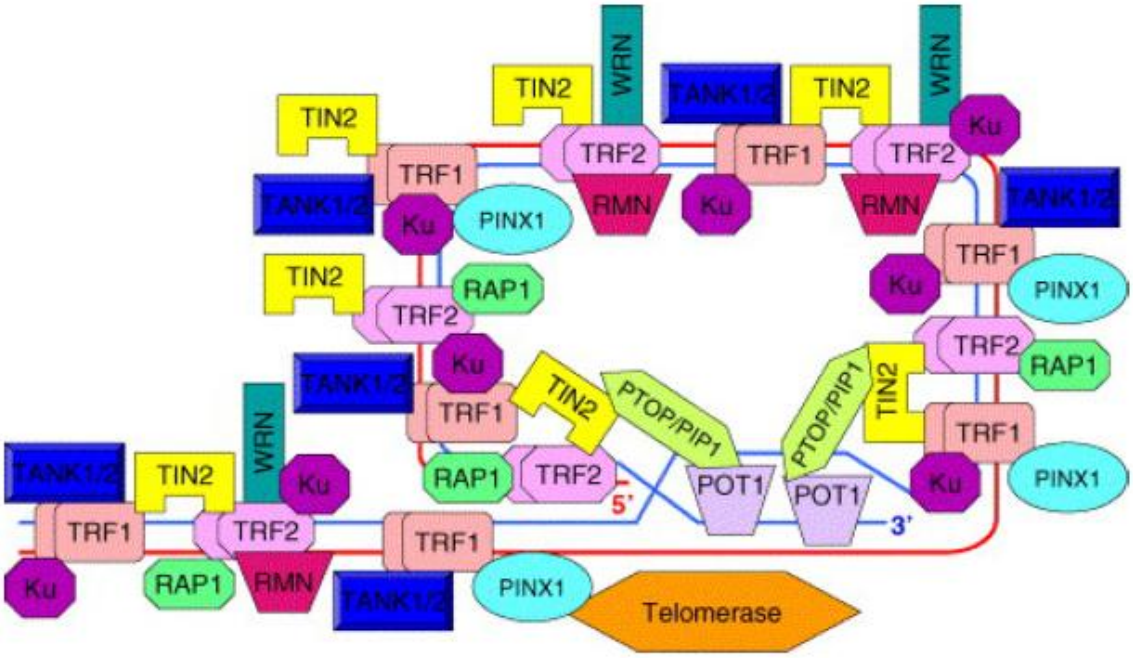


Figure 1. **Telomere capping structure.** Scheme showing the telomere in a t-loop conformation, as well as with different protein complexes found at mammalian telomeres. The 3' overhang folds back and invades the double-stranded region of the telomere forming the protective t-loop structure. The core telomere structure recruits other essential proteins that do not interact directly with telomeric DNA but are important for telomere capping: TIN2, RAP1, PINX1; TPP1 (= PTOP/PIP1). The telomere capping structure recruits many other proteins, such as tankyrases and DNA repair proteins that also have specific functions elsewhere in the cell. The TRF1 complex has been shown to influence the telomere length, while the TRF2 complex has been shown to influence both the telomere length and telomere capping [20].

Table 1. Telomere-associated proteins identified in mammalian cells

Protein	Function	References
TRF1	Binds double-stranded TTAGGG repeats; present in t-loops; negative regulator of telomere length; an excess inhibits telomere elongation; promotes pairing of telomeres	49,99, 113-116
TRF2	Binds double-stranded TTAGGG repeats; present in t-loops; has an essential role in telomere protection; plays an important role in the t-loop formation; negative regulator of telomere length; an excess inhibits telomere elongation; a loss of function leads to p53- and ATM-mediated apoptosis and senescence; possibly involved in strand-specific processing after S-phase; the TRF2 inhibition also results in a reduction in the 3' overhang signal and NHEJ-dependent chromosome-end fusions	4,50,55-57, 99,101,105, 115,117-119
POT1	An overhang binding protein-binds specifically to the single-stranded G-rich telomeric sequence; once bound to telomeres, POT1 is then able to exert its negative control on the telomerase activity; necessary for the telomere length maintenance and telomere protection	52, 53,75-77, 105,120-125,
RAP1	TRF2-binding protein; acts as a negative regulator of telomere length; plays a role in the regulation of telomere length distribution	78,79
TIN2	Simultaneously interacts with both TRF1 and TRF2, and is considered a key player in crosstalks between the TRF1 and TRF2, regulates POT1-TRF1 interaction and acts as a negative regulator of telomere length	70-73, 104,126,
TPP1 (also PTOP, PIP1, TINT1)	A negative regulator of telomere length, recruits POT1 as a terminal effector of the TRF1 complex; binds both POT1 and TIN2 *via* two distinct domains; is thought to bridge POT1 to TIN2 and ultimately to TRF1 complex	53,75,123

Tankyrase 1	Inactivates TRF1 through poly(ADP-ribosyl)ation *in vitro*; a positive regulator of telomere length	83,85, 127-129
Tankyrase 2	TRF1 binding protein; function not clear	82,127
Rif1	Implicated in the DNA damage response following DSBs and in the intra S-phase checkpoint; only associated with telomeres after induction of telomere dysfunction	130
DNA-PKcs and the Ku70 and Ku 80 proteins	Involved in DNA double-strand repair by NHEJ and HR; possibly involved in strand-specific postreplicative processing of telomeres; role in telomere capping; Ku proteins interact with TRF1 and TRF2; loss of Ku leads to aberrant telomere-telomere fusions, disruption of nuclear organization of telomeres and loss of telomeric silencing	57,88,89,91, 131-137
Ku 86	A negative regulator of telomere length; a role in telomere capping	65,138
PINX1	TRF1-TIN2 - interacting protein; implicated as a telomerase inhibitor; putative tumor suppressor	67,68
ERCC1/XPF	Structure-specific endonuclease involved in the nucleotide exchange repair; required for the removal of the 3' overhang from uncapped telomeres and for telomere fusion after TRF2 inhibition	139
MRE11/RAD 50/NBS1 (RMN)	DNA-repair complex that binds to telomeres; association with telomere mediated by TRF1 and TRF2; possible role in t-loop formation	66,93
BLM and WRN	Members of the RecQ family of DNA helicases; directly interact with TRF2, implicated in telomere structure/ function; role not clear	140, 141

1.2. Mechanism and consequences of telomere erosion

Telomere dysfunction can result from direct damage [142-144], defects in telomere maintenance proteins (Table 1), and/or a progressive decline in telomere length that occurs during each cycle of cell division [22]. As a consequence of the end replication problem [34], each cell division results in a loss of 50 to 200 bp of 3' telomeric DNA in normal human somatic cells (Fig. 2) [35,85,145-149]. Because a minimal telomere length is essential for chromosomal integrity and replication, short telomere lengths limit the replicative lifespan of cells. Thus, normal somatic cells can only undergo a limited number of cell divisions, known as the Hayflick limit [150] and are interrupted in their proliferation irreversibly when a critically short telomere length is reached [151]. Hence, telomere shortening may act as a ‘‘molecular‘‘ or ‘‘mitotic clock’’ that counts the number of cell divisions and determines the onset of replicative senescence (a cellular growth arrest, also called the mortality stage 1 - M1 stage) (Fig. 2) [35,145,152-154]. However, there are also other possible outcomes of telomere dysfunction. For example, some cell types, or some cells under certain physiological conditions, may die rather than senesce in response to telomere disruption [56]. It is likely that genetic background dictates how a cell will respond to a dysfunctional telomere.

Critically short telomeres or telomeres lacking some telomere-binding proteins lose their functionality and directly elicit a DNA damage checkpoint response [155-158]. In general, normal cells, with intact p53 and pRb checkpoints, respond to a dysfunctional telomere by undergoing an irreversible proliferation arrest, acquiring a characteristic enlarged morphology and a variety of altered functions. This response has been termed replicative senescence (M1 stage) as mentioned above (Fig. 2) [159]. Several lines of evidence suggest that telomere shortening and subsequent senescence response in the absence of other alterations may be a potent tumor-suppressor mechanism, preventing the proliferation of cells at risk for neoplastic transformation [25,160]. If only the p53 checkpoint is intact, telomere dysfunction generally promotes p53-mediated cell death [56,161]. When both p53 and pRb pathways are inactive, most human cells bypass M1 senescence and proliferate until the telomeres become extremely short. This excessive shortening eventually results in crisis (also called the M2 stage) (Fig. 2) [162]. Telomere crisis is triggered by the shortest telomere but not by

the mean telomere length [163]. Cells in crisis are characterized by many "uncapped" chromosome ends, end-to-end fusions, and a high fraction of apoptotic cells [13,164]. However, cells may survive with genomic rearrangements and instability that is considered to be a precursor to cancer [165]. In a rare M2 cell, telomerase (a cellular reverse transcriptase; chapter 2 in Introduction) can be reactivated or up-regulated, resulting in indefinite cell proliferation (Fig. 2) [149,166].

As mentioned (chapter 1.1.3. in Introduction), telomere shortening leads to changes in histone modifications (decreased H3K9 and H4K20 trimethylation, increased histone H3 and H4 acetylation) at both telomeric and subtelomeric chromatin, as well as decreased DNA methylation at subtelomeric domains [112]. Loss of DNA methylation at subtelomeric regions is accompanied by increased telomere recombination [167]. Thus, loss of heterochromatin formation at telomeres and subtelomeres could facilitate telomerase-independent pathway of telomere maintenance referred to as "alternative lengthening of telomeres" (ALT) (chapter 1.4. in Introduction) [168].

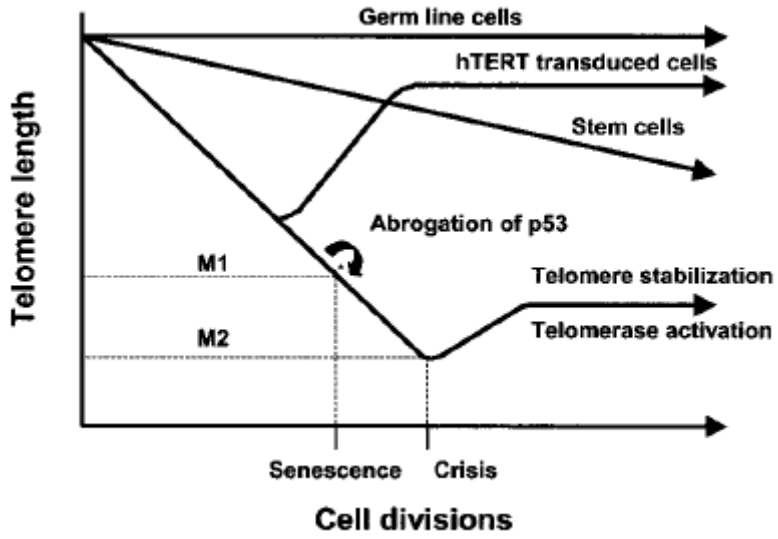


Figure 2. **Schematic view on the telomere hypothesis of cellular aging and immortalization.** The relationship between cell division (and time) and telomere length is presented. Unlike germ cells, in which telomere length is maintained by telomerase, most human somatic cells have lower levels of telomerase or are telomerase-negative and experience telomere shortening with each cell division. Pluripotent stem cells are telomerase-positive but do not maintain full telomere length. Telomere length shortens

in stem cells at rates slower than that of telomerase-negative somatic cells. Critically shortened telomeres may signal cells to enter replicative senescence at the Hayflick limit, or M1. This proliferative checkpoint can be bypassed through inactivation of pRb or p53. Such cells continue to suffer telomere erosion and ultimately enter crisis, or M2, characterized by massive cell death. Rare surviving cells acquire unlimited proliferative potential and stabilization of telomere length, almost universally by activation of telomerase (chapter 2 in Introduction) [169].

1.3. Telomere length measurement

There are a number of techniques available for measuring telomere length in cells, each with distinct advantages and disadvantages in terms of the information obtained, the amount of material needed, the time necessary to complete the test, the amount of labour involved and the accuracy of results.

The terminal restriction fragment (TRF) analysis [146] is probably the most widely used in telomere research. In this Southern blot based method, the telomere length is usually reported as a mean length of the TRFs which include the telomeres and part of subtelomeric regions. This multi-step method involves (a) cleaving purified nontelomeric DNA with a combination of frequent-cutting restriction enzymes (such as RsaI and HinfI), (b) separating the DNA fragments by size on an agarose gel, (c) denaturing and transferring the DNA fragments to a membrane, (d) hybridizing the telomere with a radioactive [^{32}P]-labeled $(CCCATT)_3$ telomere probe, (e) removing the unhybridized probe by washing the membrane, and (f) analyzing the data by autoradiography and image analysis. The telomere length is estimated from the resultant autoradiograph by densitometric analysis. Since restriction enzymes cut the DNA in the subtelomeric regions, the length of the subtelomeric DNA (which is estimated to be 2-4 kb [41] is included to a certain extent in the estimated telomere restriction fragment length. This fact represents one of the major drawbacks of this method. There may exist significant variations between individuals in the location of subtelomeric restriction enzyme cleavage sites, indicating that subtelomeric restriction site polymorphisms and/or subtelomeric length polymorphisms may occur [170]. Thus, individuals that have

the same mean length of the terminal hexameric repeat arrays may nevertheless have very different mean TRF lengths. Moreover, a determination of the TRF length value can be greatly affected by the choice of restriction enzyme(s) used to release the terminal restriction fragments (different chromosomes have different restriction sites relative to telomeric ends) [171].

TRF assays produce autoradiographic smears which have some element of subjectivity in their analyses [172]. In addition, the TRF method requires relatively large amounts of DNA (1-5 μg) and is time consuming (3-5 days). In fact, the assay is relatively cumbersome and its complexity precludes its use for large-scale analysis [173]. The other disadvantages of this method are a loss of DNA during membrane blotting, high background due to non-specific binding of telomere probe to membrane, and a loss of telomeric signal due to extensive washing. These limitations result in a low signal-to-noise ratio and, therefore, reduced sensitivity and reproducibility. Because of the problems mentioned above, the Southern blot is not fully suitable for quantitative measurements, and is usually considered adequate only for the analysis of the relative telomere length (e.g. changes with time or due to drug treatment).

A related method called **telomere amount and length assay (TALA)** [174] is a more quantitative assay based on hybridization in solution that does not require membrane blotting, prehybridization, and washing. Thus, it eliminates the problems associated with the need of immobilizing DNA on a solid support and the high background due to the non-specific binding of probes to the membrane as in the TRF analysis. The major steps are (a) DNA preparation and digestion with restriction endonucleases, (b) hybridization between DNA and telomeric probe, (c) agarose gel electrophoresis, and (d) autoradiography and data analysis. TALA was reported to be a more rapid assay with a higher sensitivity and reproducibility when compared to the TRF analysis. Both methods show comparable results of the telomere length measurement.

Single Telomere Length Analysis (STELA) [44] is a sensitive PCR-based approach that enables accurate measurement of the actual length of telomere repeats at a specific chromosome arm, such as the Xp/Yp telomere for up to several hundred individual chromosomes. This method requires knowledge of unique sequences in the subtelomeric region of the studied chromosome. Then PCR can be performed using a

combination of a reverse primer located at the end of the telomere and a chromosome-specific forward primer located in the subtelomeric region, which also can be made allele-specific. Thus, using single-allele amplification of telomeres we can even reveal the telomere length differences between homologous chromosomes. STELA was first described for the telomere at the end of the short arms of the human sex chromosomes (the XpYp telomere) in normal fibroblasts; however, it can be applied to other chromosomes as well.

Another PCR-based strategy uses **real-time quantitative PCR (Q-PCR)** [171] with ingeniously designed primers that hybridize to vertebrate telomeric regions without generating primer dimer derived products. This method measures relative telomere lengths by determining the factor by which a sample DNA differes from a reference DNA sample in its ratio of a telomere repeat copy number (T) to a single-copy gene copy number (S) (i.e. relative T/S ratio). In this approach the telomere signal is normalized to the signal from a single-copy gene in order to generate a T/S ratio which is proportional to the mean telomere length. The T/S ratio of one individual relative to the T/S ratio of another one should reflect relative telomere length differences of their DNA. The assay is simple, rapid and accurately measures the telomere length using small amounts of DNA. Furthermore, it is useful for determining interindividual differences in the telomere length within species and may also be used to measure the relative telomere length among species if those species share the same single-copy reference sequence.

Fluorescence *in situ* hybridization (FISH) has been applied to visualize telomeres. This technique was eventually used with a quantitative approach to measure the fluorescence signal and to estimate the telomere length [42,175].

Quantitative fluorescence *in situ* hybridization (Q-FISH) [42] measures the telomere length on individual metaphase chromosomes by image microscopy using directly labeled $(CCCTAA)_3$ peptide nucleic acid (PNA) probes [176]. This method is based on the principle that, at a low ionic strength, PNA (but not single-stranded DNA) can anneal to complementary single-stranded DNA sequences. Quantitative hybridization to telomere repeats is achieved using conditions that only allow labeled $(CCCTAA)_3$ PNA to hybridize to $(TTAGGG)_n$ target sequences. The use of PNA probes in Q-FISH leads to stable and reproducible results [42,43,45]. Q-FISH provides

measurements of the telomere lengths of individual chromosomes. On the other hand, only small data sets can be produced, and the requirement for metaphase chromosomes restricts analysis to cells that are still proliferating. Similarly to the TRF method, this technique is elaborate and rather time-consuming, and thus not suitable for routine purposes. With the Q-FISH method, the length of individual chromosomes can be measured in contrast to Southern blotting, where the profile of all chromosomes is analyzed.

Flow cytometry method based on **fluorescence *in situ* hybridization (flow-FISH)** [177,178] with FITC-labeled telomere peptide nucleic acid (PNA) probe. The method is optimal for telomere length estimation, as the fluorescence intensity of the cells is directly correlated to the length of telomeres. Moreover, since the probe does not recognize subtelomeric sequences (in contrast to traditional TRF measurements), it allows an estimation of the telomere length without inclusion of subtelomeres. The data obtained in the flow cytometric analysis can be used to determine the relative telomere length (RTL). The RTL value is calculated as the ratio between the telomere signal of each sample and the control cells (1301 cell line). The 1301 cells have very long telomeres and are tetraploid which enables to distinguish them from the sample cells on the dot plot diagram. Therefore, they provide a convenient reference point for telomere fluorescence measurements. Propidium iodide staining solution is used to identify the $G_{0/1}$ cells as it is important for the evaluation to look only at cells in the $G_{0/1}$-phase of the cell cycle, where the cell has one copy of its genome. Significant improvements in flow-FISH methods have included the partial automation of the procedure, the inclusion of control cells with known telomere length in every tube and limited immuno-phenotyping to measure the telomere length in subpopulations of nucleated blood cells [179].

1.4. Telomere maintenance – ALT mechanism

Immortalized cells maintain telomere length through either a telomerase-dependent process (chapter 2 in Introduction) or a telomerase-independent pathway termed “alternative lengthening of telomeres” (ALT) [180-184]. The exact mechanism by which ALT cells maintain their telomere length is unknown, though several lines of evidence suggest that homologous recombination plays a significant role [184,185].

This recombination-based mechanism is particulary common in bone and soft tissue sarcomas, including osteosarcomas, glioblastomas, and carcinomas of the lung, kidney, breast and ovary [181,186-188]. Lack of expression of the telomerase gene in ALT cells is associated with histone H3 and H4 hypoacetylation and H3K9 methylation [189]. When telomerase is available, for instance in mammalian tumor cells, telomeres are stably maintained within a relatively narrow size distribution. In contrast, ALT-positive cell lines and cancers have a longer mean telomere length and great heterogeneity of telomere size (ranging from almost undetectable to abnormally long) [180,190,191]. Other characteristics of human ALT cells include high rates of telomere exchange [192] and the presence of nuclear structures known as "ALT-associated promyelocytic leukemia bodies" (APBs) [183] that contain promyelocytic leukemia (PML) protein, extrachromosomal telomeric circles (t-circles) [193], telomere-specific binding proteins (TRF1, TRF2), and a variety of recombination proteins including RAD51 and the Werner and Bloom syndrome proteins [141,190,194,195]. RAD50/MRE11/NBS1 (RMN complex) recombination protein complex, and especially NBS1, is also required for the ALT mechanism [194,196,197]. NBS1 is required for the production of t-circles in human ALT. T-circles may be a specific marker of the ALT phenotype [197].

The type of telomere maintenance mechanism used by tumors may have a prognostic significance. For example, patients with ALT-positive high grade glioblastomas have a significantly longer survival than those that are ALT-negative [186]. Regarding treatment, an implication of the ALT existence is that tumors using this telomere maintenance mechanism (including mixed telomerase-positive/ALT-positive tumors) will be resistant to telomerase inhibitors. Repression of ALT in ALT-positive immortalized cell lines results in senescence and cell death [198,199]. This means that ALT, like telomerase, may be an attractive drug target. Combination therapy using ALT and telomerase inhibitors may help to prevent the emergence of drug resistance [183].

However, the mechanism mostly used by human tumor cells as well as several normal cells involves a specialized complex capable of elongating telomeres. This complex is referred to as telomerase.

2. Human telomerase

Telomerase is an RNA-dependent DNA polymerase which represents the most versatile and widely used mechanism of telomere maintenance [169,200]. The enzyme adds specific DNA sequence repeats (5'-TTAGGG-3' in all vertebrates) onto the 3' ends of eukaryotic chromosomes (*de novo*), thereby elongating telomeres (Fig. 3) [32]. In 1985, the telomerase activity was first identified in the ciliate *Tetrahymena thermophila* by Greider and Blackburn [201]. They first named this activity "telomere terminal transferase" for its capacity to elongate telomeric primers in the absence of a DNA template. The enzyme is now known to be almost universally conserved in eukaryotes. Telomerase is a ribonucleoprotein (RNP) complex that carries its own RNA molecule as a template, and has a reverse transcriptase activity [202,203].

In humans, telomerase activity is present in embryonic cells, germline tissues [204], and is up-regulated or reactivated in 85-90% of neoplasias [205-207]. On the contrary, it is undetectable in normal somatic tissues except for proliferative cells of renewal tissues, e.g. hematopoietic stem cells [208,209], activated lymphocytes [209,210], endothelial cells [211], hair follicle cells [212], and cells in the basal layer of the epidermis [213], where telomerase activity is present at low levels, insufficient to maintain the telomere length over many cycles of cell division [214].

It has been reported that the expression of the human telomerase catalytic subunit, hTERT, alone is sufficient to reconstitute telomerase activity, to extend the lifespan of normal human somatic cells and to allow transformed cells to escape from crisis (mortality stage 2; Fig. 2) [215-220]. Telomerase has been shown to preferentially elongate critically short telomeres, stabilize telomere length, prevent overhang loss, and permit continuous cell division [217,218,221,222]. It also protects telomeres from NHEJ [223]. Furthermore, telomerase may have possible protective capping, anti-apoptotic and survival functions [224-228]. The ectopic expression of the hTERT in combination with oncogenes results in direct tumorigenic conversion of normal human epithelial and fibroblast cells [229]. These findings indicate that telomerase plays an important role in cellular aging and tumorigenesis. Importantly, the telomerase holoenzyme complex presents multiple potential sites for the development of inhibitors [230-232]. Understanding of how telomerase functions in human cells is important for developing anti-telomerase therapies.

Telomerase is a very large complex, with a mass over 1000 kDa [233]. However, apparent mass varies with the purification strategy. Human telomerase that is affinity purified under stringent salt conditions has a molecular mass of 600 kDa, consistent with a minimal complex composed of two hTERTs and two hTRs [234]. These two essential subunits are: (*i*) the functional RNA component (in humans called hTR or hTERC) [235], which serves as a template for telomeric DNA synthesis; (*ii*) the other is a catalytic protein subunit (hTERT) with a reverse transcriptase activity [236-240]. Indeed, two molecules each of hTERT and hTR suffice to reconstitute telomerase activity *in vitro*, suggesting that these subunits form the catalytically active core of a more complex holoenzyme [241-244]. In addition, a number of telomerase-associated proteins are present in the complex that are neccessary for the proper functioning of telomerase *in vivo*. They are implicated in ribonucleoprotein assembly, processing, stability, and help telomerase to access the telomeres [230,245-248].

2.1. RNA subunit of telomerase (hTR)

hTR is one of the 2 components essential for the activity of telomerase *in vitro* [216,244,249]. In 1998, the *hTR* gene was cloned and localized on human chromosome 3q26.3 [250]. This single-copy gene does not contain any intron and it is widely expressed in both tumor and non-tumor tissues regardless of telomerase activity [235,251], with cancer cells generally having five-fold higher expression than normal cells [252-254].

The RNA component of telomerase provides the template for telomeric repeat synthesis. In humans, hTR is transcribed by RNA polymerase II and is processed at its 3' end to produce a mature transcript of 451 nucleotides, 7-methylguanosine-capped, which lacks polyA [235]. Near the 5' end of the molecule (nucleotides 46 to 56) lies the 11-nucleotide long template sequence 5'-CUAACCCUAAC-3' (Fig. 4), which is reverse transcribed by protein catalytic subunit (hTERT) to TTAGGG telomeric repeats [255]. In addition to the template (CR1; CR stands for conserved region), other conserved RNA structural elements play important parts in catalysis as well as localization, maturation and assembly of RNP telomerase complex. The predicted secondary structure of vertebrate telomerase RNAs also contains a pseudoknot domain

(CR2/CR3), a CR4/CR5 domain, a Box H/ACA (CR6/CR8) domain characteristic of small nucleolar RNPs (snoRNPs) [256], and a CR7 domain (Fig. 4) [257].

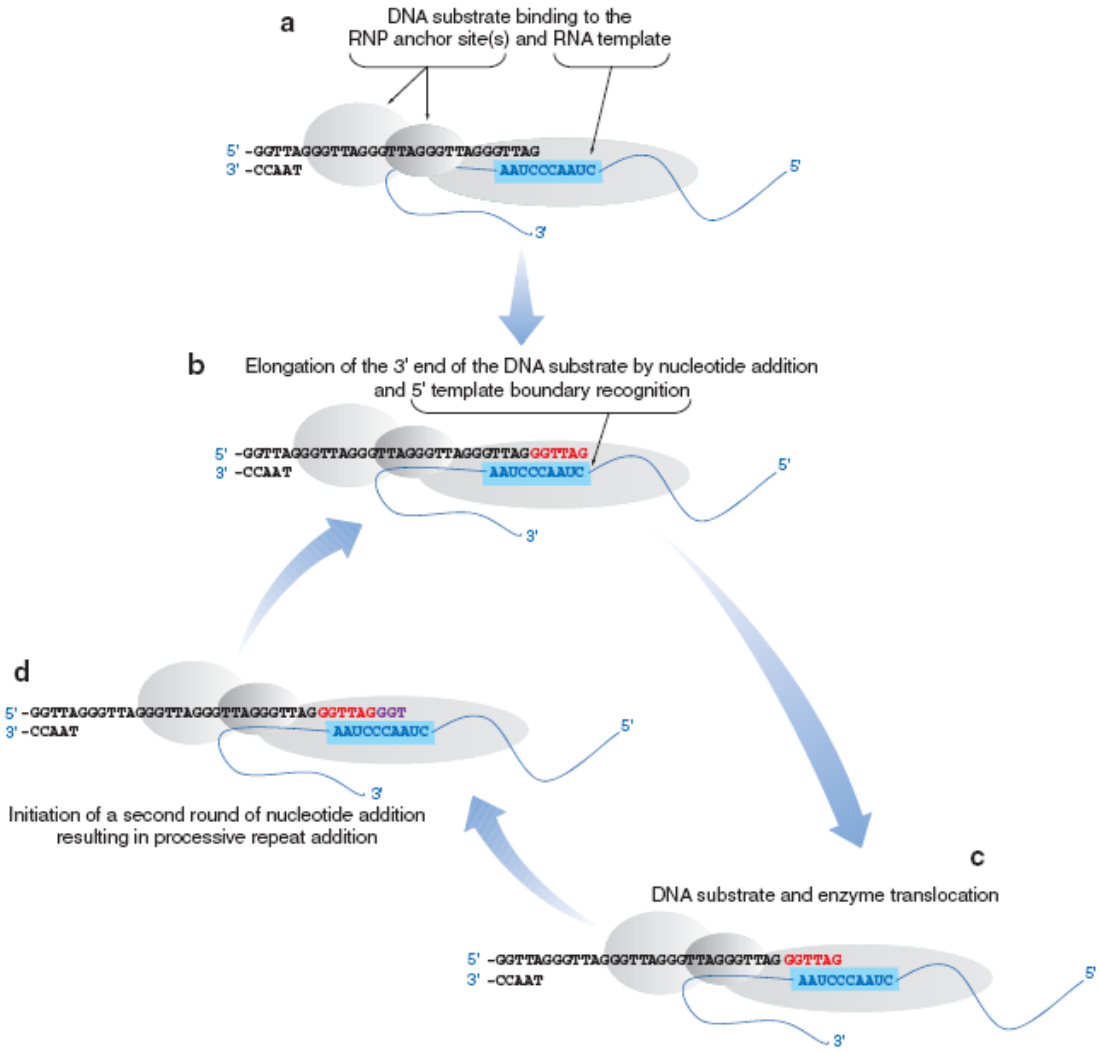


Figure 3. **Model of processive elongation by telomerase.** Processive extension of DNA by telomerase requires a number of steps, as illustrated here for the human enzyme. (*a*) First, the telomeric DNA is recognized by the telomerase ribonucleoprotein (RNP), consisting of at least the TERT protein with the anchor site(s) and the RNA subunit (depicted in *blue*). The 3' end of the DNA forms a hybrid with the RNA template (*boxed in blue*), whereas the more 5' region of the DNA is postulated to interact with the "template-proximal" and "template-distal" anchor site(s). (*b*) Next, template-directed addition of nucleotides to the 3' end of the DNA occurs sequentially until the 5' end of the template is reached. Added nucleotides (*red*) are displayed. (*c*)

Telomerase undergoes the translocation reaction and repositions the 3' end of the DNA in concert with recognition of the 3' template boundary. (*d*) Another round of nucleotide addition is initiated. Added nucleotides (*purple*) are displayed. Reiterative translocation and nucleotide addition result in the addition of multiple repeats. Repeat addition is regulated by RNA-DNA, TERT-DNA, TERT-RNA, and TERT-TERT interactions and possibly by an intrinsic nuclease activity as well as telomerase-associated proteins [200].

Two regions within the hTR molecule, the box H/ACA domain and CR4-CR5 domain, interact independently with the catalytic component of telomerase (hTERT) in a noncooperative manner [243,258]. The H/ACA box is essential for both mature hTR accumulation and and *in vivo* telomerase activity [243,256,259,260]. The CR7 domain in the terminal stem-loop of the 3'-hairpin is essential for RNA accumulation [260]. Finally, the 5' terminal part of hTR folds into a pseudoknot domain which has been also found to play a role in hTR stability [261].

2.2. Protein catalytic subunit of telomerase (hTERT)

In humans, the catalytic subunit hTERT is a specialized type of reverse transcriptase (RT) that copies the accompanying RNA template sequence onto chromosome ends to maintain telomeres. It contains motifs (1 and 2, and A-E) that are common to RT enzymes, as well as N- and C-terminal regions which are not conserved among RTs [200]. While hTR can be found in most tissues, the presence of hTERT is more restricted - it is generally repressed in normal cells and transcriptionally up-regulated in immortal cells [207,237,240]. Thus, the expression of *hTERT* is closely correlated with telomerase activity *in vitro* and *in vivo* [207,240].

The genomic sequence and the *hTERT* gene organization were described in 1999 by several groups [262-265]. The gene encompasses more than 37 kb and consists of 16 exons and 15 introns. The *hTERT* gene is located on human chromosome 5p15.33, very close to the telomere [266] and encodes a 127 kDa protein of 1132 amino acids [207,240]. This gene is present in the human genome as a single-copy sequence with a dominant transcript of ~4 kb. The cDNA sequence was determined nearly

simultaneously by four groups in 1997 [207,237,238,240]. The telomerase catalytic subunits from different organisms are phylogenetically conserved in their reverse transcriptase motifs with other reverse transcriptases [240], but are more related to each other than to other reverse transcriptases and therefore form a distinct subgroup within the reverse transcriptase family [267,268].

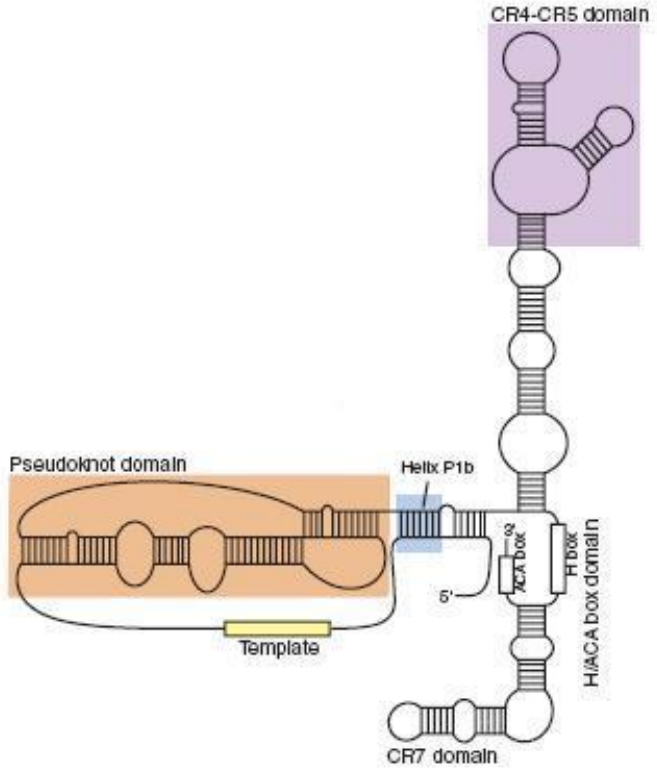


Figure 4. **Structure of the 451 nucleotide human telomerase RNA.** Universally conserved structural elements among vertebrate telomerase RNAs include the pseudoknot domain, the main TERT-binding region (CR4-CR5 domain) and the H/ACA domain. The template region consists of nucleotides 46 to 56. The RNA structure is not drawn to scale [200].

The catalytic activity of hTERT in humans is mediated through a number of protein domains. Specifically, the enzyme contains seven motifs (1 and 2, and A-E) characteristic of reverse transcriptases (located in the C-terminal half of the protein) [239,240], a conserved RNA-binding region [269] and the telomerase-specific T motif

(located just N-terminal to the RT motifs) [270-273]. Additionally, the enzyme contains four conserved N-terminal (IA, IB, II, and III) and four C-terminal (I–IV) domains necessary for catalytic activity *in vivo* [272,274].

Finally, there are two DAT regions ("Dissociate Activities of Telomerase") where mutations leave the enzyme catalytically active, but disrupt processive telomere repeat addition and possibly recruitment to telomeres. [272,274-278]. DAT domain of hTERT is implicated in telomere-telomerase associations [279]. The C-terminus of hTERT may also be implicated in functional multimerization with other hTERT molecules [280], in recruitment of the enzyme to telomeres [281], and in nuclear localization [282].

It is likely that elongation of telomeres by telomerase requires a multistep process and is precisely regulated, including the maturation, processing, and accumulation of hTR, nuclear transport, posttranslational modifications of hTERT, ribonucleoprotein assembly, substrate recognition, and coordinated synthesis of the C-strand [283]. The telomerase-associated proteins involved in each of these processes may be required for the full activity and biological function of the enzyme [230].

2.3. Telomerase-associated proteins

Numerous additional proteins bind to the telomerase complex and contribute to its activation and stabilization and may mediate or regulate the access of telomerase to its substrate, the telomeres. These proteins are not essential for catalytic activity *in vitro*; however, some of them probably regulate telomerase activity, assembly and function *in vivo*.

The Ku (Ku70/80) heterodimer, responsible for non-homologous end-joining of broken chromosomes [284], has been shown to bind directly telomerase RNA (independently of hTERT component) both *in vitro* and *in vivo* [285]. Additionally, Ku has been reported to associate with hTERT and telomerase activity [286]. It seems likely that Ku may promote telomere elongation either by recruiting hTR to chromosome ends or by stabilizing hTR/hTERT complexes once they form at the ends [285]. Furthermore, Ku has been shown to relocalize from telomeres to the sites of DNA damage [287]. Therefore, Ku is a good candidate for recruiting telomerase to multiple types of DNA ends, signifying its importance in genome preservation [137].

The putative interaction between Ku and human telomerase may be particularly important in tumor cells. Tumor cells must cope with massive amounts of genomic instability [288]. Therefore, a second reason for reactivating telomerase (beside the neccessity to overcome the telomere attrition) may be to provide tumor cells with an additional mechanism for healing DNA damage. Evidence for this assertion comes from the fact that cell lines transformed with hTERT have been shown to exhibit less radiation sensitivity than their nontransformed counterparts, although the NHEJ activities of extract made from these cell lines are identical [289].

The hTP1 (human telomerase-associated protein 1) protein can interact with both mammalian telomerase RNA [245,290] and hTERT in an RNA-independent fashion [275] but apparently is not required for telomerase function *in vitro* or *in vivo*. hTP1 deletion does not alter either telomerase activity or telomere length [291-293].

Other proteins that have been identified on the basis of their interaction with the telomerase RNA are the hStau double-stranded RNA binding protein, the L22 RNA binding protein [247], dyskerin, and the Sm proteins. These proteins are implicated in the processing and stability of ribonucleoproteins. The human telomerase RNA contains a small nucleolar RNP (snoRNP) domain. Like other snoRNP complexes, telomerase assembly occurs in the nucleolus [294]. The L22 and hStau proteins are involved in hTR transport, location, processing and telomerase assembly [247]. Dyskerin has been also proposed to play a role in telomerase assembly [256,259]. Like snoRNA, hTR contains a H/ACA motif that constitutes a dyskerin binding site (Fig. 4) [248]. It was proved that the presence of dyskerin is essential for the catalytic activity of telomerase [295]. Dyskerin mutations induce reduction of telomerase activity, and this is correlated with shorter telomeres and chromosome end fusions [248,296]. Thus, dyskerin dysfunction leads to a rare human disease referred to as dyskeratosis congenita [297].

Assembly and stability of the telomerase complex requires the molecular chaperones Hsp90 and p23. Both hsp90 and p23 were found to bind to hTERT protein and to be involved in the *in vivo* activity of telomerase complex [246].

A nuclear protein called KIP (initially characterized as a DNA-PKcs-binding protein) interacts with hTERT *in vitro* and *in vivo* even in the absence of human telomerase RNA or telomeric DNA and stimulates telomerase activity. Overexpression

of KIP in tumor cells was shown to increase telomerase activity and telomere lengths [200]. KIP may represent a novel target for anti-cancer drug development [298].

Human PINX1, first identified as a TRF1 interacting protein, negatively regulates telomerase activity and telomere length *in vivo* by binding to the assembled hTERT-hTR complex (Tab. 1) [67,299].

Binding of 14-3-3 signaling protein to hTERT has been reported to influence the subcellular localization of hTERT [282], raising the possibility that cytosolic-nuclear shuttling may be a regulatory mechanism for telomerase function *in vivo*. The 14-3-3 protein is required for efficient accumulation of hTERT in the nucleus [282]. Loss of nuclear localization could leave a protein catalytically active but unable to reach its biological substrate [272].

Other accessory factors have been found for human telomerase. Both hEST1A and hEST1B were shown to bind hTERT independently of the RNA subunit [300,301]. hEST1A contributes to telomere maintenance and protection *in vivo*; its overexpression results in telomere uncapping and resulting chromosome fusions [300]. A substantial reduction in telomere length also occurred when hEST1A levels were increased, but this effect could be reversed by co-expression of the catalytic subunit of telomerase [301].

2.4. Telomerase assays

Measurement of telomerase activity is not an easy task, considering the characteristic features of the enzyme such as the presence of labile RNA subunit and relatively low activity, even in tumor cells. However, the search for telomerase inhibitors requires a reliable method for inhibition measurement. Moreover, the detection of telomerase as a potential marker in the diagnosis of cancer has considerable potential as a noninvasive diagnostic and prognostic tool. Several methods were published for determination of telomerase activity, and several kits are also available for measurement.

2.4.1. Conventional (direct) telomerase assay

Initially, telomerase was assayed by direct measurement of the telomerase products using [α-^{32}P]-labeled deoxynucleoside 5'-triphosphates (usually [α-^{32}P]dGTP) as monomer substrates [201,295,302]. In this method, the oligonucleotide substrate,

which has the natural telomere sequence, is elongated by telomerase. The amount of incorporated radioactivity is proportional to the telomerase activity. The reaction mixture may be evaluated by electrophoresis providing information about the processivity of the enzyme. Due to the inconvenience caused by the required [^{32}P]-labeled nucleotide with high specific activity and the low sensitivity of the assay, the method is not widely used.

The improved version of this method utilizes a biotinylated oligonucleotide primer [303,304]. Similarly, [α-^{32}P]dGTP is incorporated by telomerase into telomeric repeats but, as opposed to the above mentioned, telomerase product is then separated from unincorporated label using streptavidin-coated magnetic beads and quantified by scintillation counting [304,305]. The magnetic separation shortens sample processing time and allows for easy removal of the huge excess of unincorporated label which minimizes background and sample-to-sample variation. The use of enriched telomerase instead of crude cell extracts is recommended in this method due to its low sensitivity.

2.4.2. The telomeric repeat amplification protocol (TRAP) – based assays

The polymerase chain reaction (PCR) was first applied to amplify the telomerase products for telomerase activity measurement by Kim *et al.* in 1994 [205]. The new method, referred to as the TRAP assay, solved the sensitivity problem of direct telomerase assay and has become the method of choice in the detection of telomerase activity. Many variations of this technique have been reported [306-312]. The TRAP assay is divided into three main steps that consist of extension, amplification, and detection of telomerase products. In the extension step, telomeric repeats are added to the telomerase substrate (TS), a nontelomeric oligonucleotide, by the telomerase present in a cell extract. The telomerase synthesized products are amplified by a subsequent PCR reaction using TS oligonucleotide as the forward primer and an oligonucleotide that is able to anneal to the telomeric repeats as the reverse primer (CX [205]). PCR products are detected either by radioactive or by nonradioactive methods (colorimetry, chemiluminescence, luminescence).

The accuracy of detection depends on the amplification cycles and the primers used. A problem can arise if the primer-dimers (a frequent occurrence in PCR) are indistinguishable from the actual products and would generate false-positive results. The CX reverse primer, which was the first reverse primer used in the TRAP assay, can

be attached to various sites of the amplicons in each cycle of PCR amplification. Thus, TRAP assay products do not directly reflect the distribution of telomerase products, which affects the accuracy of the results [312]. Therefore, several other reverse primers have been designed to minimize primer-dimer formation and reduce staggered annealing (ACX [306], CX-ext [313], CXa [314], RP and RP3g [309]).

Crude cell lysates [205] usually prepared from tumor cell lines (HeLa, HL-60) are good sources of the telomerase for *in vitro* inhibitory studies. Partial purification of human telomerase from the crude cell extract [308,309] could yield an enzyme with decreased concentration of endogenous inhibitors, therefore more suitable for detailed kinetic studies.

When using PCR based assays for studying telomerase inhibitors, one must realize that the telomerase is a special DNA polymerase. Thus, there is a chance that a compound, considered to be a telomerase inhibitor and/or endogenous inhibitors present in crude cell lysates may also affect PCR amplification by inhibiting *Taq* DNA polymerase. Therefore, appropriate controls are required; the standard TRAP utilizes an internal telomerase assay standard (ITAS) (Fig. 11), which enables the detection of these inhibitors in samples and serves to normalize the telomerase activity. In the detection step, after electrophoretical separation of amplified products, each lane with a typical six-base telomeric ladder (Fig. 11) is analyzed separately and the activity is measured as a ratio of the telomerase products to the internal standard. This procedure allows a comparative assessment between samples [307].

In the **conventional TRAP assay** (PAGE-TRAP), polyacrylamide gel electrophoresis and autoradiography are performed to visualize amplified telomerase products (the six-base product ladder) and to quantitate telomerase activity following densitometry. The method employs an internal control to detect a possible inhibition of the PCR step. Various modifications have been introduced to improve quantification and to simplify the time-consuming post-PCR procedures because the detection step of the TRAP assay, which requires polyacrylamide gel electrophoresis, cannot be applied to large numbers of samples.

Two-primer TRAP assay (TP-TRAP) [309] utilizes two reverse primers (RP and RP3g) instead of one. This method prevents PCR-mediated lengthening or shortening of the telomerase products caused by staggered annealing of the reverse

primer. Thus, TRAP assay products reflect the distribution of telomerase products, which improves the accuracy of quantitation.

Scintillation proximity assay (SPA-TRAP) [315] utilizes substrate oligonucleotides that are biotinylated at the 5' end and PCR amplification of the extended products is carried out in the presence of [^{3}H]-thymidine. The biotinylated [^{3}H]-labeled products are isolated by binding to streptavidin-coated fluoromicrospheres which contain a scintillant (β-emitter) that is stimulated only in the presence of tritium. Thus, TRAP products that incorporate the [^{3}H]-labeled nucleotides will stimulate the scintillant, producing a signal. As the detection following PCR is relatively faster (compared to the conventional gel-based TRAP assay), a large number of samples can be assessed, making SPA-TRAP potentially useful for high-throughput screening and analysis of telomerase inhibitors [315,316].

The real-time quantitative TRAP method (RTQ-TRAP) [317] eliminates complex post-PCR procedures and permits sensitive and linear telomerase detection. The amount of amplified telomerase products generated by PCR can be measured using either a fluorescent dye SYBR® Green I [318,319] or Amplifluor™ primers [320]. However, both of them lack the specificity of labeled hybridization probes and, therefore, are not able to distinguish specific amplicons from non-specific ones. A specific RTQ-TRAP assay that utilizes the duplex scorpion (DS) primer has been described [321]. The DS primer, which is composed of a primer-probe strand and a quencher strand [322], firstly serves as the telomerase substrate in the extension step. Subsequently, the generated telomerase products are amplified by PCR using the DS primer as a forward primer. At the fluorescence detection step during PCR amplification, the probe sequence binds to the newly synthesized complementary sequence within the same DNA strand in an intramolecular manner. The fluorescence intensity corresponds directly to the molecular number of amplified telomerase products and not repeats in them [321]. A novel DS/TP-TRAP method has been recently developed by combining the DS primer with modified TP-TRAP assay [323]. Compared to SYBR® Green I RTQ-TRAP assay, the DS/TP-TRAP assay optimizes PCR amplification efficiency and enhances the sensitivity for detection of telomerase activity. Real-time telomeric repeat amplification protocols were also developed suitable to identify and characterize telomerase inhibitors [317,323].

In the **TRAP-enzyme-linked immunosorbent assay** (TRAP-ELISA) [324], the biotinylated amplification products are denatured, hybridized with digoxigenin-labeled probe specific to telomeric repeats, and immobilized on streptavidin-coated microtiter plates. The complex is treated with a secondary antibody (antidigoxigenin-conjugated horseradish peroxidase). Finally, amplified telomerase products are detected colorimetrically. TRAP-ELISA is relatively quick when compared to conventional TRAP, however, the lack of an internal control may generate false-positive and false-negative results that affect the interpretation.

In **luminometric hybridization assay** (LHA) [325], the telomeric repeats generated by telomerase are co-amplified with a specific recombinant DNA-internal standard (DNA-IS) [326]. The DNA-IS has a similar size and the same primer recognition sites as the telomerase products and differs from them only in the central 18 bp sequence. The telomerase products are amplified by PCR in the presence of biotin-labeled nucleotides and the amplicons are separated by means of biotin-streptavidin binding [327]. The biotin-streptavidin amplicon complex is hybridized with two distinct digoxigenin-labeled oligonucleotide probes that specifically recognize the telomerase products and the DNA-IS. Detection and quantification are mediated by treatment of the complex with an antidigoxigenin antibody conjugated to alkaline phosphatase and lumiphos. The assay is highly sensitive and can be employed for large-scale evaluation of clinical samples.

In the **hybridization protection assay-TRAP** (HPA-TRAP) [312], the amplified telomerase products are amplified by the conventional TRAP assay but the amplicons generated are detected by a nonradioactive and nonelectrophoretic method that utilizes the hybridization of an acridinium ester-labeled probe to the amplicons. The probe is much more resistant to hydrolysis when hybridized to a target sequence compared to the free state [328,329].

The **transcription-mediated amplification and hybridization protection assay** (TMA/HPA) [330] differs from the TRAP methods described above. This was the first method developed that amplified telomerase products without the polymerase chain reaction. TMA/HPA is as sensitive and reproducible as conventional TRAP, but is faster, easier to perform, and does not require radiolabeled substrate. Similarly to the HPA-TRAP assay, the products formed with the TMA/HPA technique are detected by

the hybridization of a chemiluminescent probe, which allows a direct analysis of telomerase activity. This method is also applicable to a high-throughput format. Furthermore, as a non-PCR based assay, TMA/HPA has been found to be minimally influenced by TRAP inhibitors.

The **enzymatic luminometric PP_i assay** (ELIPA) [331] is a bioluminescent method for detecting telomerase activity that does not include PCR amplification. In the ELIPA, the PP_i released during synthesis of telomeric repeats (six PP_i for each TTAGGG repeat) is quantitatively converted to ATP by ATP-sulfurylase, and ATP is determined in a very sensitive luciferase luminescence system [332].

3. Regulation of human telomerase activity

Telomerase is active during embryonic development and then gradually repressed during differentiation in the majority of somatic cells. Telomerase repression is thought to act like a tumor-suppressor mechanism. For many years hTERT has been regarded as the limiting component of telomerase and most of the research in this field has focussed on its regulation. However, growing evidence has recently indicated that acquisition of telomerase activity is associated with an active regulation of the RNA component as well [333]. Since deficiency as well as overexpression is pathogenic, telomerase has to be strictly regulated. The regulation of telomerase activity occurs at various levels, including transcription, mRNA splicing, maturation and modifications of hTR and hTERT, transport and subcellular localization of each component, assembly of active telomerase ribonucleoprotein, and recruitment to the telomere terminus [169].

3.1. Regulation of telomerase at the telomere terminus

Once the active telomerase holoenzyme is assembled in an active conformation, it should be directed to the telomere end. Access to the telomere sequences is regulated in *cis* by proteins that bind telomeric DNA (chapter 1.1.2. in Introduction).

3.2. Regulation of hTR

The telomerase RNA component has an essential role in telomerase activity and telomere maintenance. Both hTR and hTERT can restrict telomerase activity and telomere length *in vitro* [242]. Recently, much more of the research has focussed on hTR regulation, especially in relation to hTERT [333]. The levels of hTR expression vary during tissue development and between normal and neoplastic cells and tissues [235,251,334-340]. Expression of hTR was shown to be up-regulated in cancer cell lines in comparison with normal cell lines and tissues [251,254,341].

Protection or stabilization of telomerase RNA by its association with the catalytic protein subunit hTERT may contribute to an increase in the half-life and steady-state levels of hTR [254]. However, the major factor determining the increased hTR levels is the up-regulation of *hTR* transcription in cells expressing endogenous hTERT. Therefore, some overlap in transcriptional regulatory control of both subunits has been suggested [254]. The *hTR* promoter contains several transcription factor binding sites including one CCAAT box and four Sp1 consensus sequences in the core promoter region of 272 bp upstream of the transcriptional start site [250]. While the Sp1 site upstream of the CCAAT box mediates positive regulation, the other three Sp1 sites downstream of the CCAAT box appear to be repressive [333,342]. Binding of nuclear factor-Y (NF-Y) complex to the CCAAT box sequence is essential for trancriptional activity. Sp1 and the retinoblastoma protein (pRb) may then activate hTR promoter, whereas Sp3 [343] and MDM2 [344] are powerful repressors. MDM2 represses transcription by binding Sp1 to prevent it from binding the promoter, while pRb is thought to alleviate this repression by sequestering MDM2 from Sp1 [345]. In contrast to Sp1 and NF-Y, pRb does not bind directly to DNA and instead mediates its effect through recruitment of additional transcriptional regulators. MDM2 may directly repress activation by both pRB and Sp1, or activation by NF-Y. Furthermore, MDM2 possess the ability to interact and interfere with components of the general transcription machinery [344].

Additionally, a role of epigenetic modifications in control of the expression of *hTR* should be mentioned. The *hTR* gene lies within a CpG island [250], indicating that DNA methylation could influence *hTR* expression, however, no common pattern has emerged [346,347]. DNA methylation has not been shown to correlate with *hTR*

expression in normal or tumor cells, regardless of the telomerase activity [346,347]. Nakamura *et al.* [348] reported that the hypomethylation of the *hTR* promoter region is not likely to be the main mechanism regulating *hTR* expression. On the other hand, a strong correlation was demonstrated between hypermethylation of the *hTR* promoter and repression of *hTR* expresssion in three ALT cell lines [346]. Furthermore, chromatin remodelling is also involved in the regulation of *hTR* expression in some ALT cell lines, where *hTR* appears to be repressed at the chromatin level and show hypoacetylation of H3 and H4 and hypermethylation of H3 lysine 9 consistent with gene repression [189].

3.3. Regulation of hTERT

Expression of the *hTERT* gene is highly regulated and correlates with telomerase activity [207,238]. Many studies indicate that transcriptional regulation of *hTERT* is the major mechanism of telomerase regulation in human cells [349-354]. It has been shown that the expression of hTERT is sufficient to restore telomerase activity in telomerase-negative cells [217,244,355].

3.3.1. Regulation at the genetic level

Genomic organization and promoter characterization of the *hTERT* gene have been described by several groups [262-265]. Four important regions involved in the *hTERT* gene regulation were identified. Firstly, the core promoter, which is essential for transcriptional activation, encompasses the proximal 283 bp region upstream of the ATG codon [263-265] and shows a bidirectional activity [356]. Secondly, the distal upstream region (-1821 to -811 bp) is involved in the splicing of the first intron. Thirdly, the intermediate promoter region (-800 to -300 bp) could play an important role in silencing the reverse promoter activity. Fourthly, the structural gene (up to +1077 bp), which contains binding sites for a transcriptional inhibitor, was reported to strongly reduce *hTERT* promoter activity. Thus, the proximal exonic region (the first two exons) plays a major role in the down-regulation of the *hTERT* promoter in telomerase positive cells [356,357].

Furthermore, the promoter sequence lacks a TATA box (or TATA-like sequences) [264] and is located in a CpG island [265]. Several groups have found

specific binding sites for activators and inhibitors of the transcription in the *hTERT* promoter sequence [351,352,358-364]. Some of them (such as c-myc) are aberrantly regulated in human cancers. Reactivation of hTERT in cancer may therefore be linked in part to hyperactivity of crucial oncogenic transcription factors [349].

Activation of *hTERT* transcription

Numerous factors able to activate *hTERT* transcription were identified, including c-myc [352,358,365-367], Sp1 [358], estrogen [368], progesterone [369], Hif-1 [370,371], Bmi-1 [372], hALP [373], USF1/2 [374], c-myb [263-265], and others (Fig. 5) [349,350,353,354,375].

Transactivation and repression by the Myc/Max/Mad network of transcription factors is mediated by binding to the two enhancer box sequences (E-boxes) within the *hTERT* promoter and seems to have a crucial role in the regulation of telomerase [349]. Max can form heterodimers with Myc and Mad, resulting in *hTERT* gene activation (Myc/Max) or repression (Mad/Max) [364]. c-Myc protein recruits histone acetyltransferases (HATs) through binding to E-boxes [352,367]. c-Myc is very often found to be up-regulated in proliferating and in many neoplastic cells (up-regulates cyclins, down-regulates *p21*) [376] and was shown to have positive effects on the *hTERT* promoter in a dose-dependent manner [365]. The *hTERT* core promoter contains beside the two E-boxes also 9 binding sites for the transcription factor Sp1 which can also activate *hTERT* promoter [264,358].

Hormones and growth factors are also crucially involved in the regulation of telomerase activity and gene expression of *hTERT* [377]. Both estrogen and progesterone activate telomerase in several cell types [368,369]. Estrogen acts through binding the estrogen response element in the distal promoter and a stimulatory sequence near the recognition site of Sp1 (Fig. 5) [359,368].

Activation of hTERT has also been observed in oncogenic viral infections. For example, the HPV E6 protein can also associate with c-myc and thereby activate the hTERT transcription [378]. HER2/Neu, Ras and Raf oncoproteins stimulate *hTERT* promoter activity *via* the ETS transcription factor ER81 and ERK mitogen-activated protein (MAP) kinases [375].

Transcriptional repressors

Negative regulators include already mentioned Mad1 [351,379], as well as Wilm's Tumor 1 protein (WT1) [360], myeloid-specific zinc finger protein 2 (MZF-2) (Fig. 5) [363], TGF-β [380], IFN-α [381], retinoic acid [382] and CTCF [357]. More general repressor candidates are E2F-1 [383], p53 [362,384] and pRB [383] (Fig. 5).

Studies showed that the down-regulation of *hTERT* transcription is partial and cell type specific. Therefore, Mad1 does not seem to be capable of completely switching off the expression of *hTERT* [349,350]. Similarly, WT1 factor downregulates *hTERT* transcription only in Wilm's tumor cells [360]. Moreover, *hTERT* repressors are preferentially expressed in telomerase-positive cells, but not in the telomerase-negative ones (E2F-1 [383,385]). Recently, it has been shown that Mad1 has a regulatory effect on *hTERT* transcription in *hTERT*-positive cells, but not in telomerase negative normal cells [365]. Although, overexpression of p53 can trigger a rapid down-regulation of *hTERT* mRNA expression [362,384], inhibition of its activity failed to reactivate *hTERT* expression [386]. It was demonstrated that p16, as well as p53, suppress telomerase activity through transcriptional regulation of *hTERT* in malignant glioma [387]. A recent study showed that p53-mediated down-regulation of *hTERT* is critical for efficient p53-dependent apoptosis [388]. Thus, the partial inhibition of *hTERT* transcription cannot explain the down-regulation of the *hTERT* expression in telomerase-negative cells. Moreover, the repression of *hTERT* transcription is not always due to a direct effect of inhibitors. A recent study suggests that the inactivation of distinct telomerase repressor genes occurs in different types of human cancers and may have implications for the tissue specific regulation of telomerase during human development and carcinogenesis [389]. Shats *et al.* [390] suggest that repression of *hTERT* by endogenous p53 is mediated by p21 and E2F. TNF-α (tumor necrosis factor alpha) was also found to indirectly inhibit the *hTERT* transcription in human myeloid normal and leukemic cells [391].

3.3.2. Regulation at the epigenetic level

The DNA methylation

As the *hTERT* promoter and the proximal exonic region are situated in a CpG island, DNA methylation has been suggested to be involved in the *hTERT*

transcriptional regulation in normal and cancer cells (Fig. 5) [262-265,392]. Methylation of most gene promoters inhibits transcription. Recently, a DNA methylation map was established on the 5' end of the *hTERT* gene. A central region, localized from -500 to +180 according to the ATG translation start, was surrounded by 2 heterogeneous hypermethylated regions in both tumor and normal tissues and cells. These hypermethylated regions did not exclude transcriptional activity of the *hTERT* promoter [393]. Moreover, several studies reported a hypermethylation of the *hTERT* promoter in telomerase-positive tumors and a hypomethylation in telomerase-negative normal tissues [392,394,395] suggesting a role for methylation in the blocking of negatively-acting transcription factors. It was shown that CTCF, an ubiquitous methylation-sensitive repressor, binds to GC-rich proximal exonic region of *hTERT* and inhibits *hTERT* gene transcription when the *hTERT* CpG island is not methylated, irrespective of the cell type [356,357]. Hypermethylation of its binding site prevents binding of CTCF and can abolish CTCF repressor activity, allowing transcription of the *hTERT* gene [396]. This was confirmed by inducing hypomethylation with 5-azadCyd, which allowed CTCF to bind the first exon and repress *hTERT* expression [396]. Several other groups observed that 5-azadCyd treatment of telomerase-positive cells inhibits hTERT transcription and reduces telomerase activity [397-399]. Conversely, Renaud *et al*. [357] hypothesizes that the *hTERT* first exon demethylation restores CTCF binding.

On the other hand, methylation of the *hTERT* promoter is also observed in differentiated and senescent cells that do not express hTERT [400,401]. Moreover, hypermethylation of the *hTERT* promoter correlated with repression of telomerase activity in B-cell lymphocytic leukemia [402] and normal human oral cells [401]. In ovarian and cervical cancers no correlation was found between the hypermethylation of *hTERT* and *hTERT* mRNA expression [403]. In some transformed and neoplastic cells, *hTERT* is reactivated and transcribed regardless of a methylated promoter [347,394]. Zinn *et al.* [404] has recently shown that in many cancer cell lines the region surrounding the transcription start site should remain unmethylated for *hTERT* to be expressed. And Devereux *et al.* [392] found no common methylation pattern that correlated with hTERT expression in a variety of human normal, immortalized, and cancer cell lines. The authors also reported that the cell line SUSM-1 is completely

methylated in the region around the transcription start and does not express *hTERT*. Treatment of this cell line with the DNA hypomethylating agent 5-azadCyd resulted in restored expression of *hTERT*.

The activity of the *hTERT* promoter depends on the final balance between all the involved factors. Although DNA methylation has been shown to participate in *hTERT* regulation, mechanisms and factors involved in this context remain to be identified.

The DNA packaging

Changes in chromatin structure of the native *hTERT* locus have been shown to play an important role in *hTERT* regulation (Fig. 5). Histone deacetylation, leading to chromatin condensation, is implicated in *hTERT* repression [405-409]. During differentiation, as shown in HL-60 cells, the switch of c-myc to Mad1 can induce active deacetylation at the *hTERT* promoter, resulting in a rapid decrease in *hTERT* mRNA [364]. Conversely, histone acetylation was found to induce hTERT expression in a telomerase-negative cell line [405]. Furthermore, histone modifications have been implicated in telomerase repression in ALT cells: lack of expression of hTR and hTERT seems to be associated with histone H3 and H4 hypoacetylation and methylation of Lys9 on histone H3 [189]. Finally, recent findings suggested the possibility that human tumors may be able to reversibly interconvert their telomere maintenance phenotypes by chromatin structure-mediated regulation of *hTERT* expression [399].

Telomere position effect (TPE)

Interesting questions have been raised as the *hTERT* gene has been mapped to chromosome 5p15.33, with only a few hundred kb from the telomere, *hTERT* is the most distally located gene on 5p [207,266]. The close association to the telomere makes the *hTERT* gene a potential candidate for a gene silencing by the TPE. The TPE is dependent on a specific higher-order organization of the telomeric chromatin [410] and the extent of this effect is proportional to telomere length [17]. Therefore, TPE could form an autoregulatory feedback loop on *hTERT* expression [411].

3.3.3. Regulation at the post-transcriptional and post-translational level

Alternative splicing of *hTERT* transcripts seems to be another regulatory mechanism for telomerase (Fig. 5) [412-419]. Studies of RNA processing revealed

complex splicing patterns in different cell types [238]. Besides the full length *hTERT* transcript, at least 7 alternatively spliced mRNA variants (3 deletion-type [α, β and α/β] and 4 insertion-type molecules) have been reported [414-416]. Presence of these alternative forms is regulated during human development and depends on the tissue type [265,414,415]. However, only full length mRNA permits translation into a protein with catalytic activity [238,294]. Interestingly, the full-length *hTERT* mRNA is significantly less abundant than the β-spliced variant. Expression of the α-spliced variant leads to telomerase inhibition and, to some extent, to the cell death [412,413]. As this splice variant still contains the *hTR* binding site, it would compete with full length mRNA and thus could lead to a weak concentration of active telomerase complex [269]. The presence of alternative splicing of *hTERT* in osteosarcoma cell lines has been correlated with the lack of telomerase activity [420]. In most lung carcinoids, which are telomerase-negative, *hTERT* transcription and alternative splicing play a negative regulatory role [421]. Moreover, alternatively spliced *hTERT* mRNA tends to be less abundant in tissues with high telomerase activity [422]. These results support a role for *hTERT* spliced-variants in the regulation of telomerase activity.

Posttranslational modifications of the hTERT protein are used in some cell types to control hTERT activity. For example, phosphorylation of hTERT by protein kinase Cα, by PP2A (protein phosphatase 2A) or by c-Abl tyrosine kinase are involved in modulation of telomerase activity (Fig. 5) [423,424]. Phosphorylation may alter either hTERT enzymatic activity or subcellular localization (for instance, cytoplasmic or nuclear) [424-426] Indeed, the regulation of subcellular localization of hTERT may regulate the biological functions of telomerase by controlling access of the telomerase complex to the telomeres or by modulating the efficiency of telomerase RNP assembly [427]. A previous report showed that hTERT distribution between nucleoli and the nucleoplasm is regulated in a cell cycle-dependent manner in normal cells, and deregulation of the proper localization of hTERT in cancer cells correlates with the growth advantage observed in transformed cells [428]. Aisner *et al.* [283] proposed that hTERT is present in the cytoplasm in an inactive, unphosphorylated state in unstimulated cells but upon stimulation, phosphorylation permits nuclear localization of hTERT, thereby allowing for assembly of active telomerase and function on telomeres. Moreover, hTERT shuttles between nuclear compartments during the cell cycle.

Catalytically active human telomerase has a regulated intranuclear localization that is dependent on the cell-cycle stage, transformation and DNA damage. In T lymphocytes, which express hTERT constitutively, phosphorylation causes activation and the nuclear translocation. This process seems to be important for the telomerase activation that accompanies clonal expansion [429].

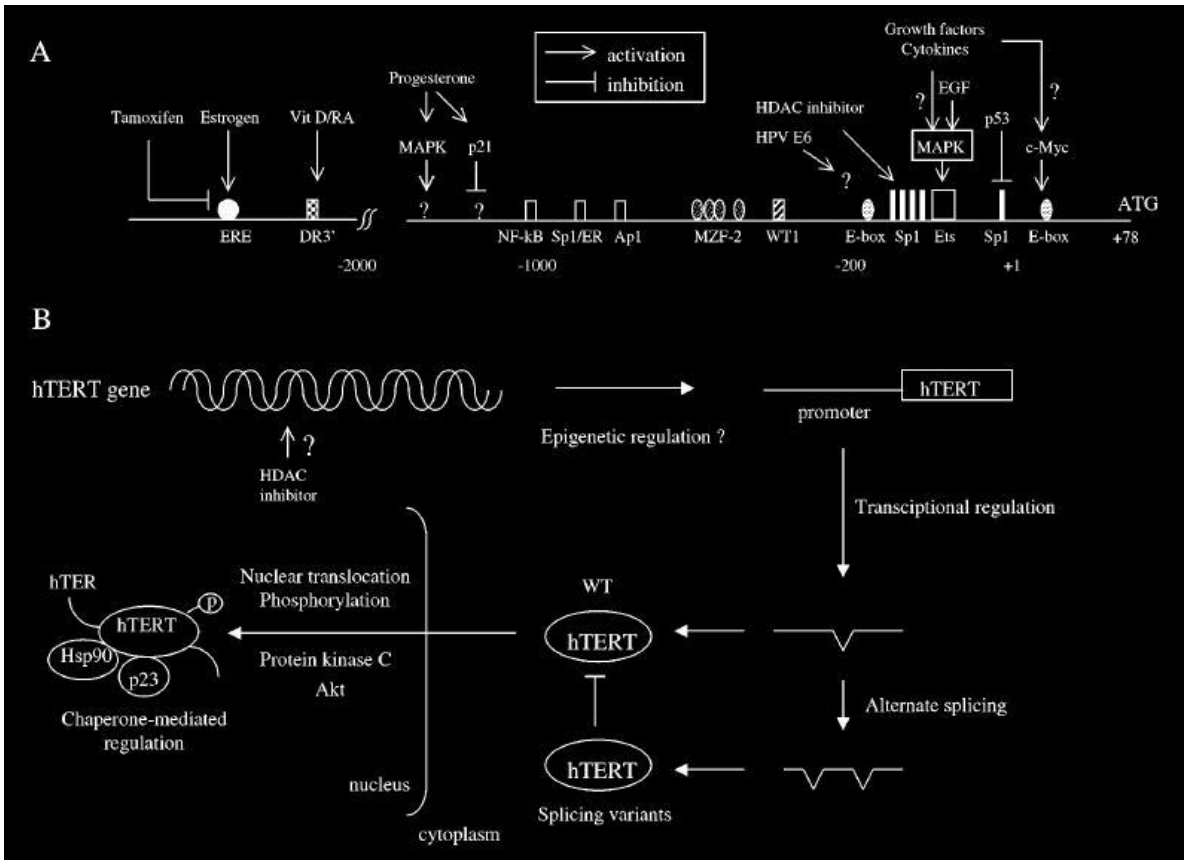


Figure 5. **Schematic view on the putative regulatory mechanisms of telomerase activity.** (A) Potential cis-acting elements in the hTERT promoter as well as factors that interact with them are shown. The +1 indicates the start site of transcription. ERE: estrogen responsive element. DR3': degenerated vitamin D3 receptor/retinoid X receptor binding site. WT1: Wilms' tumor 1 tumor suppressor gene product. (B) Possible regulatory mechanisms of telomerase activity at various steps [425].

4. Targeting telomerase as anticancer strategy

As mentioned above, telomerase confers immortality on cells in most types of cancer. Telomerase activity is present in almost 90% of the malignant tumors in humans and restricted to a few types of normal cells [205,206]. This feature have made telomerase and the telomere structure attractive targets for the development of new anti-cancer agents [430]. The telomerase holoenzyme complex presents multiple potential sites for the development of inhibitors (Fig. 6). The approaches for telomerase inhibition include interaction with *hTERT* mRNA and hTR (antisense oligonucleotides, ribozymes, siRNA), telomeres (G-quadruplex ligands) and telomerase holoenzyme (nucleoside analogues, non-nucleoside small molecules, chaperone inhibitors, protein kinase inhibitors), transcriptional repression of *hTERT* and *hTR*, gene therapy directed at telomerase positive cells (*hTERT* promoter-driven strategies) and immunotherapy (Fig. 6). Numerous approaches for targeting telomeres and telomerase activity have been studied [431] and several reviews concerning telomerase inhibition have been published in the last few years [432-438].

Concentrations of compounds that inhibit 50% of telomerase activity (IC_{50}) are listed in the text below. However, direct comparisons of the IC_{50} values should be made with caution because many laboratories use related but not identical protocols for telomerase activity measurement and/or the source of the telomerase enzyme. On the other hand, high-throughput screening tests have already been applied, allowing the simultaneous and systematic evaluation of thousands of compounds [316].

4.1. Inhibition of the telomerase catalytic protein subunit (hTERT)

Validation of hTERT targeting as a potentially powerful site for anticancer drug design was demonstrated in human tumor cells using dominant-negative mutant forms of hTERT [439,440]. In these experiments, telomerase activity was abolished and it was accompanied by continuous telomere shortening leading to senescence or apoptosis. Various nucleosides, nucleoside triphosphates and their analogues, non-nucleoside reverse transcriptase inhibitors, and some antisense strategies have been investigated as anti-hTERT agents for clinical drug development. A major difficulty of such approaches

lies in the existence of a lag phase between the initiation of telomerase inhibition and an impact on the proliferative capacity of investigated cells.

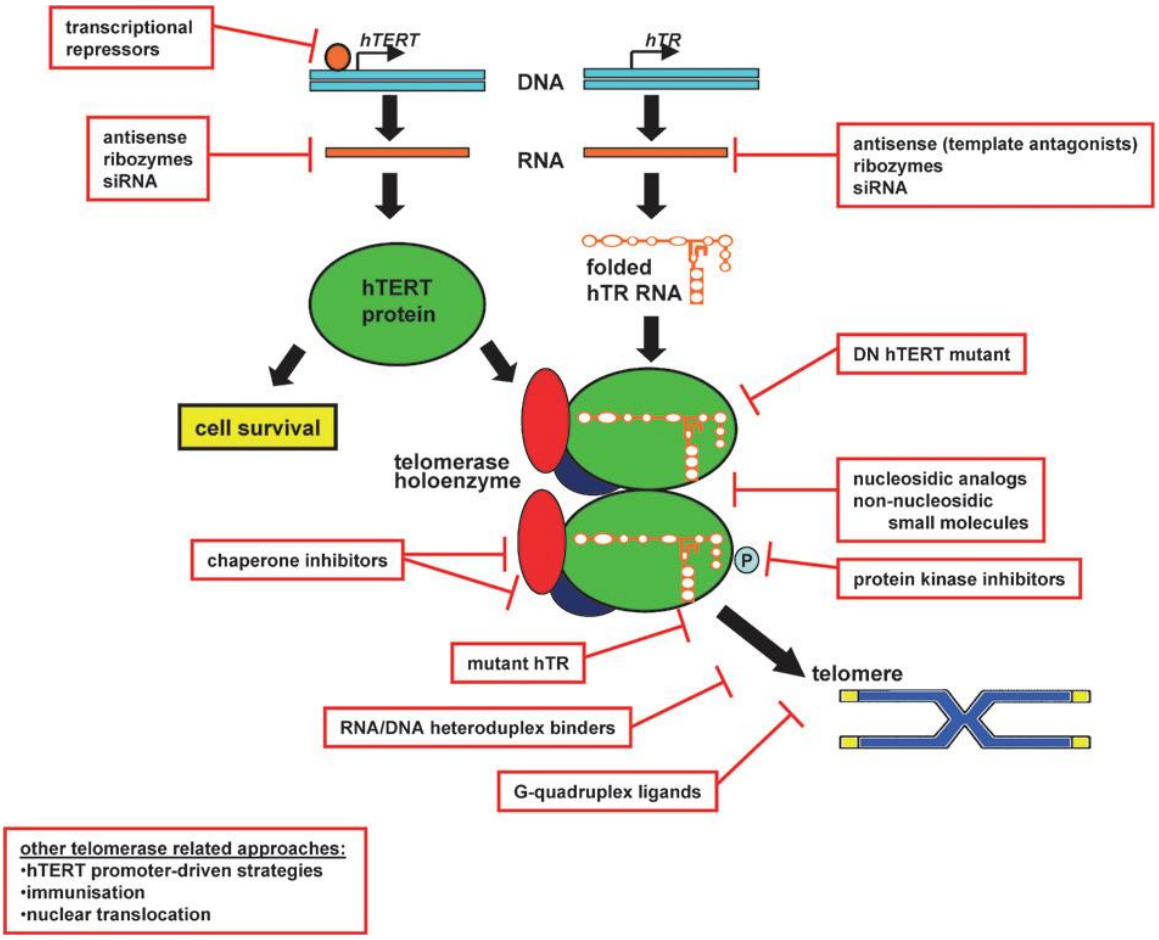


Figure 6. **Telomerase targeting** [432].

4.1.1. Nucleotide analogues and nucleoside-type reverse transcriptase inhibitors

The identification of the hTERT component of telomerase [240] as a functional catalytic reverse transcriptase (RT) [236,239,441] prompted many groups to study inhibition of telomerase with established HIV reverse transcriptase inhibitors (RTIs), such as the chain terminating 3'-azido-3'-dideoxythymidine (AZT) and 2',3'-dideoxycytidine (ddC) [442-444]. These parent nucleosides are transformed *in vivo* in

the form of 5'-triphosphates, which inhibit reverse transcriptases by acting as competitive substrates and/or terminate DNA synthesis *de novo* [442].

Among the RTIs, the anti-HIV-1 drug AZT has been the most extensively studied for the telomerase-inhibitory activity [445]. Like other nucleoside analogues, AZT inhibits telomerase after activation to its 5'-triphosphate (AZT-TP) with IC_{50} value of 30 $\mu mol.l^{-1}$ [442,446,447]. However, AZT-TP is not specifically targeted to telomerase, and acts as a general inhibitor of DNA polymerase activity [448].

In order to obtain more potent and selective inhibitors of telomerase, other nucleoside/nucleotide-based inhibitors, both purine and pyrimidine derivatives, have been evaluated with IC_{50} values in the micro-molar range [447]. Beside others, the inhibitory effects of purine analogues of 3'-azido-2',3'-dideoxynucleosides and their 5'-triphosphates have been investigated: 3'-azido-2',3'-dideoxy-2-aminoadenosine (AZddAA), 3'-azido-2',3'-dideoxyadenosine (AZddA), 9-(3-azido-2,3-dideoxy-β-D-ribofuranosyl)-2-aminopurine (AZddAP), 3'-azido-2',3'-dideoxy-2-chloroadenosine (AZddClA) [449], 3'-azido-2',3'-dideoxyguanosine (AZddG) [450], 3'-azido-2',3'-dideoxy-6-thioguanosine (AZddSG) [451], and their 5'-triphosphate derivatives (Table 2). Of these, AZddGTP has been shown to be the most potent and selective telomerase inhibitor. AZddGTP was incorporated into 3' terminus of DNA and did not exhibit significant inhibitory activity against DNA polymerases α and δ [451].

Fletcher *et al.* [452] reported the use of deazadeoxypurines as inhibitors of telomerase with one compound, 6-thio-7-deaza-2'-deoxyguanosine 5'-triphosphate (TDG-TP) showing an IC_{50} value of 60 $nmol.l^{-1}$. Series of the other nucleotide analogues [ddGTP, ddATP, ddTTP, 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-triphosphate (d4TTP), 7-deaza-2′-deoxyadenosine 5′-triphosphate (deazadATP), 7-deaza-2′-deoxyguanosine 5′-triphosphate (deazadGTP), arabinofuranosylguanine 5'-triphosphate (araGTP), 6-thio-2'-deoxyguanosine 5'-triphosphate (thiodGTP), 2′,3′-didehydro-2′,3′-dideoxyguanosine 5′-triphosphate (CBV-TP), 2'-fluoro-2'-deoxy-arabinofuranosylthymine 5'-triphosphate (FaraTTP), and 2'-fluoro-2'-deoxy-5-methyl-arabinofuranosyluracil 5'-triphosphate (FMAU-TP)] have previously been shown to inhibit telomerase activity [302,442, 446,447,453-455]. A direct comparison of dideoxynucleoside 5'-triphosphates ddGTP, ddATP, ddTTP and ddCTP revealed that

ddGTP (IC_{50} 5 μmol.l^{-1}) was the most potent inhibitor while ddCTP did not inhibit telomerase activity [447].

Several „structure-activity“ *in vitro* studies have been performed. Arabinofuranosylthymine 5'-triphosphate (araTTP), FMAU-TP and 1-[(2-hydroxyethoxy)methyl]thymine 5'-triphosphate (acycloTTP) showed weaker telomerase inhibitory activity than the corresponding guanine analogues [456]. Similarly, the guanine analogues AZddGTP and AZddSGTP are more potent telomerase inhibitors than their adenine and thymine counterparts AZddATP, AZddAATP and AZTTP [451]. When comparing inhibitory potency of AZddATP (IC_{50} 4 μmol.l^{-1}) and AZddAATP (IC_{50} 9 μmol.l^{-1}), it seems that the 2-amino group of the purine moiety increases the telomerase inhibitory activity [449]. These results suggest that dGTP analogues may be promising as telomerase inhibitors. The comparison of the inhibitory effects of D-CdG-TP (IC_{50} 11 μmol.l^{-1}) and L-CdG-TP (IC_{50} 80-160 μmol.l^{-1}) (carbocyclic 2′-deoxyguanosine 5′-triphosphate) indicates that telomerase can distinguish between D and L enantiomers of nucleotide substrates [453]. The similar observation was made with D and L enantiomers of FMAU-TP [447].

4.1.2. Inhibition by non-nucleoside molecules

The growing family of potent telomerase inhibitors is being identified through the large-scale screening of chemical small-molecule libraries using a telomerase assay [316,435,460-462]. This group includes chemically diverse small molecules with non-nucleoside structures acting mostly on the hTERT protein subunit of telomerase. Some of these agents, regarding their mode of action, resemble to the non-nucleoside RTIs used in the treatment of HIV infections.

The isothiazolone derivative 2-[3-(trifluoromethyl)phenyl]isothiazolin-3-one (TMPI) (Fig. 7) demonstrated non-competitive inhibition with the DNA primer and mixed inhibition with the substrate dNTPs (with IC_{50} value of 1 μmol.l^{-1} at 50 μM each of dNTPs) [316]. The isothiazolone moiety of TMPI most likely acts at cysteine residues in or near the active site of the enzyme. Additionally, the inhibition by TMPI appears selective for telomerase since TMPI has not been shown to inhibit eukaryotic DNA polymerase α, β, or HIV reverse transcriptase [316].

Table 2. Nucleoside/nucleotide analogues as telomerase inhibitors

Compounds	**Notes**	**References**
AZT	High concentration of AZT induced irreversible telomere shortening in HeLa cells; no evidence of senescence was detected	445
AZT	AZT treatment induced inhibition of telomerase activity, telomere loss, senescence and growth delay in human breast cancer cells MCF-7; decrease in telomerase activity was preceded by down-regulation of *hTERT* and *c-myc* expression.	457
Acyclovir, ganciclovir, penciclovir	Antiviral acyclic nucleosides were found to be potent inhibitors of telomerase	458
AZddG	Causes a significant telomere shortening; more potent effects on telomere shortening in HL-60 cells than treatment with AZT	450
AZddAA	Causes only a moderate telomere shortening in HL-60 cells, however, its triphosphate AZddAATP is a potent inhibitor of telomerase (IC_{50} 9 μM)	449
TDG-TP	A very potent inhibitor of human telomerase with an IC_{50} of 60 nM	452
deazadGTP, deazadATP	Potent telomerase inhibitors IC_{50} values 11 and 8 μM for deazadGTP and deazadATP, respectively; both deazadGTP and deazadATP were incorporated into telomeric DNA by telomerase	454
ddGTP, CBV-TP, T-dGTP	Potent inhibitors of telomerase	453
ACV-TP	A relatively strong telomerase inhibitor when compared to AZT-TP and ddTTP	459

Rhodacyanine derivatives MKT077 and FJ5002 (Fig. 7) have also been reported to inhibit telomerase activity *in vitro* with 50% inhibition at ~5 $\mu mol.l^{-1}$ and ~2 $\mu mol.l^{-1}$, respectively [460]. However, the selective toxicity of MKT077 to telomerase-positive cancer cells was not accompanied by inhibition of telomerase activity or telomere shortening *in vivo* [463]. In contrast, FJ5002 was effective in inducing telomere erosion and cellular senescence on U937 human leukemia cells [460]. Telomerase inhibition by FJ5002 was substrate-dependent and competitive and FJ5002 was proposed to inhibit telomerase by a direct interaction [460].

Another compound, designated BIBR1532 {2-[(E)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid)} (Fig. 7), is a potent and selective telomerase inhibitor capable of inducing telomere shortening and senescence in human cancer cell lines derived from fibrosarcoma, lung, breast and prostate carcinoma [461]. A proliferation arrest was observed after a sustained period of treatment with hallmarks of senescence, including morphological, mitotic and chromosomal aberrations and altered patterns of gene expression. BIBR1532 proved to be active *in vivo* [464]. BIBR1532 is a mixed-type non-competitive inhibitor and with a drug binding site distinct from the sites for 5′-deoxyribonucleotides and the DNA primer, respectively [464]; therefore, its mode of action may be considered as a very similar one to the non-nucleosidic inhibitors developed against HIV-1 reverse transcriptase. The compound inhibits the *in vitro* processivity of telomerase in a dose-dependent manner, with the IC_{50} value of 93 $nmol.l^{-1}$ (for the purified enzyme) [464].

A similar compound 2,3,7-trichloro-5-nitroquinoxaline (TNQX) (Fig. 7) is a potent and selective telomerase inhibitor [465]. TNQX exhibited inhibition of human telomerase in a dose-dependent manner, with a mean IC_{50} of 1.4 $\mu mol.l^{-1}$ (at 100 μM of each dNTP), and did not inhibit DNA and RNA polymerases, including retroviral reverse trancriptase. Like BIBR1532, TNQX is a mixed-type non-competitive inhibitor, with an inhibitor-binding site distinct from the binding sites for the telomeric substrate (TS) primer and the dNTPs, yet influences the binding of the substrates [465]. Long-term cultivation of the breast cancer MCF7 cell line with a TNQX concentration that did not cause acute cytotoxicity resulted in progressive telomere erosion followed by an increased incidence of chromosomal abnormalities and induction of the senescence phenotype [465].

Additionally, the quinone antibiotic β-rubromycin (Fig. 7) appeared to be a potent telomerase inhibitor, with an IC_{50} of 3 μmol.l^{-1} (at 100 μM each of dNTPs), as well as an inhibitor of retroviral RTs. A substantial decrease of its telomerase inhibitory potency was observed after opening the spiroketal system of β-rubromycin and converting it to α-analogue. A kinetic study of the inhibition by β-rubromycin revealed a competitive interaction with respect to the telomerase substrate primer, whereas a mixed type inhibition was observed with respect to the nucleotide substrate [466]. The potency of this compound to induce senescence in human cancer cells has not yet been confirmed.

Helenalin, a natural sesquiterpene lactone, can directly inhibit telomerase *in vitro*, possibly through alkylating the cysteine residues of hTERT (similarly to TMPI, see above). Helenalin also inhibits telomerase activity in hematopoietic cancer cells Jurkat and HL-60. This inhibition appears to be non-reversible and at least partly can be attributed to the inhibition of *hTERT* expression (chapter 4.1.4. in Introduction) [467].

Another alkylating agent, a phospholipase C inhibitor designated U-73122 (Fig. 7), was proved to be a potent and selective inhibitor of telomerase *in vitro* with an IC_{50} value of 0.2 μmol.l^{-1}. Similarly to helenalin, it was demonstrated that U-73122 inhibits telomerase in Jurkat and HL-60 cells. Similarly to TMPI and helenalin, the inhibitory effect of U-73122 can be protected by the presence of thiol-containing compounds such as dithiothreitol (DTT) [468].

A nitrostyrene derivative 3-(3,5-dichlorophenoxy)-nitrostyrene (DPNS) (Fig. 7) showed a potent inhibitory effect with an IC_{50} of 0.4 μmol.l^{-1} (at 100 μM each of dNTPs), while no inhibition of DNA and RNA polymerases, including retroviral RT, was observed. Treatment of telomerase-positive HeLa cells with DPNS resulted in progressive telomere erosion followed by the induction of senescence phenotype [469].

Finally, natural products including tea catechins [470], alterperylenol [471], and diazaphilonic acid [472] have also been reported to be telomerase inhibitors.

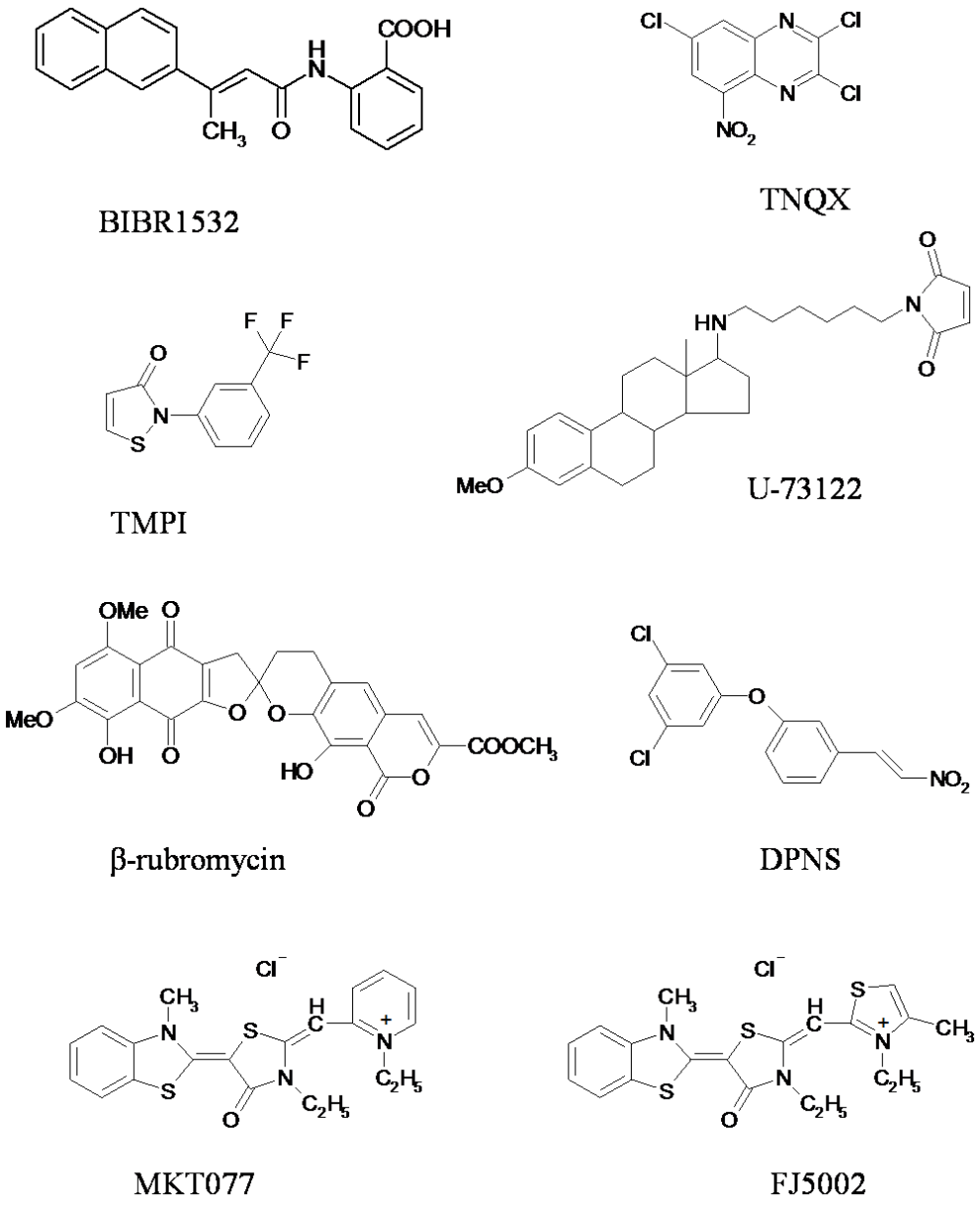


Figure 7. **Structures of selected non-nucleoside telomerase inhibitors.**

4.1.3. hTERT phosphorylation inhibitors

Phosphorylation of hTERT protein may be another mechanism of the post-transcriptional control of telomerase. Some protein kinases, such as Akt kinase (protein kinase B) [473] and protein kinase C (PKC) [423], increase telomerase activity *via* hTERT phosphorylation. PKC inhibitors were shown to inhibit telomerase activity in

nasopharyngeal [474] and cervical [475] cancer cells. Nonsteroidal anti-inflammatory drugs (NSAIDs) might also interfere with any of these pathways to down-regulate telomerase. For example, SC-236 (chapter 4.1.4. in Introduction) inhibits PKC-β_1 expression and activity [476], which is involved in gastric carcinogenesis [477]. However, the inhibition of protein kinases is not specific way to suppress telomerase activity.

4.1.4. Inhibiting hTERT transcription

Since regulation of the *hTERT* transcription plays a crucial role in the modulation of telomerase activity during carcinogenesis, a complex network of regulatory elements has been identified to act in the promoter region of *hTERT* [431] (chapter 3.3.1. in Introduction). In this regard, several studies reported compound capable of inhibition of *hTERT* expression.

Besides helenalin (chapter 4.1.2. in Introduction), another natural sesquiterpene lactone, costunolide, also inhibits *hTERT* expression. In fact, costunolide was reported to inhibit telomerase activity by down-regulation of hTERT and transcriptional factors c-myc and Sp1 in human breast carcinoma cell lines [478]. This compound was also observed to induce differentiation in HL-60 cells (to granulocytes and monocytes/ macrophages) [479].

Ceramides have been demonstrated to have a repression effect on Sp1/Sp3, positive regulators of hTERT transcription [480]. Genistein shows double effect on telomerase activity: it blocks Akt activation (and thereby hTERT phosphorylation) and is able to repress c-myc, a known activator of *hTERT* transcription, as well [481]. Interestingly, some NSAIDs, namely aspirin, indomethacin, and SC-236 (a specific cyclooxygenase-2 inhibitor), have been reported to inhibit telomerase activity through suppresssion of *hTERT* transcription in colon carcinoma cells [482].

4.1.5. Targeting hTERT mRNA

Antisense oligonucleotides (chapter 4.2.1. in Introduction), RNA interference (RNAi) and ribozymes may disrupt *hTERT* mRNA (Fig. 6). For example, hTERT RNAi treatment of carcinoma cells increased their sensitivity chemotherapeutic agents and ionizing radiation [483]. The phosphothiorate anti-hTERT oligomers can cause a rapid loss of tumor cell viability and induce apoptosis independent of telomerase enzymatic

function [484]. Anti-hTERT hammerhead ribozymes have pro-apoptotic effects, again mostly independent of telomere shortening [485], and can also chemosensitize cancer cells [486].

Overall, *hTERT* mRNA disruption seems to prevent not only telomerase-mediated telomere maintenace but also potential hTERT downstream pathways. Thus, two essential cancer cells properties, unrestricted proliferation and protection from apoptosis, can be targeted simultaneously.

4.2. Targeting the RNA component of telomerase (hTR)

The hTR subunit is a core component of telomerase holoenzyme necessary for its catalytic activity. In contrast to hTERT, hTR is constitutively expressed in most cells, but does not seem to have a function in telomerase-negative cells. This makes it a potential target for telomerase inhibition (Fig. 6).

4.2.1. Antisense oligonucleotides

Targeting the template region of the telomerase RNA subunit with antisense oligonucleotides directly inhibits the enzymatic activity of telomerase (Fig. 6). This approach offers several advantages for inhibitor development since the 11-base hTR template sequence (nucleotides +46 to +56) is known [235], easily accessible for oligomer hybridizaton [487,488], and its role in binding and extending telomeres requires the template to be single stranded. Therefore, the inhibition of telomerase by template antagonists is very attractive area of the antitumor research [489]. The other way is to design non-template specific oligonucleotides that disrupt the assembly of the telomerase holoenzyme.

The major drawbacks of the use of oligonucleotides as therapeutic agents are their poor cellular uptake and their low stability in biological environment (they are subjected to degradation by a variety of exo- and endonucleases inside the cell). A number of chemically modified oligomers with improved stability and bioavailability have been synthesized. In spite of their non-natural structural modifications, they still retain most of their biological activity [490-493].

Peptide nucleic acids (PNAs) are DNA or RNA analogues, in which the pentose-phosphate backbone is replaced by an oligomer of N-(2-aminoethyl) glycine,

making them resistant to endo- and exonucleases. This neutral pseudopeptide backbone enables PNAs to bind complementary sequences with high affinity [494,495]. Complementary PNAs have been designed to target specific regions in the telomerase template (hTR) [496]. They can inhibit telomerase activity in cell extract with IC_{50} values in the pico- and nanomolar range [497]. By contrast, inhibition by non-template-directed PNAs has been shown to be dependent on the state of holoezyme assembly. When a non-template-directed PNA was added to the RNA component prior to holoenzyme assembly, inhibition was characterized by a relatively low IC_{50} value (in nanomolar concentrations), whereas no inhibition was detected upon addition of the same PNA toward holoenzyme [498]. Different strategies of enhancing the cellular uptake of PNAs have been examined in order to improve the extent of telomerase inhibition in various cell lines [496,498-501].

2′,5′-Oligoadenylate (2-5A) antisense oligomers were also shown to be potent inhibitors of telomerase. The 2-5A moiety covalently attached to antisense oligonucleotides may enhance the efficacy of the antisense treatment [502,503]. Once the antisense oligomer is hybridized to the targeted hTR, 2-5A tail recruits and activates RNase L along with RNase H, which in turn degrades the telomerase RNA componet [504]. This 2-5A antisense telomerase RNA therapy was used *in vitro* and *in vivo* in several cancer models including intracranial malignant glioma [505], prostate cancer [503], bladder cancer [506], and cervical cancer [507] with promising results.

2′-O-alkyl ribo-oligonucleotides that are complementary to the hTR template with either 2′-O-methyl- [508] or 2′-O-(2-methoxyethyl)-substituted ribose were proved to be highly potent telomerase inhibitors, with IC_{50} values (in cell lysate) at nanomolar concentrations, and inhibited proliferation of prostate cancer cells [509]. The pharmacokinetic properties of these molecules were enhanced by introduction of phosphorothioate (PS) internucleotide linkages at both 3′ and 5′ end because they protected the molecule against the degradation by exonucleases [508,509].

Another class of oligomeric telomerase inhibitors contain **N3′-P5′ phosphoramidate** (NP) and **N3′-P5′ *thio*-phosphoramidate** (NPS) linkages with a variety of 2′-methoxy, 2′-deoxy, 2′-hydroxy, 2′-ribo-fluoro and 2′-arabino-fluoro substituents in the ribose rings [510]. These compounds demonstrated sequence-specific and dose-dependent activity, with the IC_{50} values in the sub-nanomolar concentration

range [510]. An extremely successful representative of this class, the template antagonist oligonucleotide designated GRN163, and its lipid-modified version (GRN163L) were developed by Geron Corporation (California) [511]. GRN163L carries a palmitoyl group at its 5′ end that is attached covalently with a linker to the *thio*-phosphoramidate. This modification increases the cellular uptake and the lipid moiety could interact with the protein subunit of the enzyme enhancing the telomerase inhibitory activity [512]. GRN163L has recently received clearance by the Food and Drug Administration (FDA) to enter human I/II clinical testing (safety and dose studies) for chronic lymphocytic leukemia. Another clinical trial against solid tumors was initiated very recently with GRN163L.

4.2.2. Other approaches to hTR targeting

The other modalities of targeting hTR involve agents that bind RNA/DNA heteroduplex [513], hammerhead ribozymes cleaving the hTR template [514-515], over-expression of mutant hTR template [516], and siRNA-mediated hTR knockdown [517,518]. These directions show interesting progress and could reach the clinical stage in the near future.

4.3. Targeting the telomere

Recently, El-Daly *et al.* [519] reported that high-dose BIBR1532 (30 - 80 $\mu mol.l^{-1}$) interferes with the capping function of telomeres. This direct damage of the structure of individual telomeres must be distinguished from telomerase inhibition accompanied with overall gradual telomere shortening caused by the same compound at lower concentrations (chapter 4.1.2. in Introduction). Moreover, high-dose BIBR1532 (Fig. 7) has a direct cytotoxic effect in leukemia cells but not in normal hematopoietic stem cells or fibroblasts [519], suggesting that longer telomere lengths exert a protective function toward treatment with high-dose BIBR1532. This mechanism that induces immediate rather than delayed growth arrest might be a novel approach for cancer therapy.

Blocking the access of telomerase to the telomere by altering its structure has the potential for more rapid growth-inhibitory effects in tumor cells than “classic“ telomerase inhibition [56,118]. Here lies the role of G-quadruplex stabilizing ligands,

namely trisubstituted acridines (BRACO 19) [520,521], cationic porphyrins (TMPyP4) [522], perylenes (PIPER) [523], bisacridines [524], ethidium derivatives [525], natural products (telomestatin) [526], etc. In particular, telomestatin appears very promising due to its high selectivity toward quadruplexes (compared to other nucleic acid conformations) [527]. This compound most likely causes dissociation of telomere-binding proteins TRF2 and POT1, resulting in a rapid decrease of 3′ overhang and double-stranded telomeric repeats [528,529].

4.4. Targeting telomere-associated proteins

The enzyme tankyrase 1 prevents TRF1 from binding to the telomere (chapter 1.1.2. in Introduction) This eventually results in the disruption of the t-loop structure allowing telomerase to act on the telomere. Seimiya *et al.* [530] demonstrated that pharmacological targeting of tankyrase 1 enhances telomere shortening by means of a telomerase inhibitor and results in earlier crisis of human cancer cells. Thus, simultaneous telomerase and tankyrase inhibition might represent a way to circumvent the problem of the lag phase by application of a telomerase inhibitor alone and tankyrase may be considered as an attractive target for cancer therapy.

Considering the hypothesis that telomerase may represent a suitable target for specific anticancer therapies, several strategies for the inhibition of telomerase have been designed and evaluated. Apart from the above mentioned approaches based on the use of RTIs including nucleoside 5′-triphosphate analogues [442], non-nucleoside molecules (chapter 4.1.2. in Introduction), antisense oligonucletides [503,508], strategies employing the telomerase immunotherapy directed against telomerase positive cells, as well as *hTERT* promoter-driven therapeutic genes (apoptosis-inducing, toxin-encoding, etc.) are currently under investigation [531,532]. These modalities have the advantage of abolishing the lag phase that is required with the classic mode of telomerase inhibition. However, these treatments might also prove to be more toxic to normal cells expressing telomerase. Going into more detailed description of these and other possible ways of telomerase inhibition is beyond the scope of this chapter. For more information about this topic, you can read several up-to-date articles and comprehensive reviews [231,232,431-438,462,533].

6. Nucleoside and nucleotide analogues

6.1. Acyclic nucleoside phosphonates

Acyclic nucleoside phosphonates (ANPs) are nucleotide analogues in which a phosphonate group is linked to a purine or pyrimidine through an aliphatic chain *via* an ether linkage (Fig. 8). Numerous ANPs possess excellent antiviral activities against a broad spectrum of DNA viruses and retroviruses [reviewed in 534,535,536] as well as a significant antiproliferative potency [537-541]. Moreover, their antiparasitic and immunomodulatory effects are subject of an increasing interest [542-550].

Antiviral, antiprotozoal, and antineoplastic activities of ANPs are determined by their structural features, including the type of side-chain structure, heterocyclic base and phosphonate linkage. According to their side-chain structure, PME {N-[2-(phosphonomethoxy)ethyl]}, PMP {N-[2-(phosphonomethoxy)propyl]}, HPMP {N-[3-hydroxy-2-(phosphonomethoxy)propyl]}, and FPMP {N-[3-fluoro-2-(phosphono-methoxy)propyl]} series can be distinguished, each with a different spectrum of biological activity. Compounds of PMP, HPMP, and FPMP series have a chiral carbon atom at the position 2 of the aliphatic chain, therefore, they can form (*S*)- and (*R*)-enantiomers. Their structure-activity relationships are comprehensively reviewed by Holý *et al.* [534].

HPMP-derivatives are active against all DNA viruses [551,552]. They have no *in vitro* effect against RNA viruses and retroviruses. In the HPMP series, the antiviral activity is usually connected with (*S*)-enantiomers. PME-derivatives display activity against DNA viruses [551,552] and retroviruses [553,554]. (*R*)-enantiomers of PMP and FPMP-derivatives show pronounced and selective activity against retroviruses and HBV [555,556]. By contrast, these two structural groups of ANPs are characterized by little or no activity against DNA viruses [557].

All important biological activities found for the ANPs reside in compounds that contain a purine base such as adenine, 2,6-diaminopurine, 2-aminopurine and guanine [534,558,559]. From cytosine derivatives, only HPMP-derivative (*S*)-HPMPC (cidofovir) inhibits the DNA viruses replication [560-562]. The related cytosine ANPs, PMPC and FPMPC, are inactive against all viruses tested. Also uracil and thymine ANPs have no antiviral activity.

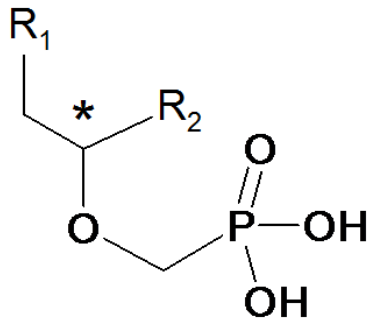
6.1.1. Cellular uptake and activation of ANPs

Biological activity of ANPs depends on their transport through the cellular membrane and subsequent intracellular activation. In cells, ANPs are activated by conversion to their diphosphates, target antimetabolites, which inhibit the viral and/or cellular replicative DNA polymerases and/or terminate nascent DNA chain [552,563,564]. ANP diphosphates are analogues of natural nucleoside 5′-triphosphates since their nonphosphorylated parental forms already bear chemically stable O-phosphonomethyl ether group resistant to enzymatic degradation. Thus, ANPs circumvent the first phosphorylation step that is necessary for the activation of the other nucleoside analogues such as acyclovir or ganciclovir [565]. On the basis of the structural resemblance to natural 2′-deoxynucleoside 5′-triphosphates, ANP diphosphates act as selective substrate/inhibitors of replicative DNA polymerases [563,566-568].

It was reported that the cellular uptake of PMEA, (*S*)-HPMPC and HPMPA occurs *via* an endocytosis-like process, characterized by slow kinetics and temperature-dependence [569-572]. The negative charge of the phosphonate moiety of the ANP compounds significantly impairs their cellular uptake. In order to improve the cellular uptake (and oral bioavailability) of the ANP analogues, ester derivatives have been synthesized that contain a lipophilic group attached to the phosphonate moiety. For example, the bis(pivaloyloxymethyl) ester of PMEA [bis(POM)-PMEA] shows more than 100-fold increase in cellular uptake when compared to parent PMEA [573,574]. Similarly, the problem with the low oral bioavailability of PMPA was solved by conversion to a lipophilic diester bis(isopropyloxycarbonyloxymethyl) PMPA [bis(POC)-PMPA] [575].

In the cells, the cellular nucleoside monophosphate (NMP) kinases, which are disctinct for the pyrimidine and for the purine derivatives, catalyze the first step of the ANP phosphorylation to ANP monophosphates (ANPp). This process is enantiospecific, *i.e.* the absolute configuration at the side chain is important for this reaction [576-579]. The enantiospecificity of nucleoside kinases may result in the enantioselectivity of biological effect [556]. For example, (*S*)-HPMP derivatives of adenine and cytosine are active against DNA viruses. Their (*R*)-counterparts do not exert any biological activity and, indeed, are not substrates for cellular NMP kinases. Following the second

phosphorylation to the corresponding diphosphates (ANPpp) by nucleoside diphosphate (NDP) kinases, they can be incorporated into DNA [576,578-580]. In contrast to PMEApp, PMPApp or PMEDAPpp, which are DNA-chain terminators, (*S*)-HPMPApp or (*S*)-HPMPCpp contain the hydroxy group which permits certain DNA-chain elongation *de novo* associated with the incorporation of several analogue molecules [566,567].



R_1 - adenine, guanine, cytosine, diaminopurine

R_2 - CH_3 (PMP series)
- CH_2OH (HPMP series)
- H (PME series)

Figure 8. **General structure of ANPs.**

6.1.2. Biological activity of selected ANPs

PMEA {9-[2-(phosphonomethoxy)ethyl]adenine} (adefovir, ADV) (Fig. 10) displays activity against DNA viruses, such as herpes simplex virus type 1 (HSV-1), HSV-2, vaccinia virus (VV) [581], hepatitis B virus (HBV) [582] and a broad variety of retroviruses, such as HIV type 1 (HIV-1), HIV-2, simian immunodeficiency virus (SIV) [553,554,583]. Moreover, its significant cytostatic potency against various neoplasias [537,538] and immunomodulatory effects [547] have been also reported. Although originally developed as an anti-HIV drug, its oral prodrug, adefovir dipivoxil (Hepsera™), was approved for treatment of hepatitis B in 2002. PMEA inhibits relatively poorly DNA polymerases alpha and epsilon and exerts only moderate inhibition of polymerase delta [563]. Additionally, PMEA, along with PMEG and PMEDAP, perturb DNA replication by terminating the growing DNA chain and suppress thereby the cell growth at low concentrations while inducing apoptosis at higher concentrations [584]. PMEA has been shown to be a strong inducer of differentiation in several tumor cell lines including human erythroleukemia K562 and

human HL-60 myeloid leukemic cells [585] Furthermore, this compound was found to have a strong differentiation-inducing effect on rat choriocarcinoma (RCHO) cells *in vitro* [586] and to inhibit growth of choricarcinoma tumor *in vivo* [587].

PMEG {9-[2-(phosphonomethoxy)ethyl]guanine} (Fig. 10) possesses significant antiviral [588] and antitumor properties [539]. It has demonstrated anticancer activity in a number of *in vitro* and *in vivo* animal model systems [539]. On the other hand, this compound was shown to be the most cytotoxic of the ANP analogues studied [589]. Its diphosphate (PMEGpp) is the most efficient inhibitor of cellular DNA polymerases α and ε [563,590].

PMEDAP {9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine} (Fig. 10) displays extensive antiviral and cytostatic activity [591,592]. PMEDAP is phosphorylated by cellular kinases to its diphosphate [580,593], which strongly inhibits DNA polymerase δ [563]. Antitumor activity of PMEDAP was examined *in vivo* on a model of spontaneous T-cell lymphoma in inbred Sprague-Dawley (SD/cub) rats [540]. Another study reported that the therapeutic effect of PMEDAP depends on the phenotype of the individual SD/cub neoplasia [594]. In the same model, the antitumor effect of the combined treatment of docetaxel with PMEDAP was significantly higher than that of docetaxel or PMEDAP alone [595]. The effect of PMEDAP on the cell cycle [584] and its capability to induce apoptosis [584,594,596] was also investigated. Unlike PMEDAP, its N^6-mono and disubstituted congeners are less effective or exhibit the same antitumor efficacy in spontaneous T-cell lymphoma in inbred SD/cub rats as PMEDAP [597].

(*R*)-PMPA {(*R*)-9-[2-(phosphonomethoxy)propyl]adenine} (tenofovir, TDV) (Fig. 10) acts as a reverse transcriptase inhibitor. Tenofovir proved selective inhibitory effect on HIV-1 and 2, feline immunodeficiency virus and Moloney murine sarcoma virus (MSV) in cell cultures; its selectivity is higher than that of PMEA [598]. Tenofovir has also demonstrated a potent anti-SIV activity in rhesus macaques [599]. Its oral prodrug, tenofovir disoproxil fumarate, was approved for treatment of HIV infection in 2001 (Viread™, Truvada™, Atripla™). (*R*)- and (*S*)-enantiomers of PMPA greatly enhance the secretion of tumor necrosis factor-alpha (TNF-α) and interleukin-10, factors known to play a role in HIV virus replication [544].

(*S*)-HPMPA {(*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine} (Fig. 10) is very active against DNA viruses [551,600] and against higher cellular parasites - protozoa (Plasmodium, Leishmania, Trypanosoma spp., etc) [548-550]. It is not significantly active against RNA viruses, including human immunodeficiency virus (HIV). HPMPApp is a selective and potent inhibitor of DNA polymerases δ and ε [563].

(*S*)-HPMPC {(*S*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine} (cidofovir, CDV, Vistide™) is a potent and selective anti-DNA virus agent, suppresses the *in vitro* growth of all human and animal DNA viruses thus far examined (herpesviruses, adenoviruses, papillomaviruses, etc.) [560,601]. (*S*)-HPMPC proved to be a selective and potent anti-cytomegalovirus (CMV) agent and was officially approved for intravenous treatment of CMV retinis in AIDS patients [561]. Several groups have shown that treatment with cidofovir restores cellular p53 and pRb levels in HPV-associated cervical cancers, thus slowing cell proliferation and increasing the susceptibility of the cancer cells to radiation and apoptosis [602,603].

6.2. Nucleoside-type DNA methylation inhibitors

Epigenetic modifications, like DNA methylation, play an important role in the regulation of gene expression in normal and cancer cells. DNA methylation patterns are established during development and maintained throughout the life of an individual by a family of enzymes called DNA methyltransferases (DNMTs) [604]. They methylate DNA at the 5-position (C5) of the cytosine ring. This is the only common covalent modification of human DNA and occurs almost exclusively at cytosines that are followed immediately by a guanine (so-called CpG dinucleotides). Promoter regions of approximately 50% of genes are located within small stretches of DNA rich in CpG dinucleotides, known as CpG islands, which are nearly always free of methylation [605]. This pattern is disrupted in cancer cells where CpG islands in the promoter regions of various genes (especially tumor-suppressor genes) become hypermethylated and the corresponding genes become transcriptionally silenced [606,607]. Treatment of cancer cells with hypomethylating agents can re-establish normal DNA methylation

patterns and reactivate genes that are involved in cell cycle regulation, apoptosis, DNA repair, differentiation, etc [605,608-611].

The most well characterized and widely used drugs to inhibit DNA methylation and reactivate silenced genes are nucleoside analogues 5-azacytidine (5-azaCyd) and 5-aza-2'-deoxycytidine (β-5-azadCyd, decitabine) (Fig. 9) [608,612,613]. Both compounds have a nitrogen in place of a carbon at position 5 of the pyrimidine ring (Fig. 9) and are unstable in neutral aqueous solutions [614]. β-5-AzadCyd has been recently approved for the treatment of myelodysplastic syndromes (MDS) [615]. This compound, after activation by cellular kinases to its 5'-triphosphate, is incorporated into DNA, where it produces an irreversible inactivation of DNA methyltransferase [616]. Recent work has also suggested that DNMT1 becomes a target for proteasomal degradation following β-5-azadCyd treatment [617]. Drug resistance to β-5-azadCyd occurs primarily by reduction in deoxycytidine kinase activity or increase in the activity of cytidine deaminase, the enzyme that inactivates this analogue [618]. An approach to overcome this obstacle is to use β-5-azadCyd in combination with zebularine, a potent inhibitor of cytidine deaminase [619]. Less cytotoxic and more stable alpha anomer (α-5-AzadCyd) appeared to hypomethylate genomic DNA to a similar extent as the widely used beta form [620]. α-5-AzadCyd itself is not incorporated into DNA and is not degraded by cytidine deaminase. Its biological activity is based on the spontaneous conversion into the beta anomer that enters the DNA synthesis pathway [621]. α-5-AzadCyd exhibits significant antileukaemic activity, although at higher concentrations than beta anomer [620].

In contrast to α- and β-5-azadCyd, a cytidine analogue zebularine [1-(beta-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one] (Fig. 9) is a chemically stable DNA demethylating agent of low toxicity and the first drug in its class able to reactivate an epigenetically silenced gene by oral administration [611,622]. Zebularine is a potent inhibitor of cytidine deaminase with anticancer properties [623]. Zebularine and 5-fluoro-zebularine (F-PymRf) have been shown to bind at the active site of cytidine deaminase as covalent hydrates [624]. F-PymRf has been shown to be the most potent inhibitor of cytidine deaminase among a number of zebularine analogues [623]. Up to now, structurally related (*S*)-HPMPazaC and F-PymRf have not yet been studied for

their DNA hypomethylating capabilities. Both of them are putative DNA methylation inhibitors.

Both non-specific methylation inhibitors (*S*)-DHPA and (*R,S*)-AHPA-*ibu* (Fig 9) hypomethylate DNA *via* SAH-hydrolase inhibition [625]. SAH-hydrolase is essential to maintain the methylation capacity of the cell. The enzyme eliminates S-adenosyl-L-homocysteine (SAH), the product of methyltransferase reactions, which acts as methyltransferase inhibitor. S-adenosyl-L-methionine (SAM) mediated methylations represent most of all methylation processes in proliferating cell. (*S*)-DHPA is a catabolically stable open-chain adenosine analogue which acts as a reversible competitive inhibitor of SAH-hydrolase [626]. This compound showed potent antiviral activity [627] - it inhibits mainly RNA viruses by interfering with capping of viral mRNA [628]. In contrast to (*S*)-DHPA, (*R,S*)-AHPA-*ibu* is an irreversible inhibitor of SAH-hydrolase [629].

Several groups suggested that DNA methylation participates in regulation of telomerase activity (chapter 3.3.2. in Introduction). For example, in cancer cell lines with hypermethylated *hTERT* promoter, treatment with β-5-azadCyd led to promoter hypomethylation up to 95%, which strongly decreased *hTERT* mRNA [397]. Several other groups also observed that β-5-azadCyd and/or 5-azaCyd treatment of telomerase-positive cells caused a down-regulation of *hTERT* expression in several cancer cell lines [395,398,399]. This compound was also shown to activate *p16* and other methylated tumor-suppressor genes [609]. Kitagawa *et al.* [398] indicated that up-regulation of *p16* and subsequent down-regulation of c-*myc* might be another possible pathway for *hTERT* repression by 5-azaCyd.

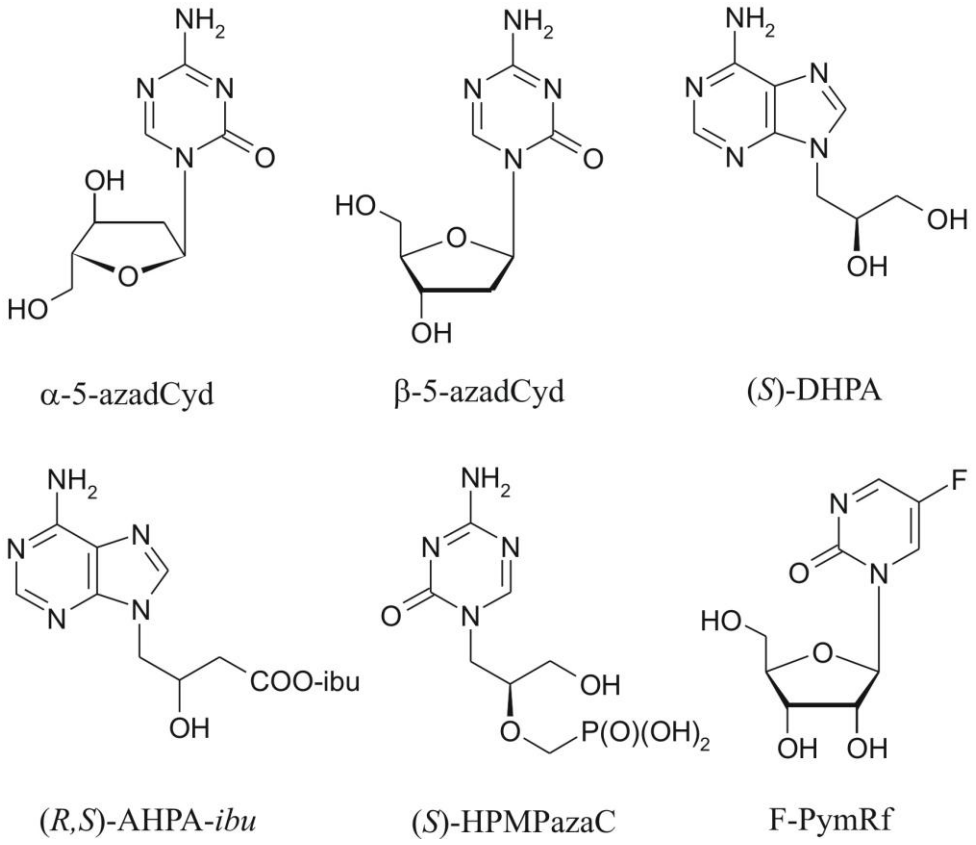


Figure 9. **Structures of hypomethylating agents.**

OBJECTIVES OF THE THESIS

(1) Evaluation of inhibitory potency of ANP diphosphates towards human telomerase *in vitro* using the PCR-based TRAP assay.

(2) With regard to a significant influence of several ANP diphosphates on telomerase activity/processivity in *in vitro* experiments, I further aimed to investigate whether the observed effects have consequences on actual telomere length which represents a limiting factor of the cell proliferative capacity.

(3) Comparison of the capability of tested nucleoside analogues to down-regulate *hTERT* expression in human leukemia HL-60 cells with a special focus on α-5-azadCyd as a compound with a potential antileukemic activity.

MATERIALS AND METHODS

1. Compounds

The following compounds were used for evaluation of their telomerase inhibitory potency (Fig. 10): (*S*)-HPMPApp, (*R*)-HPMPGpp, PMEApp, PMECpp, PMEGpp, PMETpp, PMEDAPpp, PMEO-DAPypp, 6-Me_2PMEDAPpp, (*R*)-6-cyprPMPDAPpp, (*R*)-PMPDAPpp, (*R*)-PMPApp, (*S*)-PMPApp, (*R*)-PMPGpp, (*S*)-PMPGpp. (*S*)-HPMPA and (*R*)-HPMPG diphosphates were synthesized according to Otmar *et al*. [630]. Other ANP diphosphates were synthesized by the modified morpholidate method. In a typical experiment, a mixture of ANP (free acid, 1 mmol), N,N'-dicyclohexylcarbodiimide (1.3 g) and morpholine (2 ml) in 80% aqueous *tert*-butanol (20 ml) was refluxed under stirring for 6-8 hours and evaporated in vacuo. The residue in water (100 ml) was filtered over Celite®, the filtrate extracted with ether (3 x 50 ml) and the aqueous phase was taken down in vacuo. The residue was transferred into 100-ml flask, evaporated, codestilled with ethanol (2 x 20 ml) and dried overnight at 15 Pa over phosphorus pentoxide. Bis(tributylammonium) monophosphate or tris(tributylammonium) diphosphate solution in dry dimethylsulfoxide (1 $mol.l^{-1}$, 2.5 ml) was added and the mixture was stirred at room temperature in a tightly closed flask for 4 - 6 days. Reaction mixture was then acidified with 6 M HCl to pH 3 and an appropriate amount of activated charcoal was added. After an exhaustive washing of pelleted activated charcoal with HPLC water, desalted nucleotides were eluted by 5% NH_4OH in 50% methanol. The eluate was evaporated at 30 ^oC, dissolved in 0.05 M triethylammonium bicarbonate and purified by chromatography on POROS® 50HQ anion exchanger (Applied Biosystems, Foster City, CA, USA) in the linear concentration gradient of triethylammonium bicarbonate (0.05-0.4 $mol.l^{-1}$). Peak corresponding to ANPpp (triethylammonium salt) was collected, evaporated in vacuo at room temperature and then converted to the ANPpp sodium salt on DOWEX™ 50X8 (Na^+) (SERVA Electrophoresis GmbH, Heidelberg, Germany).

The synthesis of the hypomethylating agents, α- and β-5-azadCyd, (*S*)-DHPA, (*R,S*)-AHPA-*ibu*, F-PymRf, and (*S*)-HPMPazaC (Fig. 9), has been described previously [621,623,631-633].

All other chemicals and materials were commercial products, e.g. activated charcoal, N,N'-dicyclohexylcarbodiimide, morpholine, *tert*-butanol, Celite®, dimethylsulfoxide, ddGTP, streptomycin, penicillin G, CHAPS, □-mercaptoethanol, RNase A, proteinase K, propidium iodide, Triton X®-100, PBS and RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA), fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), vitamin B_{12} (Léčiva a.s., Prague, Czech Republic), Pefabloc-SC, Protector RNase inhibitor (Roche Diagnostics GmbH, Mannheim, Germany), [γ-^{32}P]ATP (MP Biomedicals GmbH, Germany), T4 Polynucleotide Kinase Buffer and T4 Polynucleotide Kinase (TaKaRa Bio, Inc., Shiga, Japan), HEPES, deoxynucleoside triphosphates (dNTPs), Taq polymerase reaction buffer, Taq DNA polymerase (Promega, Madison, WI, USA) and TS, ACX, NT, TSNT primers and primers listed in Table 3 (Invitrogen Ltd, Paisley, United Kingdom). Telomere length measurement was performed using Telomere PNA Kit/FITC for Flow Cytometry (DakoCytomation, Denmark).

2. Cell culture

The human acute promyelocytic leukemia HL-60 cells (ATCC CCL 240) were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, antibiotics (200 μg/ml of streptomycin and 200 units/ml of penicillin G), 10 mM 2-mercaptoethanol, and vitamin B_{12} at 37 ºC in a humidified atmosphere containing 5% CO_2.

The human T-cell acute lymphoblastic leukemia CCRF-CEM cells (ATCC CCL 119) were cultivated in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, antibiotics (200 μg/ml of streptomycin and 200 units/ml of penicillin G) and 3 mM glutamine at 37 ºC in a humidified atmosphere containing 5% CO_2.

3. Measurement of telomerase activity using a PCR-based TRAP asssay

3.1. Preparation of cell lysates

Extracts with telomerase activity were prepared and analyzed as described [205] with some modifications. Briefly, after harvesting in log-phase growth, cells were pelleted, washed in PBS and incubated on ice for 30 min in CHAPS lysis buffer containing 0.5% CHAPS, 10 mM HEPES-NaOH (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 5 mM 2-mercaptoethanol, 2 mM Pefabloc-SC, 10% glycerol and 1 μl (40 U) of Protector RNase inhibitor. 200 μl of CHAPS lysis buffer was used to lyse one million cells. In order to ensure lysis of HL-60 cells, these cells were subjected to two freeze/thaw cycles during the incubation. This additional step did not affect telomerase activity in the HL-60 cells. Cell debris was pelleted (20 min, 16 000g, 4 ºC) and the supernatant was removed, aliquoted, frozen on dry ice, and stored at -70 ºC. The protein concentration of the supernatant was determined by the Bradford assay.

3.2. TRAP asssay

Telomerase activity was determined using the TRAP assay as described by Kim *et al.* [306] with the modifications described below. In the present study, the sensitivity of the TRAP assay was increased by the prolongation of incubation time from 10 to 15 min and by increasing the number of PCR cycles from 27 to 33. The dependence of the amount of amplified telomerase product versus number of PCR cycle was linear in the range of 30 to 35 cycles (data not shown). An aliquot of 800 pmol of TS substrate primer (5'-AATCCGTCGAGCAGAGTT-3') was labeled in 100 μl reaction mixture containing 2.22 MBq [γ-^{32}P]ATP (2.22 GBq/ml, 2.59 x 10^5 GBq/mmol), T4 polynucleotide kinase buffer and 40 U T4 polynucleotide kinase. After 30 min incubation at 37 ºC and then 2 min at 85 ºC, an excess of unincorporated [γ-^{32}P]ATP was removed from reaction mixture on MicroSpin™ G-25 Column (Amersham Biosciences, Piscataway, NJ, USA). Forty microlitre TRAP reactions contained Taq polymerase reaction buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25 ºC), 1.5 mM $MgCl_2$, 0.1% Triton X®-100), dNTPs (30, 60 and 125 $\mu mol.l^{-1}$), 18 pmol of end-labeled TS substrate primer, an appropriate amount of studied ANPpp, ANPp and ANP

respectively. Reaction was started by cell extract addition (0.15 μg protein). Each TRAP reaction mixture was placed in a thermocycler block preheated to 30 ºC and incubated at 30 ºC for 15 min and then heated at 95 ºC for 2 min (for one cycle) to stop telomerase reaction. After addition of 10 μl of mixture containing 6 pmol ACX reverse primer (5'-GCGCGG[CTTACC]$_3$CTAACC-3'), 3 pmol NT internal control primer, 0.01 amol TSNT internal control and 1.25 U Taq DNA polymerase, the reaction was cycled 33 times at 94 ºC for 20 s, 52 ºC for 30 s and 72 ºC for 20 s.

To ensure that the observed telomerase activities were really dependent on telomerase, numerous of inactivation experiments were performed. Aliquot of cell lysate was incubated with RNase A (50 μg/ml) at 37 ºC for 30 min. Proteinase K and heat-inactivated cell extracts were prepared by incubating the cell extract with proteinase K (50 μg/ml) at 37 ºC for 30 min and by heating 10 μl extract at 75 ºC for 10 min prior to assaying of 3 μl by TRAP assay. HL-60 cell extract showed telomerase activity with the characteristic primer extension-binding pattern on the autoradigraphs. We considered the sample being positive for telomerase activity if the signal had disappeared after RNase A treatment and if no signal was detected in the lysis buffer alone (negative control). RNase A and proteinase K treatments abolished the PCR product ladder bands and confirmed both the protein and RNA dependence of the enzyme activity.

3.3. Analysis of reaction products and quantitation of telomerase activity

The amplified telomerase products were analyzed on a denaturating 15% polyacrylamide - 7 M urea sequencing gel at 1,900 V for 2 h with Tris-borate-EDTA. Dried gels were exposed to a PhosphorImager storage screen and the amount of reaction products was evaluated using TYPHOON™ 9410 imager and ImageQuant™ software (Molecular Dynamics, Sunnyvale, CA, USA). To compare relative telomerase activity in the presence of inhibitors, the TRAP assay signals of the telomerase ladder in each lane were normalized to the signal of the corresponding internal standard after background substraction. The normalized total intensity of the telomerase products from ANPpp, ANPp and ANP - treated samples was expressed as a percentage of the signal intensity detected in the control. Their relative intensities were calculated with the ImageQuant™ software. In this manner, the concentration of each ANP required to

produce a 50% reduction of telomerase activity (IC_{50}) relative to the control was determined. All results were expressed as mean ± SD of the four independent determinations.

4. Analysis of *hTERT* mRNA expression in HL-60 cells

4.1. Treatment of HL-60 cells with hypomethylating agents

24 h after seeding at a concentration of 100,000 cells/ml, HL-60 cells were treated with various concentrations of freshly prepared solutions of hypomethylating agents, α- and β-5-azadCyd, (*S*)-DHPA, (*R,S*)-AHPA-*ibu*, F-PymRf, and (*S*)-HPMPazaC (Fig. 9). After the 72 h treatment, the cells were pelleted, washed with phosphate-buffered saline (PBS) and collected for RNA extractions (as described below). Three independent treatments with each compound were performed.

4.2. RNA extraction

Total cellular RNAs were extracted from 1-1.5 million of control (untreated) and treated HL-60 cells using the RNeasy Mini isolation kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Genomic DNA was eliminated by RNase-free DNase I digestion (Qiagen) during the isolation procedure. The concentration and purity of the RNA samples was assessed by measurement of the UV absorption at 260 nm and by the absorption ratio of 260 to 280 nm, respectively. The exact quantification of the RNA was carried out in triplicate using the Quant-iT™ RiboGreen® RNA assay kit (Invitrogen, Eugene, Oregon, USA) as described by the manufacturer. RNA samples were stored at -70 °C.

4.3. One-step real-time qRT-PCR

This assay was based on TaqMan methodology. Primers (Invitrogen) and probe (Generi-Biotech, Hradec Králové, Czech Republic) nucleotide sequences for *hTERT* (GenBank accession number **AF015950**) were: forward primer 5'-CACGCGAAAACC-TTCCTCA-3' (placed in exon 10; nt 2,690 to 2,708), reverse primer 5'-CAAGTTCACCACGCAGCC-3', and TaqMan probe 5' (FAM)-CTCAGGGACACC-TCGGACCAGGGT-(BHQ1) 3' (both placed in exon 11; nt 2,755 to 2,738 and 2,734 to

2,711, respectively) [634]. SuperScript™ III Platinum® One-Step Quantitative RT-PCR system (Invitrogen) was used to amplify the *hTERT* mRNA according to the manufacturer's protocol. PCR was performed in a total volume of 50 μl containing 1x TaqMan buffer, 4 mM $MgSO_4$, 200 nM each primer, 100 nM probe, 40 ng of total RNA, 20 U of Protector RNase inhibitor. The thermal cycling conditions included 15 min at 50 °C and 2 min at 95 °C for the reverse transcription step, followed by 40 cycles of 95 °C for 15 s and 60°C for 1 min. All samples were amplified in triplicate using a DNA Engine Opticon® 2 (Bio-Rad, Hercules, CA, USA) and the mean Ct value was obtained for futher calculations. Normalization of *hTERT* mRNA levels was performed against total RNA in the reaction mixture.

4.4. cDNA synthesis

Total RNA (500 ng) was reverse transcribed using SuperScript™ II Reverse Transciptase kit (Invitrogen) according to the manufacturer's instructions in a total volume of 20 μl containing 0.5 μg oligo$(dT)_{12-18}$ primer, 0.5 mM deoxynucleotides, 10 mM DTT, and 40 U of Protector RNase inhibitor. Tubes were heated to 65 °C for 5 min to denature the secondary RNA structure. The RT reaction was completed by adding 200 U SuperScript™ II Reverse Transciptase before incubation at 42 °C for 50 min and then 70 °C for 15 min. cDNA samples were stored at -70 °C.

4.5. Two-step real-time qRT-PCR

Quantification of c-*myc* and *hTERT* mRNA expression was performed by qRT-PCR using the DNA Engine Opticon® 2 and DyNAmo™ SYBR® Green qPCR kit (Finnzymes Oy, Espoo, Finland). Reactions were done in triplicate, each 20 μl PCR reaction mixture contained 4 μl (<10 ng/μl) of cDNA template, 6 pmol of each primer (primer sets listed in Table 3) [634] and 10 μl of DyNAmo™ SYBR® Green qPCR mix supplemented with the kit. The thermocycling program included: an initial denaturation at 95 °C for 15 min to ensure a complete reactivation of the hot start DNA polymerase; 40 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The specifity of an amplified product was checked by melting curve analysis. The melting protocol called for heating from 65 °C to 90 °C, holding for 10 s at each temperature, with increases of 0.5 °C per step. *hTERT* and c-*myc* expression levels were normalized by dividing the

raw *hTERT* and c-*myc* quantities for each sample by the appropriate normalization factor calculated as the geometric mean of the 4 most stable housekeeping genes (GAPDH, RPII, TBP and PLA) selected using a geNorm Visual Basic Application (VBA) for Microsoft Excel (freely available at http://medgen.ugent.be/~jvdesomp/genorm/). The underlying principles and calculations are described in Vandesompele *et al.* [635].

Table 3

Sequences of PCR primers in two-step real-time qRT-PCR.

	Sequence 5'-3'	
Gene	Sense	antisense
GAPDH	gAAggTgAAggTCggAgTC	gAAgATggTgATgggATTTC
RPII	gCACCACgTCCAATgACAT	gTgCggCTgCTTCCATAA
TBP	TTCggAgAgTTCTgggATTgTA	TggACTgTTCTTCACTCTTggC
G6PDH	ATCgACCACTACCTgggCAA	TTCTgCATCACgTCCCggA
PBGD	ggCTgCAACggCggAA	CCTgTggTggACATAgCAATgATT
PLA	AAgTTCTTgATCCCCAATgCTT	gTCTgATAggATgTgTTggTTgC
β-actin	TCCTTCCTgggCATggAg	AggAggAgCAATgATCTTgATCTT
hTERT	TgACACCTCACCTCACCCAC	CACTgTCTTCCgCAAgTTCAC

5. Determination of SAH and SAM levels

For determination of S-adenosyl-L-homocysteine (SAH) and S-adenosyl-L-methionine (SAM) levels cells were harvested 72 h after the addition of SAH-hydrolase inhibitors [(*S*)-DHPA, (*R,S*)-AHPA-*ibu*] into the culture medium. The PBS-washed cell biomass (6 x 10^6 cells) was extracted using 0.25 M perchloric acid at 4 °C and clarified by centrifugation. The acid-soluble extract was analyzed in the Aliance Waters HPLC system (996 PDA Detector, PDA Software Millenium, version 4.0) equipped with 15 cm x 4.6 mm SUPELCO Discovery C8, 5 μm reverse-phase column. A three-step gradient at a flow rate 0.9 ml/min was used. With (1) solvent A, 50 mM sodium phosphate pH 3.2, 10 mM heptanesulfonic acid, 50% acetonitrile; (2) solvent B, 50 mM

sodium phosphate pH 3.2, 10 mM heptanesulfonic acid. The program consisted of (1) 9-11% A, 5 min (convex curve no. 3); (2) 11-17% A (concave curve no. 8), 10 min; (3) 100% A, 5 min (curve no. 11, isocratic). Peaks of SAH and SAM were identified (UV-spectra library) and quantified with the aid of external standards. The concentration of both cofactors was determined relative to the protein content.

6. Flow cytometric methods

6.1. Analysis of DNA content

HL-60 cells were plated in 25 cm^2 flasks at a density of 1 x 10^5 cells per mililiter of media. 24 h after seeding at a concentration of 100,000 cells/ml, cells were treated with various concentrations of nucleoside analogues and incubated for additional 72 h. They were then collected by centrifugation, and resuspended with PBS. Cells were centrifuged at 1,000g for 5 min, washed again with PBS, and then fixed with 70% ice-cold ethanol for 30 min. To stain with propidium iodide, fixed cells were sedimented by centrifugation, washed in PBS and treated with RNase A (500 μg/ml) at 37 °C for 30 min, and finally incubated for 1 h with a staining solution containing 0.1% Triton X®-100 and propidium iodide (100 μg/ml) in PBS at a final cell concentration of 1 x 10^6 cells/ml. Cellular DNA content was determined using a flow cytometer FACSAria (BD Biosciences, San Jose, CA, USA). At least 30,000 cells were used for each analysis, and the results were displayed as histograms. Cell cycle distribution was analyzed using ModFit LT 3.0 (Verity Software House, Topsham, ME, USA) program.

6.2. Flow-FISH analysis of telomere length

Cells were passaged for 11 weeks in the presence of either 0.75 μM PMEG, 20 μM PMEDAP and 100 μM (*S*)-PMPA in the growth medium. Then, the cells were centrifuged and resuspended in the fresh medium without ANPs for additional 2 weeks.

The telomere length analysis was performed using a Telomere PNA Kit/FITC for Flow Cytometry (Cat. No. K5327; DakoCytomation, Denmark) according to manufacturer's instructions. The procedure consisted of fluorescence *in situ* hybridization

(FISH) and subsequent flow cytometric analysis. As control cells we used the cell line 1301. The 1301 cells serve as an internal standard and are very easy to distinguish from most other cell types because of its characteristic features: Tetraploidy (seen in PI channel) and very long telomeres (> 30 kb) (seen in FITC channel). In principle, other cell types that are easily distinguished from the sample cells can be applied.

Fluorescence *in situ* hybridization (FISH)

After harvesting, the cells were pelleted, washed twice in PBS and counted in Bürker chamber. For each sample, 2 x 10^6 test cells (CCRF-CEM) and 2 x 10^6 control cells 1301 were mixed and PBS was added to a total of 6 ml. The mixture was divided into four 1.5 ml aliquots (1 x 10^6 cells) and centrifuged at 500g for 5 min. Then, the cell pellets were properly resuspended in the hybridization solution with (2 samples) and without (2 samples) the fluorescein-conjugated PNA telomere probe. After 10 minutes of incubation in water bath at 82 °C, the samples were hybridized in the dark at room temperature overnight. Next day, the cells were washed twice at 40 °C with 1 ml of washing solution and finally, 0.5 ml of PI-staining solution was added to each of the 4 tubes. They were kept in dark at + 4° C for at least 3 hrs until flow cytometric analysis.

Flow cytometry

The flow-FISH analysis was performed using a FACSAria flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). No compensation was set on the instrument. List mode data from 10^4 cells were collected in each experiment and analyzed using the DIVA software (Becton Dickinson). To get the correct probe signal value, the autofluorescence of each cell population, detected in parallel runs with no probe added, was subtracted from the fluorescence signal with the PNA probe. The RTL (relative telomere length) is an arbitrary value for the telomere length of the test sample derived from the ratio between the signal intensity in G_0/G_1 gates from the test sample and the 1301 cell line after compensation for autofluorescence and DNA index.

The RTL value was calculated using the following formula:

RTL value = [MF(test)-AF(test)/MF(1301)-AF(1301)] x DI (1301)/DI (test)

MF(test) Mean fluorescence for test sample in G_0/G_1

MF(1301) Mean fluorescence for 1301 cell line in G_0/G_1

AF(test) Mean autofluorescence for test sample in G_0/G_1

AF(1301) Mean autofluorescence for 1301 cell line in G_0/G_1

DI(test) DNA index for test sample

DI(1301) DNA index for 1301 cell line

RESULTS

1. Inhibition of telomerase activity by ANPs

Cytostatic and antiviral effects of ANPs result from DNA polymerase and/or reverse transcriptase inhibition by their diphosphates (chapter 6.1. in Introduction). In this study, representatives of PME, PMP and HPMP series were also evaluated for their human telomerase inhibitory potency. To measure telomerase activity in the HL-60 cell extract, an advanced TRAP assay was used that could also estimate the processivity (the total number of telomeric repeats added to a DNA substrate) of telomerase [306]. This is a reliable and reproducible protocol that has several advantages over the original TRAP assay [205] (chapter 3 in Materials and Methods). First of all, the method employs so-called "anchored return primer" designated ACX, which prevents 3' elongation of telomerase products by capping the 3' end of a telomerase product after the first PCR cycle. Thus the length of products produced in a TRAP assay accurately reflects the processivity of the telomerase activity being tested. Moreover, primer ACX is relatively resistant to primer dimer artifacts formation and if they incidentally form, then they are easily distinguished from the genuine telomerase products. IC_{50} values represent 50% telomerase inhibitory concentrations and are determined as described under Materials and Methods, chapter 3.3.

1.1. Inhibition by PME-derivatives

The approximate IC_{50} values for the PME-derivatives are shown in Table 4, where they are listed in the order of their telomerase inhibitory potency: PMEGpp > PMEDAPpp > PMEO-DAPypp > PMEApp > PMECpp ≥ PMETpp > 6-Me_2PMEDAPpp.

The guanine derivative PMEGpp is the most potent telomerase inhibitor among all acyclic nucleotide analogues studied with the IC_{50} 12.7 ± 0.5 $\mu mol.l^{-1}$ at 125 μM dNTPs (Table 4, Fig. 11). Its inhibitory potency towards telomerase is comparable to that of ddGTP (IC_{50} 8.1 ± 0.4 $\mu mol.l^{-1}$ at 125 μM dNTPs), which is known to be one of the most effective nucleotide analogue based telomerase inhibitors. PMEGpp inhibits telomerase activity by 50% when it is present in the dGTP concentration ratio of 0.08 to

0.12 only, depending on the dNTPs concentration. The PMEG monophosphate and PMEG itself do not show at 125 μM dNTPs any effect on telomerase activity up to the concentration of 300 μM PMEG and/or PMEGp, respectively.

PMEDAPpp, which selectively inhibits DNA polymerase δ and exerts significant cytostatic effects [540,563], inhibits the activity of telomerase with IC_{50} 76 ± 13.5 $\mu mol.l^{-1}$ (at 125 μM dNTPs). Surprisingly, its N^6-dimethyl derivative 6-Me_2PMEDAPpp increases repeat addition processivity of the enzyme (Fig. 12A). 6-Me_2PMEDAP has no effect on telomerase ladder pattern.

Neither, PMEApp, PMETpp and PMECpp, which inhibit retroviral reverse transcriptases [534], show any significant inhibitory potency towards telomerase. An inhibition was observed also with PMEO-DAPypp; this open ring ANP is considered to be a PMEDAPpp analogue (Fig. 10).

Inhibitory potency of PMEGpp and PMEDAPpp towards telomerase is consistent with the capability to induce apoptosis, strong cytostatic efficiency, and anticancer activity of their parental compounds PMEG and PMEDAP [539-541,584,591,594,596]. The growth of HL-60 cells is inhibited *in vitro* by PMEG by 50% at the concentration of 2.5-3 $\mu mol.l^{-1}$, while the GI_{50} (growth inhibition concentration) for PMEDAP is 15 - 20 $\mu mol.l^{-1}$. The GI_{50} values for (*S*)-PMPG, PMEO-DAPy and PMEA are 22, 30 and 30 $\mu mol.l^{-1}$, respectively. The lymphoid cell line CCRF-CEM is more susceptible to these agents than the myeloid cell line HL-60. Incubation of CCRF-CEM leukaemia cells with PMEG, PMEDAP, PMEO-DAPy, PMEA and (*S*)-PMPG inhibits cell growth at GI_{50} of 1, 7, 14, 25 and 29 $\mu mol.l^{-1}$, respectively [534].

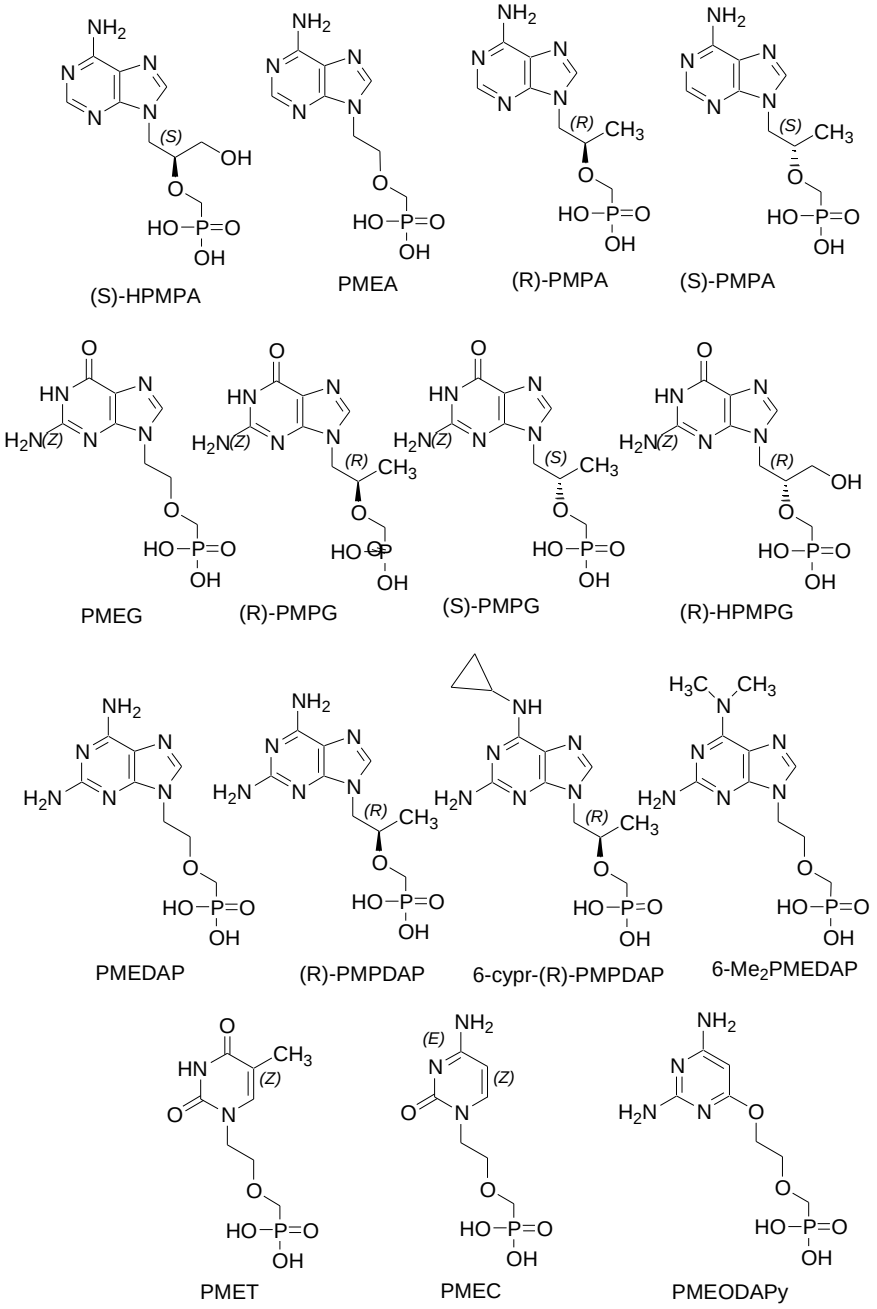


Figure 10. **Structure of PME, PMEO, PMP, and HPMP purines and pyrimidines.**

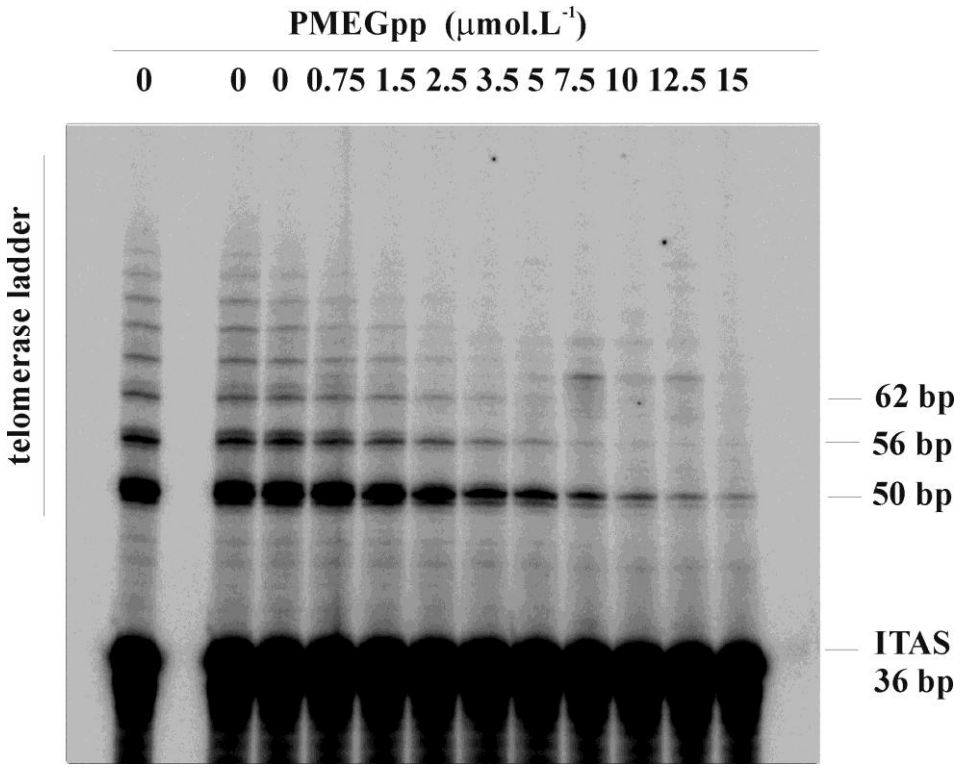


Figure 11. **Inhibition of telomerase by PMEGpp.** Telomerase activity was measured in HL-60 cell extract in the presence of 30 μmol.l^{-1} dNTPs and increasing concentrations of PMEGpp (0 - 15 μmol.l^{-1}). ITAS - internal standard. The primer dimer PCR artifacts derived from TS and ACX primers, which are present in lanes corresponding to PMEG concentrations of 7.5, 10 and 12.5 μmol.l^{-1}, lack the typical 6 bp periodicity and can be easily distinguished from the telomerase products.

Table 4

Inhibition of HL-60 telomerase by PME type of ANPpp (IC_{50})[a]

	dNTPs, [μmol.l^{-1}]		
Compound	125	60	30
PMEGpp	12.7 ± 0.5	7.1 ± 1.7	2.30 ± 0.04
PMEDAPpp	76.0 ± 13.5	41.6 ± 10.1	23.3 ± 3.2
PMEO-DAPypp	133 ± 15	N.D.[b]	N.D.
PMEApp	380 ± 28	N.D.	110 ± 11
PMECpp	N.I.[c]	N.D.	106 ± 16
PMETpp	N.I.	N.D.	N.I.
6-Me$_2$PMEDAPpp	P.E.[d]	N.D.	N.D.

[a] Values are means ± SD of the four independent determinations; [b] not determined; [c] no inhibition; [d] processivity enhancement.

1.2. Inhibition by PMP-derivatives

The approximate IC_{50} values for the diphosphates of PMP-derivatives, listed in the order of their efficacy as telomerase inhibitors, are shown in Table 5: (*R*)-PMPGpp > (*S*)-PMPGpp > (*R*)-6-cyprPMPDAPpp > (*R*)-PMPApp > (*R*)-PMPDAPpp > (*S*)-PMPApp.

The most potent inhibitor among the PMP-type analogues is the guanine derivative (*R*)-PMPGpp, which inhibits the enzyme activity with the IC_{50} at 5 to 8 times lower concentration compared to that of the natural substrate dGTP. (*S*)-PMPGpp is less inhibitory compared to (*R*)-enantiomer: its IC_{50} exceeds almost 5 times that of (*R*)-PMPGpp. This indicates that absolute configuration plays a significant role in the telomerase inhibition and that the enzyme distinguishes between the (*R*)- and (*S*)-enantiomers. (*S*)-PMPApp increases repeat addition processivity of the enzyme (Fig. 12B), while no significant inhibition of telomerase activity is found for (*R*)-PMPApp (IC_{50} of 224 ± 30 μmol.l^{-1} at 125 μM dNTPs), which is very efficient as a chain-terminating inhibitor of retroviral reverse transcriptases [534,598]. These results are

consistent with the observation of Pai *et al.* [447] on discrimination between the D and L enantiomers of FaraTTP and Tendian and Parker [453] concerning inhibition efficiency difference in the pair of D and L enantiomers of CdG-TP.

Table 5
Inhibition of HL-60 telomerase by PMP type of ANPpp (IC_{50})[a]

	dNTPs, [μmol.l^{-1}]		
Compound	125	60	30
(*R*)-PMPGpp	17.3 ± 2.8	11.3 ± 1.6	3.6 ± 1.0
(*S*)-PMPGpp	81.7 ± 19.6	47.6 ± 5.2	16.7 ± 2.9
(*R*)-6-cyprPMPDAPpp	152 ± 3	95 ± 6	41 ± 12.7
(*R*)-PMPApp	224 ± 30	125 ± 24	51.3 ± 6.7
(*R*)-PMPDAPpp	376 ± 24	224 ± 30	103 ± 19
(*S*)-PMPApp	P.E.[b]	N.D. [c]	N.D.

[a] Values are means ± SD of the four independent determinations; [b] processivity enhancement; [c] not determined.

Neither (*S*)-PMPA nor the corresponding monophosphate (*S*)-PMPAp increase repeat addition processivity of telomerase. Similarly to PMEG and 6-Me_2PMEDAP, activity of (*S*)-PMPA on telomerase is limited to its diphosphate only. Very high concentration of (*R*)-PMPDAPpp (376 μmol.l^{-1}) is required to reach 50% inhibition (at 125 μM dNTPs).

1.3. Inhibition by HPMP-derivatives

The approximate IC_{50} values of the diphosphates of purine HPMP-derivatives are shown in Table 6. In accordance with the PME and PMP series, the adenine derivative (*S*)-HPMPApp is less inhibitory than the guanine derivative (*R*)-HPMPGpp; however, the effect of stereoisomerism of the aliphatic chain plays the same role as it is in the case of PMP-derivatives and may thus contribute to higher inhibitory efficiency of (*R*)-HPMPGpp.

Table 6
Inhibition of HL-60 telomerase by HPMP type of ANPpp (IC_{50})[a]

	dNTPs, [$\mu mol.l^{-1}$]		
Compound	125	60	30
(*R*)-HPMPGpp	43.6 ± 5.9	28.3 ± 9.5	23.3 ± 5.9
(*S*)-HPMPApp	104 ± 15	61 ± 25	N.D.[b]

[a] Values are means ± SD of the four independent determinations; [b] not determined.

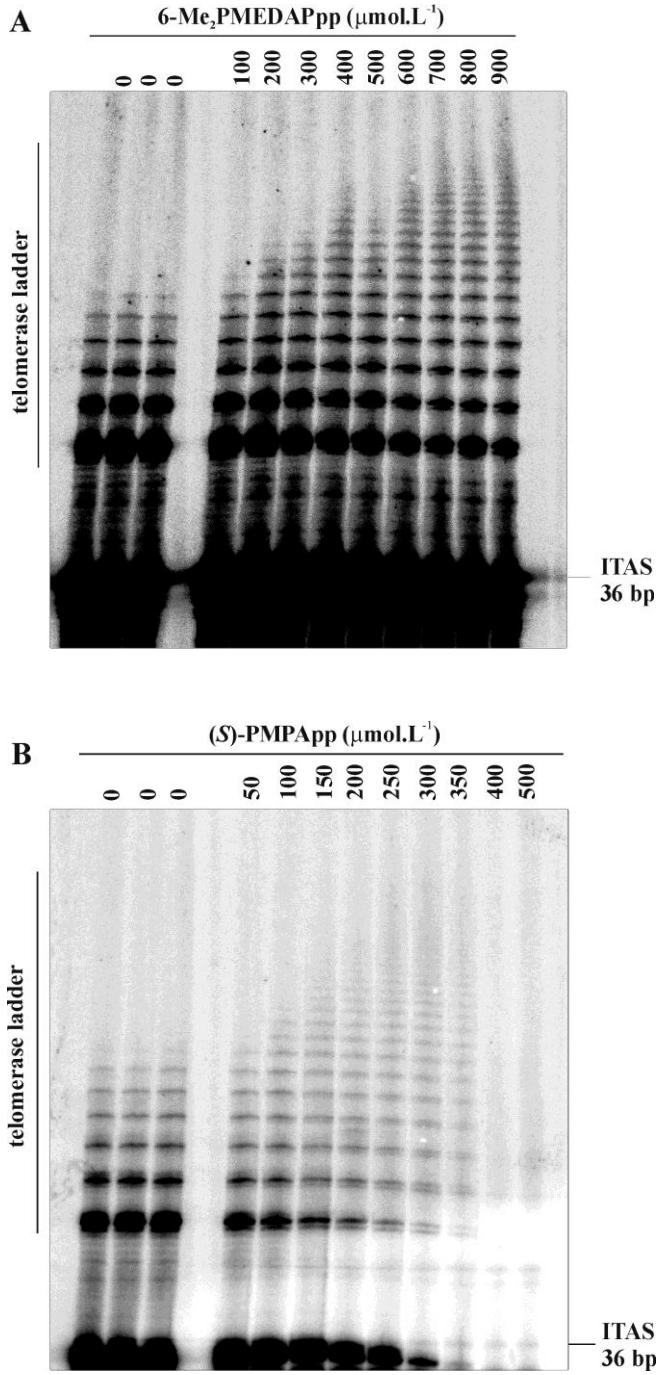


Figure 12. **Enhancement of telomerase processivity by 6-Me_2PMEDAPpp (A) and (*S*)-PMPApp (B).** Telomerase activity was measured in HL-60 cell extract in the presence of 125 μM dNTPs and increasing concentrations of 6-Me_2PMEDAPpp (0 - 900 μmol.l^{-1}) and (*S*)-PMPApp (0 - 500 μmol.l^{-1}). ITAS - internal standard.

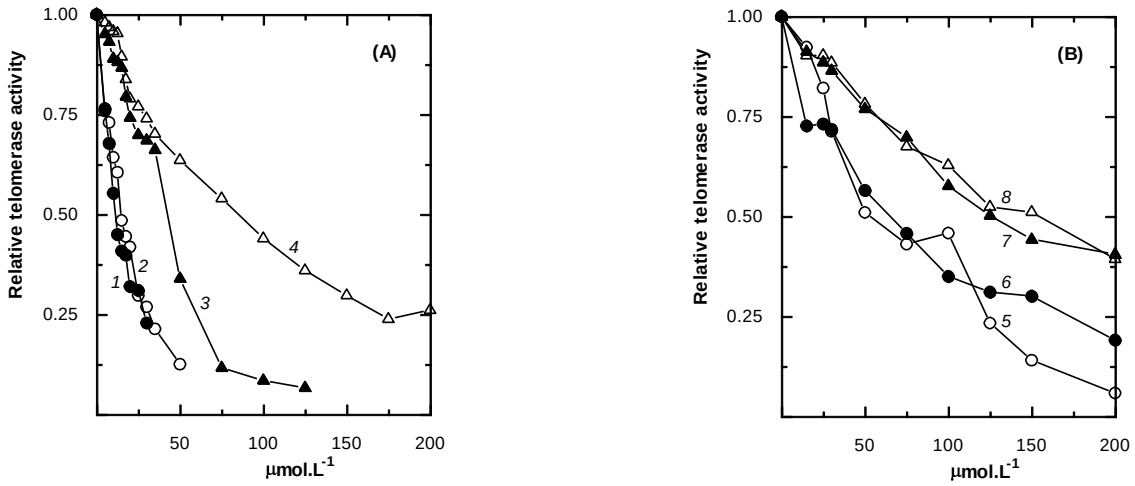


Figure 13. **(A) Inhibition of HL-60 telomerase by guanine derivatives** PMEGpp (1), (*R*)-PMPGpp (2), (*R*)-HPMPGpp (3), (*S*)-PMPGpp (4) **and (B) by adenine and 2,6-diaminopurine derivatives** PMEDAPpp (5), (*S*)-HPMPApp (6), PMEO-DAPypp (7) and (*R*)-6-cyprPMPDAPpp (8). The enzyme activity was measured in HL-60 cell extract in the presence of 125 μmol.l^{-1} dNTPs.

2. Effects of (*S*)-PMPA, PMEG and PMEDAP on the mean telomere length in CCRF-CEM cells

ANP diphosphates PMEGpp and PMEDAPpp were shown to inhibit telomerase activity *in vitro* with IC_{50} of 12.7 μmol.l^{-1} and 76 μmol.l^{-1} (at 125 μM dNTPs), respectively (Table 4). On the contrary, the diphosphate of PMP-derivative (*S*)-PMPA increased the processivity of telomerase *in vitro* (Fig. 12B). These findings prompted me to assess their effects on telomere length in human T lymphoblastoid CCRF-CEM cell line after the long-term treatment. Since the transport of studied nucleotide

analogues PMEGpp, PMEDAPpp and (*S*)-PMPApp across the cell membrane is very limited and accompanied by dephosphorylation, the parental nonphosphorylated compounds bearing enzymatically resistant phosphonomethyl ether group were supplied in the growth medium of dividing cells. The activation essential for their biological activity was enabled by conversion to their diphosphoryl derivatives (PMEGpp and PMEDApp) in the cells.

To examine the changes in telomere lengths in CCRF-CEM cells treated with either PMEG, PMEDAP or (*S*)-PMPA, I used flow cytometry method based on quantitative fluorescence *in situ* hybridization (flow FISH) with FITC-labeled telomere peptide nucleic acid (PNA) probe. The method is optimal for estimation of telomere length, as the fluorescence intensity of the cells is directly correlated to the length of the telomeres. Moreover, since the probe does not recognize subtelomeric sequences (in contrast to traditional TRF measurements) it allows an estimation of the mean telomere length without inclusion of subtelomeres. After the flow cytometric analysis, the data obtained can be used for determination of a relative telomere length (RTL). The RTL value is calculated as the ratio between the telomere signal of each sample and the internal standard (1301 cell line) after substraction of the autofluorescence of the corresponding populations (chapter 6.2. in Materials and Methods). The 1301 cells have very long telomeres and are tetraploid which enables to distinguish them from the sample cells on the dot plot diagram (Fig. 14). Propidium iodide staining solution is used for identification of $G_{0/1}$ cells because it is important for the evaluation to look only at cells in the $G_{0/1}$ phase of the cell cycle where the cell has one copy of its genome (Fig. 14).

The telomerase inhibitory activity of both PMEGpp and PMEDAPpp observed in the cell-free assay can result in telomere shortening in *in vitro* growing cells. Although PMEGpp is a much more potent human telomerase inhibitor than any of the other ANPs tested, only a moderate and reversible telomere shortening can be achieved by exposure to 0.75 μM PMEG over a period of 9 weeks of treatment (Fig. 15). The prolonged passaging in the presence of 0.75 μM PMEG caused approximately 50% decrease in population doubling rate compared to control (non-treated) cells. Cells lost about 20% of their mean telomere length after 9 weeks of treatment with 0.75 μM PMEG. Then, since the beginning of the 10^{th} week of treatment I observed a gradual

elongation of telomeres until the end of 11th week when treated cells had restored their initial mean telomere length. Then, PMEG was removed from the growth media in the end of 11th week and no significant change in the mean telomere length was observed during the following 2 weeks.

To keep the same population doubling rate as in case of cells treated with 0.75 μM PMEG, I had to increase the concentration of PMEDAP in the media from 7 μmol.l^{-1} to 20 μmol.l^{-1} after the first 4 weeks of treatment. Subsequently, a progressive telomere shortening was observed and cells lost more than 60% of their initial mean telomere length until the removal of PMEDAP from the growth media in the end of 11th week (Fig. 15). In contrast to PMEG treated cells, telomere shortening was irreversible and no increase in the mean telomere length was observed during the following 2 weeks after the removal of PMEDAP.

On the other hand, (*S*)-PMPA did not cause any changes in telomere length in CCRF-CEM cells when supplied in the growth medium for 11 weeks at concentration of 100 μmol.l^{-1} (Fig. 15). This data correspond with the fact that (*S*)-PMPA diphosphate does not inhibit telomerase activity. However, the increase of the processivity of telomerase activity *in vitro* has not been shown to be manifested in telomere elongation in the growing cells.

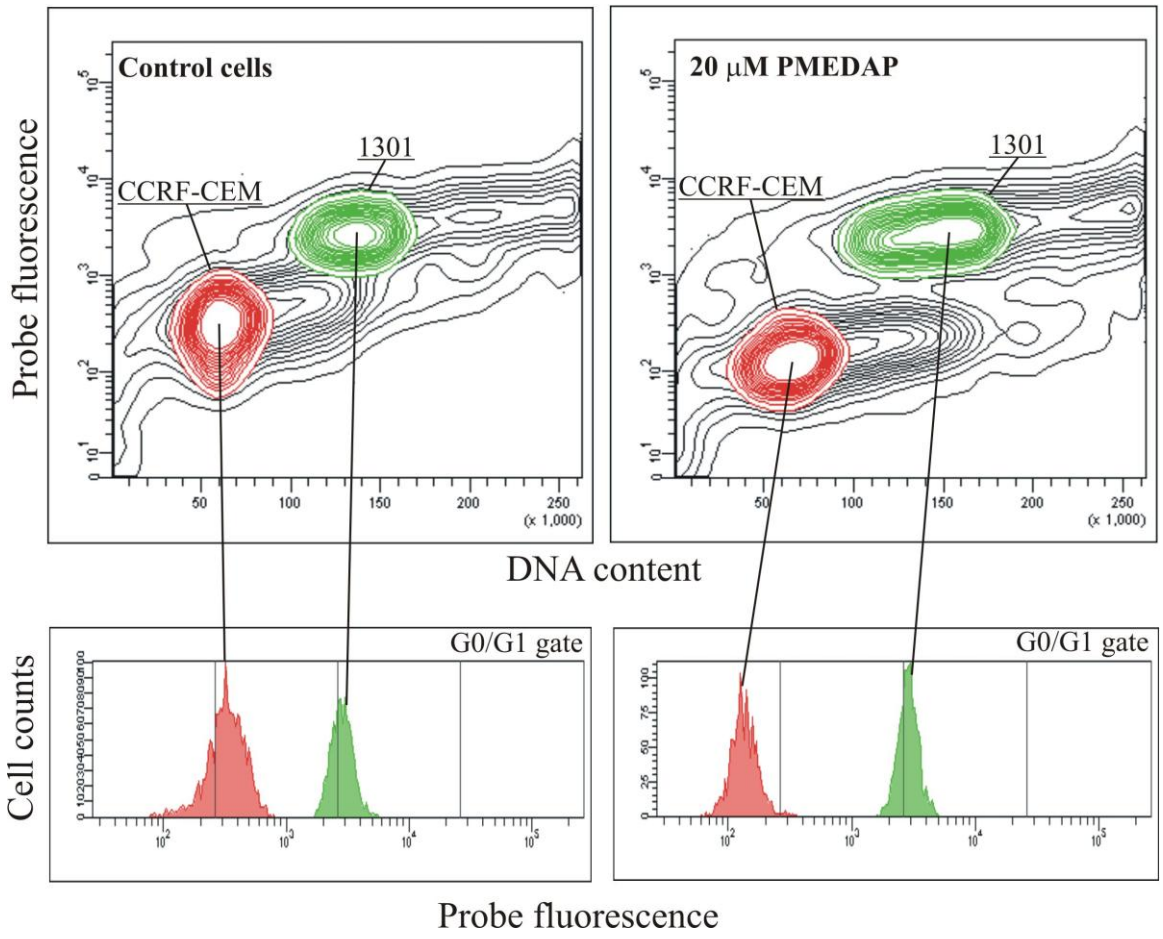


Figure 14. **Flow-FISH analysis of control (non-treated) and PMEDAP-treated CCRF-CEM cells** with the 1301 cells as an internal standard after 11 weeks of treatment with 20 μM PMEDAP. The contour plots show telomere probe fluorescence vs. DNA content and in the histograms the effect of G_0/G_1 gating is displayed. The mean fluorescence signal values obtained for each cell population from the histrograms are used for calculation of RTL value (Materials and Methods, chapter 6.2.). The 1301 cells have long telomeres and are tetraploid which enables to distinguish them from the sample cells (CCRF-CEM) on the contour plot. Propidium iodide staining solution is used for identification of $G_{0/1}$ cells.

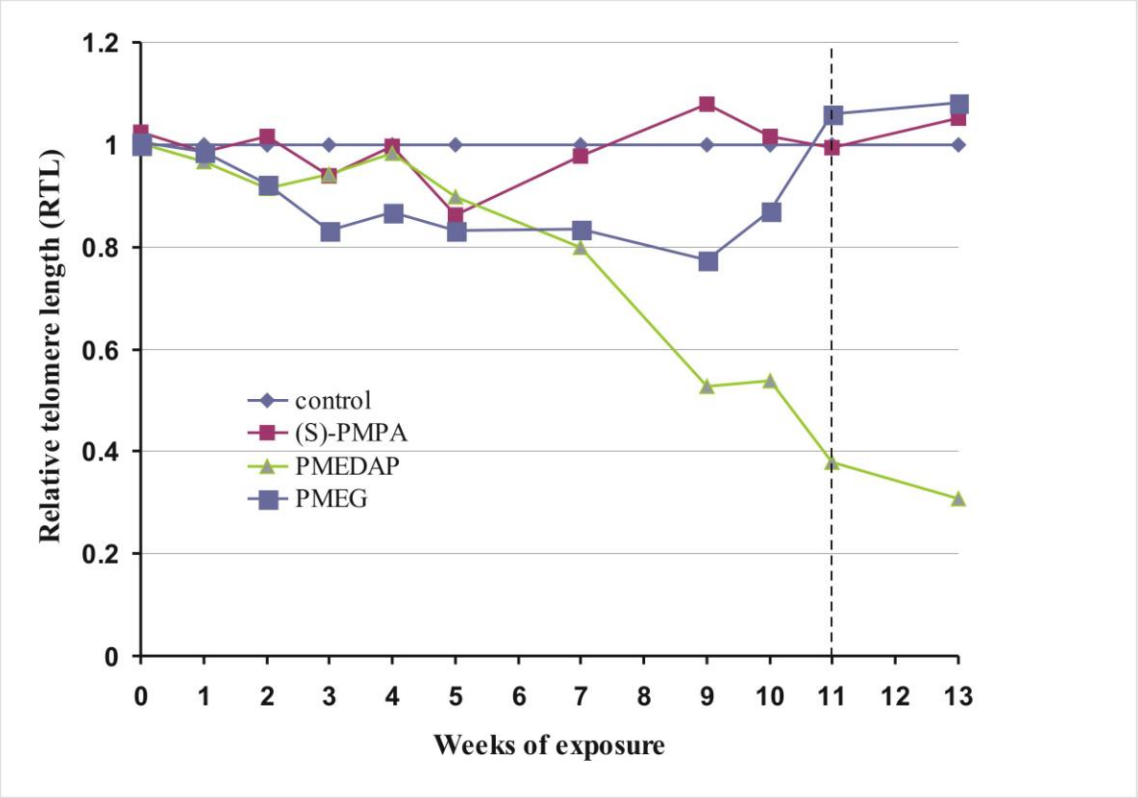


Figure 15. **The relative telomere length in CCRF-CEM cells** treated with either 0.75 μM PMEG, 7 μM PMEDAP for the first 4 weeks and 20 μM PMEDAP (from the beginning of the 5th week until the end of the 11th week) or 100 μM (*S*)-PMPA. ANPs were removed from the growth media at the beginning of the 12th week (dotted line). The RTL value of each sample is compared to the RTL value in untreated control cells, which are set to 1.

3. Modulation of *hTERT* expression by nucleoside-type DNA methylation inhibitors

hTERT expression was quantified by both one-step quantitative reverse transcription-PCR (qRT-PCR) based on TaqMan methodology and two-step qRT-PCR using SYBR® Green I. To obtain reliable results I applied two different strategies for normalization of real-time PCR data. In case of one-step qRT-PCR, *hTERT* expression was normalized against total RNA in the reaction mixture and in the latter case,

normalization was performed to a set of housekeeping genes that exhibited the most stable expression in tested samples. Advantages and pitfalls of both approaches have been discussed previously [636].

Because of relatively small differences in *hTERT* mRNA levels between treated samples and untreated controls, the requirement for a proper reference (housekeeping) gene for normalization was stringent. As numerous studies reported that the housekeeping gene expression can vary considerably [637,638] I decided to use multiple housekeeping genes rather than a single one to ensure accurate and reliable normalization of gene expression data. The expression stability of seven housekeeping genes from different abundance and functional classes [634], potentially useful as reference genes to study changes in *hTERT* and c-*myc* expression in HL-60 cells after treatment with hypomethylating agents, was investigated. Validation of their expression stability was performed by the geNorm VBA applet using data from 3 independent treatments with each nucleoside analogue. The program ranked the tested candidate genes from most stable to least stable as follows: GAPDH, RPII, TBP, PLA, PBGD, Act, G6PDH. The combination of 4 reference genes (GAPDH, RPII, TBP and PLA) was identified as being most suitable for normalization of samples obtained after treatment with all tested compounds. Their mean expression was used for normalization factor calculation and subsequent normalization of *hTERT* and c-*myc* mRNA levels. A guidance for determination of the optimal number of housekeeping genes for normalization factor calculation is described by Vandesompele *et al.* [635].

3.1. α-5-AzadCyd and β-5-azadCyd

Both α-5-azadCyd and β-5-azadCyd down-regulated *hTERT* expression; however, treatment with these compounds induced a distinct pattern of *hTERT* expression in HL-60 cells. α-5-AzadCyd inhibited *hTERT* expression in the whole range of tested concentrations (Fig. 17A) whereas the beta anomer (decitabine) caused a transient elevation of *hTERT* mRNA at low micromolar concentrations followed by subsequent *hTERT* down-regulation at higher concentrations of β-5-azadCyd (Fig. 17B). The increase of *hTERT* expression correlated with up-regulation of c-*myc* and was obvious even at a concentration as low as 0.05 μM β-5-azadCyd, which is about 70

times below its GIC_{50} value (Table 7). Under these conditions c-*myc* mRNA levels were increased 3-fold while *hTERT* expression increased for about 50% compared with control. However, the subsequent decrease in *hTERT* expression seems to be independent of c-*myc* expression since c-*myc* remained up-regulated to some extent even at higher concentrations of β-5-azadCyd.

After treatment with α-5-azadCyd c-*myc* mRNA levels slightly decreased; however, no dose-dependent manner was observed as it was for *hTERT* (Table 10). *hTERT* expression has been shown to decrease gradually within the whole range of concentrations and at 10 μM α-5-azadCyd reached approximately 30% of control (Fig. 17A). The real-time qRT-PCR results on *hTERT* expression after normalization to reference genes were in accordance with those after normalization against total RNA. As shown in Table 9, Fig. 16, α-5-azadCyd exerts similar effect on cell cycle as the beta anomer. Both compounds caused an inhibition of cell cycle progression, resulting in an increase of the percentage of cells in the G_0/G_1 phase.

Table 7

Cytostatic activity of tested compounds on HL-60 cell line after 72 h of exposure.

Compound	GIC_{50} (μmol/l)*
α-5-azadCyd	12.5 ± 2.1
β-5-azadCyd	3.4 ± 1.3
(*R,S*)-AHPA-*ibu*	174 ± 32
(*S*)-DHPA	650 ± 108
(*S*)-HPMPazaC	1050 ± 107
F-PymRf	520 ± 85

* Values are means ± SD of the three independent determinations.

3.2. (*S*)-DHPA and (*R,S*)-AHPA-*ibu*

I also intended to evaluate the effect of (*R,S*)-AHPA-*ibu* and (*S*)-DHPA on *hTERT* expression. The levels of methylation intermediates SAM and SAH after 72 h treatment with (*R,S*)-AHPA-*ibu* and (*S*)-DHPA were examined. The results show a significant and concentration dependent increase in SAH/SAM ratio for both compounds (Table 8), which is caused predominantly by an increase in the intracellular concentration of SAH. The character of the increase in SAH/SAM ratio predicts a reduced methylation capacity of HL-60 cells after treatment with both (*R,S*)-AHPA-*ibu* and (*S*)-DHPA.

The prevalent effect of reversible SAH-hydrolase inhibitor (*S*)-DHPA is the up-regulation of *hTERT* within a broad range of concentrations up to 1000 μM. The increase in *hTERT* mRNA levels is obvious even at a concentration as low as 5 μM (*S*)-DHPA and reaches its peak at 100 μM when *hTERT* expression is elevated more than 2-fold (Fig. 17D). c-*Myc* expression is significantly elevated at all tested concentrations (Table 10). In accordance with the higher hypomethylation capacity, the irreversible SAH-hydrolase inhibitor (*R,S*)-AHPA-*ibu* exhibited stronger potency to inhibit *hTERT* expression when compared to (*S*)-DHPA. In contrast to (*S*)-DHPA, we observed a significant decrease in *hTERT* mRNA levels (compared to control) from concentration corresponding to its GIC_{50} value (174 μM, Table 7, Fig. 17C). Again, there is an up-regulation of *hTERT* expression at lower concentrations of (*R,S*)-AHPA-*ibu* and c-*myc* remains overexpressed within the whole range of tested concentrations. Similar to β-5-azadCyd and (*S*)-DHPA, the down-regulation of *hTERT* seems to be independent of c-*myc* expression.

Table 8

Intracellular changes of SAH and SAM concentrations in (*S*)-DHPA and (*R,S*)-AHPA-*ibu* treated human leukemia HL-60 cells.

	μmol/l	SAH pmol/mg*	SAM pmol/mg*	SAH/SAM
(*S*)-DHPA	Not added	18.68	696.86	0.027
	50	238.47	877.70	0.272
	250	730.17	1048.64	0.696
	500	853.85	830.81	1.028
	5000	1817.44	706.33	2.573
(*R,S*)-AHPA-*ibu*	Not added	1.88	79.17	0.024
	25	30.07	84.92	0.354
	50	40.14	78.85	0.509
	100	53.37	56.17	0.950
	250	97.66	60.35	1.618

* Per mg of cellular protein

3.3. (*S*)-HPMPazaC and F-PymRf

(*S*)-HPMPazaC and 5-fluoro-zebularine (F-PymRf) exert a distinct effect on cell cycle distribution. In contrast to the other compounds studied, these antimetabolites most probably interfere with DNA replication and cause S-phase arrest (Table 9; Fig. 16). Treatment with (*S*)-HPMPazaC, compound with a potent and selective activity against several DNA viruses, results in the decrease of *hTERT* mRNA levels (Fig. 17F). The effect of (*S*)-HPMPazaC on expression of *hTERT* and c-*myc* is similar to that of α-5-azadCyd, however, at considerably higher concentrations. The down-regulation of *hTERT* expression is apparent already at 25 μM (*S*)-HPMPazaC and the subsequent decrease of *hTERT* mRNA levels is slow within the broad range of concentrations up to 1000 μM (*S*)-HPMPazaC. No transient elevation of *hTERT* mRNA levels and no c-*myc* overexpression is observed.

F-PymRf significantly up-regulates *hTERT* and c-*myc* (Table 10, Fig. 17E) from concentrations well below its GIC_{50} value (520 μM, Table 7). From the studied compounds, F-PymRf was shown to have the highest potency to increase c-*myc* and *hTERT* mRNA levels (Fig. 17E).

Table 9

The cell cycle distribution after 72 h treatment with concentrations corresponding to GIC_{50} values on HL-60 cells for each studied compound.

Compound	Concentration	%		
	(μmol/l)	G_0/G_1	S	G_2/M
Control	Not added	46.5	44.2	9.3
α-5-azadCyd	12.5	58.0	31.0	11.0
β-5-azadCyd	3.4	56.3	32.4	11.3
(*R,S*)-AHPA-*ibu*	174	48.9	42.3	8.8
(*S*)-DHPA	650	50.4	42.6	7.0
(*S*)-HPMPazaC	1050	13.5	76.5	10.0
F-PymRf	520	39.5	52.4	8.1

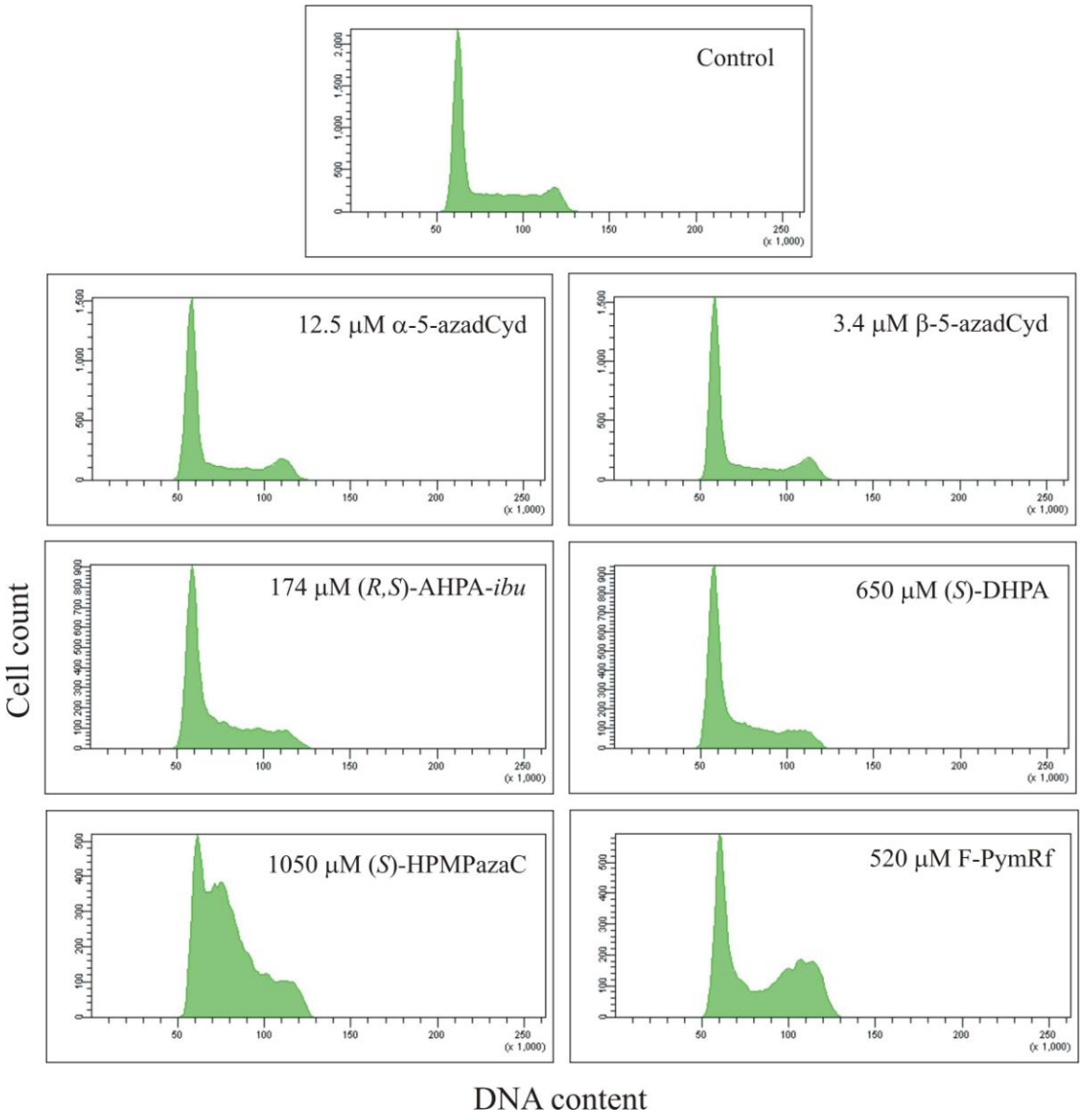


Figure 16. **DNA histograms displaying the cell cycle distribution** after 72 h treatment with concentrations corresponding to GIC_{50} values on HL-60 cells for each studied compound. Cellular DNA content was determined using a flow cytometer FACSAria. At least 30,000 cells were recorded for each analysis. Cell cycle distribution was analyzed using the cell cycle analysis program ModFit LT 3.0. When analyzing the data in the ModFit, the single cell gate was used to exclude debris and aggregates. The resulting percentual distribution of cell cycle phases G_0/G_1, S and M is shown in Table 9.

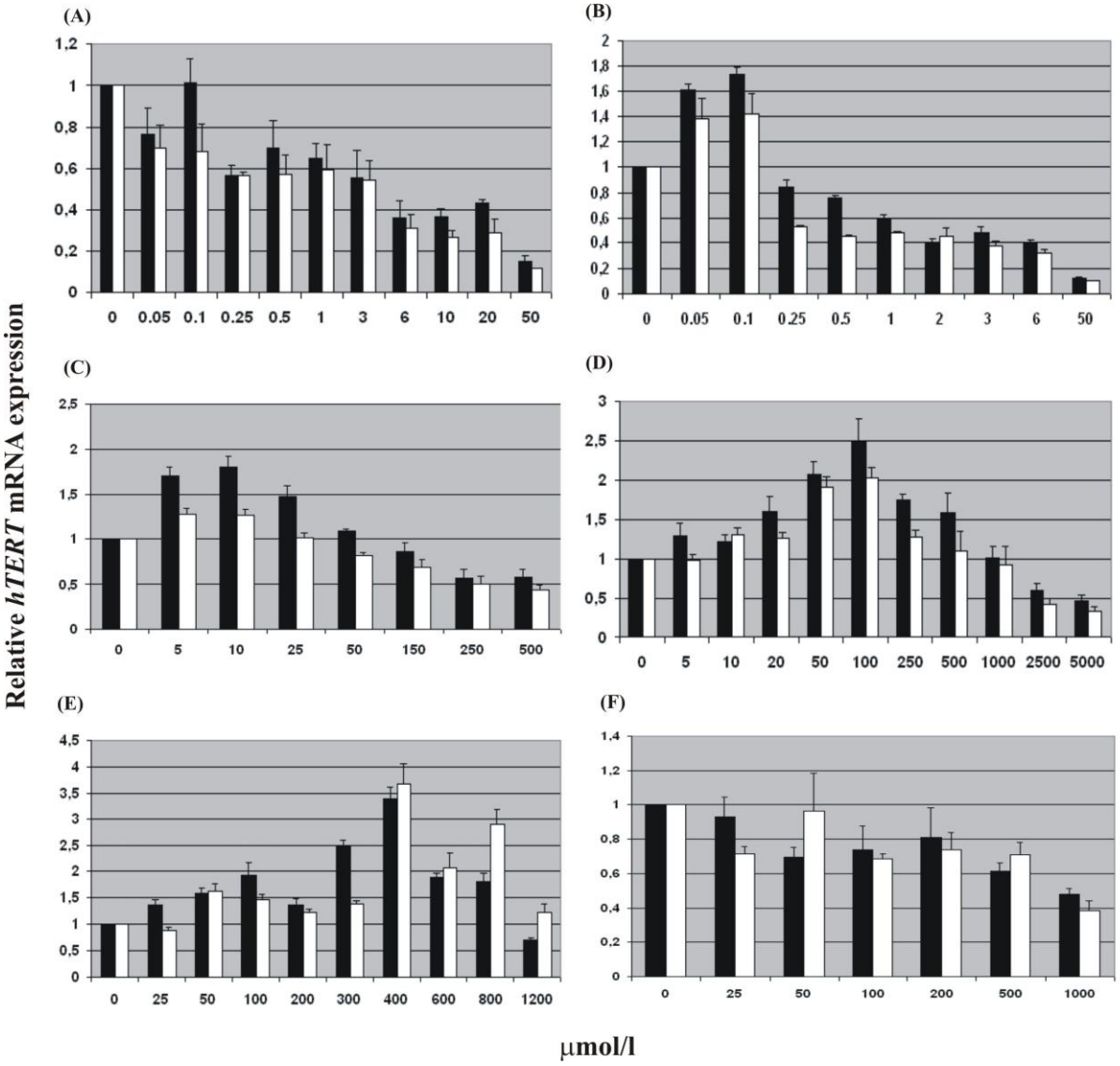


Figure 17. **Relative *hTERT* gene expression** after 72 h treatment with α-5-azadCyd (A), β-5-azadCyd (B), (*R,S*)-AHPA-*ibu* (C), (*S*)-DHPA (D), F-PymRf (E), (*S*)-HPMPazaC (F) as measured by real-time qRT-PCR using SYBR® Green I dye (black columns, normalization to housekeeping genes) and TaqMan probe (white columns, normalization to total RNA). The gene expression levels in each sample are compared to the expression levels in untreated controls, which are set to 1. Graphs show the mean values of three independent experiments in duplicate. Error bars represent standard deviation (SD).

Table 10. Comparison of relative *hTERT* and c-*myc* expression after 72 h treatment. Data obtained by two-step qRT-PCR - normalization to reference genes. The gene expression levels in each sample are compared to the expression levels in controls.

	μmol/l	*c-Myc*[*]	*hTERT*[*]
	0	1	1
α-5-azadCyd	0.1	0.78 ± 0.05	1.02 ± 0.11
	0.25	0.70 ± 0.06	0.56 ± 0.05
	1	0.80 ± 0.06	0.65 ± 0.08
	10	0.97 ± 0.29	0.37 ± 0.04
β-5-azadCyd	0.05	2.80 ± 0.13	1.60 ± 0.05
	0.1	3.05 ± 0.25	1.73 ± 0.06
	1	1.70 ± 0.08	0.59 ± 0.03
	6	1.11 ± 0.02	0.39 ± 0.03
(*R,S*)-AHPA-*ibu*	5	4.19 ± 0.19	1.70 ± 0.11
	10	4.84 ± 0.27	1.81 ± 0.12
	50	2.05 ± 0.07	1.09 ± 0.02
	250	3.89 ± 0.45	0.56 ± 0.10
(*S*)-DHPA	5	1.30 ± 0.17	1.30 ± 0.16
	50	3.81 ± 0.41	2.07 ± 0.17
	250	5.14 ± 0.44	1.76 ± 0.06
	1000	3.50 ± 0.47	1.01 ± 0.14
(*S*)-HPMPazaC	50	0.73 ± 0.11	0.69 ± 0.06
	200	0.61 ± 0.09	0.81 ± 0.18
	500	0.67 ± 0.10	0.61 ± 0.05
	1000	0.34 ± 0.05	0.48 ± 0.04
F-PymRf	300	6.21 ± 0.40	2.49 ± 0.11
	400	5.13 ± 0.62	3.40 ± 0.21
	600	4.16 ± 0.97	1.90 ± 0.08
	800	3.88 ± 0.47	1.81 ± 0.16

[*]Values are presented as the mean ± SD for at least 3 independent experiments in duplicate.

DISCUSSION

Although the telomerase active site was shown to be related to that of other reverse transcriptases [639], the ability of reverse transcriptase inhibitors to interfere with telomerase activity is not general as shown in this work. (*R*)-PMPApp, (*R*)-PMPDAPpp and PME-derivatives PMETpp, PMECpp and PMEApp known to be chain-terminating inhibitors of retroviral reverse transcriptases [reviewed in 534], do not inhibit human telomerase activity.

From these reverse transcriptase inhibitors only the guanine derivative PMEGpp shows strong inhibitory potency towards human telomerase (Fig. 13). Although the rate of telomerase inhibition by ANP diphosphates seems to depend on the side-chain structure and absolute configuration of the aliphatic chain in the case of PMP-derivatives, the presented data imply that the character of the base is probably more important. The data comprised in Tables 4, 5 and 6 show that the IC_{50} values for inhibition of telomerase activity by all guanine derivatives studied [PMEGpp, (*R*)-PMPGpp, (*S*)-PMPGpp and (*R*)-HPMPGpp] are 2 - 10 times lower than the dGTP concentration in the assay.

Interestingly, of all the compounds tested, the behaviour of two differ substantially from the other: (*S*)-PMPApp and 6-Me_2PMEDAPpp do not inhibit telomerase, on the contrary, they increase the repeat addition processivity of telomerase in a dose-dependent manner at 125 μM dNTPs concentration (Fig. 12).

The increase of telomerase repeat addition processivity was also observed in the presence of high concentrations of dGTP as previously reported [640]. dGTP seems to mediate its processivity-stimulatory effect by binding at the active site, which harbours a specific increase of dGTP binding affinity compared to other dNTPs [641]. Previous studies on *Tetrahymena* telomerase revealed that the enzyme requirements for dGTP structure features allowing the enhancement of repeat addition processivity are very strict. Stimulation of telomerase repeat addition processivity was demonstrated to be specific for dGTP base and sugar constituents. The addition of hydroxyl group at the C-2' position or loss of the C-2 amino group both prevent dGTP from stimulating repeat addition processivity [641]. Reduced repeat addition processivity was also observed for

human telomerase when deazadGTP and/or deazadATP were used as substrates [454]. It has been also shown that dGMP promotes as much repeat addition processivity as dGTP [641]. In contrast, (*S*)-PMPA and 6-Me_2PMEDAP, the analogues of nucleoside 5'-phosphates, as well as (*S*)-PMPAp, do not increase repeat addition processivity of telomerase.

Similarly to dGTP, increased repeat addition processivity requires high micromolar concentrations of both (*S*)-PMPApp and 6-Me_2PMEDAPpp. However, due to a very low structural resemblance to dGTP and chain-terminating properties of both (*S*)-PMPApp and 6-Me_2PMEDAPpp [642], we can speculate that their ability to stimulate repeat addition processivity could be mediated more likely by interaction with a site distinct from the active site.

Nevertheless, both (*S*)-PMPApp and 6-Me_2PMEDAPpp may cause rearrangements in active site, DNA anchor site or RNA template by conformational change in the ribonucleoprotein and prevent the enzyme from dissociation from the growing DNA strand. This ability could stimulate processive repeat addition. According to “anchor site model”, the RNA template of one subunit could be used primarily for substrate binding, while the second template would be copied during telomere repeats addition [270]. So-called DNA anchor site, which is distinct from the catalytic site, could be affected by both (*S*)-PMPApp and/or 6-Me_2PMEDAPpp and might thereby facilitate processivity during the synthesis of telomere repeats most likely through enhancing of interactions between DNA product and telomerase. However, these putative aspects must be confirmed or disapproved by further experimental work.

The changes in the mean telomere length in CCRF-CEM cells treated with either PMEG, PMEDAP or (*S*)-PMPA were examined using the well established flow-FISH technique. The method (previously described in [177,643] and reviewed in [178]) is more suitable for estimation of telomere length than the commonly used TRF analysis because the PNA probe binds quantitatively to the telomeric sequences and does not recognize subtelomeric region. Thus, it allows an estimation of the mean telomere length without inclusion of subtelomeres.

Effects of acyclic phosphonates PMEG and PMEDAP on telomere length in CCRF-CEM cells are consistent with the capability to induce apoptosis, strong cytostatic efficiency, and anticancer activity of these nucleotide analogues

[540,594,596,597,644]. In contrast to telomerase inhibitory potency of PMEGpp and PMEDAPpp in a cell-free assay (IC_{50} 12.7 μmol.l^{-1} and 76 μmol.l^{-1}, respectively at 125 μM dNTPs), PMEDAP is more potent in its ability to shorten telomeres than PMEG (Fig. 15). The mean terminal restriction fragment (TRF) length in CCRF-CEM cell line revealed using *Eco*RI, *Alu*I and *Hinf*I restrictases was shown to be maintained around 10.8 kb (data not shown). Then, taking into account the half population doubling rate of treated cells compared to control, the loss of approximately 60% of their initial mean telomere length from the beginning of the 5th week until the end of the 11th week of PMEDAP treatment (Fig. 15) would mean the loss of approximately 250 bp per cell division within this period. Although it is premature to speculate as to the exact mechanism of this rapid telomere shortening in the presence of 20 μM PMEDAP in the growing media (Fig. 14; chapter 2 in Results), it should be noted that the inhibition of telomerase catalytic activity by the corresponding diphosphate is not the only possible explanation. Ramírez *et al.* [645] showed that cells undergoing apoptosis upon DNA damage also exhibit a rapid and dramatic loss of telomeric sequences. This telomere loss occurs at early stages of apoptosis, because it does not require caspase-3 activation, and it is induced by loss of the mitochondrial membrane potential and production of reactive oxygen species. PMEDAP, a strong inhibitor of replicative DNA polymerase δ [563], was shown to reduce the proportion of G_1 cell cycle phase, which was compensated by an increase of S-phase and/or G_2/M-phase [584]. Moreover, it has been reported that suppression of proliferation by PMEDAP is accompanied by induction of apoptosis at higher concentrations of the analogue [594,644]. Therefore, it may be possible that the observed shortening of the telomeres is a primary effect of PMEDAP-induced apoptosis in CCRF-CEM cells rather than a direct inhibition of telomerase catalytic activity.

The moderate telomere shortening achieved by exposure to 0.75 μM PMEG was reversible and treated cells had restored their initial mean telomere length even before the removal of PMEG from the growth media in the end of the 11th week (Fig. 15). It might be possible that CCRF-CEM cells had developed resistance against PMEG as it was recently reported for PMEDAP [646]. The increased resistance against PMEDAP during long-term treatment was found to be associated with overexpression of MRP4 and MRP5 transporters [646]. Moreover, other studies showed that *in vitro* resistance

against structurally related adenine congener PMEA was due to overexpression and amplification of the *MRP4* gene and correlated with ATP-dependent efflux of the drug from cells [647-649].

The ability of (*S*)-PMPA to increase the processivity of human telomerase in a cell-free assay (Fig. 12B, chapter 1.2. in Results) was not projected into any significant changes in the mean telomere length in *in vitro* growing cells after 11 weeks of treatment with 100 μM (*S*)-PMPA (Fig. 15). This finding might be explained by poor cellular uptake and/or low ability of cellular kinases to activate the compound into its diphosphate (analogue of nucleoside 5'-triphosphate) since the activity of (*S*)-PMPA on telomerase is limited to its diphosphate only (neither (*S*)-PMPA nor the corresponding monophosphate (*S*)-PMPAp increase repeat addition processivity of telomerase; chapter 1.2. in Results). Cellular kinases are stereospecific and phosphorylation of HPMPA was shown to be limited to its (*S*)-enantiomer [576]. In terms of absolute configuration due to altered priority of the substituents of the asymetric center, (*S*)-enantiomer of HPMPA corresponds to (*R*)-PMPA and conversely, (*S*)-enantiomer of PMPA corresponds to (*R*)-enantiomer of HPMPA. Thus, similarly to (*R*)-HPMPA, (*S*)-PMPA was shown to be a poor substrate for cellular kinases [576]. The first phosphorylation of (*S*)-PMPA by NMP kinase is a limited step in its intracellular activation [593]. Furthermore, in the second phosphorylation step, nucleoside diphosphate (NDP) kinase was shown to phosphorylate (*R*)-enantiomers of the PMP series more efficiently than the corresponding (*S*)-enantiomers [580]. This findings correlate with the more potent biological activity of (*R*)-PMPA compared to (*S*)-PMPA.

Beside the modulation of telomerase by inhibitors of its catalytic activity, telomerase can be regulated at the transcriptional level as well, because expression of the catalytic subunit hTERT is fundamental for telomerase activity. The presence of abundant CpG sites in the *hTERT* promoter region has triggered an increasing interest in examining the possible role of DNA methylation in regulation of *hTERT* transcription in normal and cancer cells. In cancer cell lines with a hypermethylated *hTERT* promoter, treatment with the hypomethylating agent 5-aza-2'-deoxycytidine led to *hTERT* promoter hypomethylation, which strongly decreased *hTERT* mRNA [397]. Here, the effect of hypomethylating agents on *hTERT* expression was evaluated with the aid of

real-time qRT-PCR using SYBR® Green I dye and TaqMan probe based detection of PCR products with normalization to housekeeping genes and to total RNA, respectively.

Real-time PCR data normalization is an important step in gene expression studies. It is now generally accepted that gene expression levels should be normalized to an invariably expressed reference gene that reflects differences in cellular input, RNA quality, and RT efficiency. Many other factors in real-time PCR may affect the results, including the selection of the reference genes. A number of studies have shown that their transcription levels can be affected by the experimental treatment and vary significantly between different individuals, different cell types, and different developmental stages as well. Therefore, thorough validation of candidate housekeeping genes is critical for accurate analysis of gene expression. We prefer using the mean expression of multiple housekeeping genes rather than a single one for normalization.

Normalization to total RNA ensures the same reverse transcriptase input, but does not correct for differences in reverse transcriptase and PCR efficiencies between samples [636]. It is also assumed that rRNA:mRNA ratio does not change between samples. However, this assumption can be wrong since increased c-*myc* mRNA levels in treated samples compared to control were found in this study. The effect on rDNA transcription is not clear, but we can not rule out the possibility that rRNA:mRNA ratio is increased in treated samples with upregulated c-*myc* expression [650]. Furthermore, it has been shown that even a particular cell type may contain different quantities of total RNA and/or mRNA under various physiological conditions [651,652]. The above mentioned considerations on normalization strategies led me to involve both commonly used methods of normalization to obtain reliable *hTERT* expression data.

Both anomers of 5-azadCyd are chemically unstable in aqueous solution. HPLC analysis of chemical stability revealed that the half-life of alpha and beta anomer in RPMI 1640 media (pH 7.2) supplemented with 10% fetal calf serum at 37 °C is 12 h and 3.5 h, respectively. Under these conditions, a relatively slow spontaneous conversion of alpha anomer to beta anomer occurred in the medium, reaching a maximum concentration of β-5-azadCyd after 6 h incubation (initial velocity $v_0 \sim 4$ pmol/min at 2 mM α-5-azadCyd) [Votruba, personal communication]. Surprisingly, treatment with α-5-azadCyd causes a distinct expression pattern of c-*myc* and *hTERT* than treatment with β-5-azadCyd. α-5-AzadCyd does not induce c-*myc* overexpression

and transient *hTERT* up-regulation as it is observed for beta anomer. The reason is unclear because α-5-azadCyd is supposed to be a prodrug of beta anomer [621]. With regard to relatively short half-life of alpha anomer in the media (12 h) we might expect that after 72 h treatment all α-5-azadCyd is converted into beta anomer which is supposed to exert its biological activity. However, more stable alpha anomer most likely exhibits a prolonged action compared to beta anomer.

The mechanism of action of (*S*)-HPMPazaC remains to be elucidated, however, it could be similar to that of HPMPC [534] and 5-azadCyd. Due to the presence of 5-azacytosine ring, (*S*)-HPMPazaC when incorporated into DNA might probably covalently trap DNA methyltransferase. The lower potency of (*S*)-HPMPazaC to inhibit *hTERT* expression might be explained in analogy with (*S*)-HPMPC [653] by weaker ability to incorporate into DNA and/or by lower uptake into the cells compared to 5-azadCyd.

The results show that all tested compounds [β-5-azadCyd, F-PymRf, (*R,S*)-AHPA-*ibu*, (*S*)-DHPA] except for α-5-azadCyd and (*S*)-HPMPazaC cause the increase in c-*myc* expression which is accompanied with transient increase of *hTERT* mRNA levels. The correlation was found between c-*myc* overexpression and transiently elevated *hTERT* expression, indicating that the up-regulation of *hTERT* observed might be conferred through the transactivation of *hTERT* by c-*myc*. Indeed, association between c-*myc* overexpression and induction of telomerase activity has been previously reported [367]. The *hTERT* promoter contains numerous c-myc-binding sites that mediate *hTERT* transcriptional activation. c-Myc-induced *hTERT* expression is rapid and independent of cell proliferation [352]. Similarly, *hTERT* expression has been shown to correlate with c-*myc* overexpression in human prostate cancer [654].

Subsequent decrease in *hTERT* expression at higher concentration of hypomethylating agents seems to be independent of c-*myc* expression. We can speculate that more extensive hypomethylation of DNA caused by treatment with high doses of hypomethylating agents enables binding of CTCF to GC-rich proximal exonic region [356,357]. Then the effect of transcriptional repressor CTCF might prevail over the activation of *hTERT* expression by c-myc. However, the methylation status of the *hTERT* promoter was not examined. This is why we can not state whether inhibition of *hTERT* expression was due to *hTERT* promoter hypomethylation or it originated from

altered expression of transcription factors affecting the *hTERT* transcription. Most likely the regulation of *hTERT* expression is a complex process and both aspects contribute to some extent.

CONCLUSIONS

Considering human telomerase as a promising target of anti-cancer therapy, the thesis deals with the study of inhibitory potency of selected ANP diphosphates towards telomerase, and the capability of nucleoside-type DNA methylation inhibitors to inhibit *hTERT* expression, knowing that *hTERT* expression closely correlates with telomerase activity *in vitro* and *in vivo*. The results can be summarized as follows:

• All the purine ANP diphosphates except for (*S*)-PMPApp and 6-Me_2PMEDAPpp show dose-dependent inhibition of human telomerase in cell-free assay, the adenine derivatives are less effective inhibitors than the guanine derivatives. The only two pyrimidine ANP diphosphates tested (PMECpp and PMETpp) do not show any significant inhibitory potency towards telomerase.

• Activity of tested ANPs on telomerase is limited to their diphosphates (ANPpp) only.

• (*R*)-enantiomers are more inhibitory compared to (*S*)-enantiomers. This indicates that absolute configuration plays an important role in the telomerase inhibition and that the enzyme distinguishes between the (*R*)- and (*S*)-enantiomers.

• PMEGpp is the most potent human telomerase inhibitor among all ANPs studied with the IC_{50} value of 12.7 ± 0.5 $\mu mol.l^{-1}$ at 125 μM dNTPs. Its inhibitory potency towards telomerase is comparable to that of ddGTP (IC_{50} value of 8.1 ± 0.4 $\mu mol.l^{-1}$ at 125 μM dNTPs), which is known to be one of the most effective nucleotide analogue based telomerase inhibitors.

• (*S*)-PMPApp and 6-Me_2PMEDAPpp do not inhibit telomerase, on the contrary, they increase the repeat addition processivity of telomerase in a dose-dependent manner in cell-free assay.

• Although PMEGpp is a much more potent human telomerase inhibitor than any of the other ANPs tested, only a moderate and reversible telomere shortening can be achieved

by exposure to 0.75 μM PMEG over a period of 9 weeks of treatment – CCRF-CEM cells lost about 20% of their initial mean telomere length. In contrast, treatment with 20 μM PMEDAP caused a progressive and irreversible telomere shortening in CCRF-CEM cell line. Cells lost more than 60% of their initial mean telomere length until the removal of PMEDAP from the growth media in the end of 11^{th} week.

• The increase of telomerase processivity *in vitro* caused by (*S*)-PMPApp has not been shown to be manifested in telomere elongation in the growing cells - (*S*)-PMPA does not cause any significant changes in telomere length in CCRF-CEM cells when supplied in the growth medium for 11 weeks at concentration of 100 $\mu mol.l^{-1}$.

• Both α-5-azadCyd and β-5-azadCyd down-regulate *hTERT* expression, however, treatment with these compounds induces a distinct pattern of *hTERT* expression in HL-60 cells. α-5-AzadCyd inhibited *hTERT* expression in the whole range of tested concentrations whereas the beta anomer (decitabine) causes a transient elevation of *hTERT* mRNA at low micromolar concentrations followed by subsequent *hTERT* down-regulation at higher concentrations of β-5-azadCyd. The increase of *hTERT* expression correlates with up-regulation of c-*myc*, however, the subsequent decrease in *hTERT* expression seems to be independent of c-*myc* expression.

• The reversible SAH-hydrolase inhibitor (*S*)-DHPA causes up-regulation of *hTERT* within a broad range of concentrations up to 1000 μM. c-*Myc* expression is significantly elevated at all tested concentrations.

• The irreversible SAH-hydrolase inhibitor (*R,S*)-AHPA-*ibu* exhibites stronger potency to inhibit *hTERT* expression when compared to (*S*)-DHPA. In contrast to (*S*)-DHPA, we observed a significant decrease in *hTERT* mRNA levels from concentration corresponding to its GIC_{50} value (174 μM). Again, there is an up-regulation of *hTERT* expression at lower concentrations of (*R,S*)-AHPA-*ibu* and c-*myc* remains overexpressed within the whole range of tested concentrations. Similar to β-5-azadCyd and (*S*)-DHPA, the down-regulation of *hTERT* seems to be independent of c-*myc* expression.

• Treatment with (*S*)-HPMPazaC results in the decrease of *hTERT* mRNA levels. The effect of (*S*)-HPMPazaC on expression of *hTERT* and c-*myc* is similar to that of α-5-azadCyd, however, at considerably higher concentrations. No transient elevation of *hTERT* mRNA levels and no c-*myc* overexpression is observed.

• From the studied compounds, F-PymRf was shown to have the highest potency to increase c-*myc* and *hTERT* mRNA levels.

ABBREVIATIONS

Act	β-actin
acycloTTP	1-[(2-hydroxyethoxy)methyl]thymine 5'-triphosphate
ADV	adefovir
(*R,S*)-AHPA-*ibu*	isobutyl ester of (*R,S*)-3-(adenin-9-yl)-2-hydroxypropanoic acid
ALT	alternative lengthening of telomeres
ANP	acyclic nucleoside phosphonate
ANPpp	ANP diphosphate
APBs	ALT-associated PML Bodies
araGTP	9-β-D-arabinofuranosylguanine 5'-triphosphate
araTTP	arabinofuranosylthymine 5'-triphosphate
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia- and Rad3-related
5-azaCyd	5-azacytidine
5-azadCyd	5-aza-2'-deoxycytidine
AZddA	3'-azido-2',3'-dideoxyadenosine
AZddAA	3'-azido-2',3'-dideoxy-2-aminoadenosine
AZddAP	9-(3-azido-2,3-dideoxy-β-D-ribofuranosyl)-2-aminopurine
AZddClA	3'-azido-2',3'-dideoxy-2-chloroadenosine
AZddG	3'-azido-2',3'-dideoxyguanosine
AZddSG	3'-azido-2',3'-dideoxy-6-thioguanosine
AZT-TP	3'-azido-3'-deoxythymidine 5'-triphosphate
bp	base pair
CBV-TP	2',3'-didehydro-2',3'-dideoxyguanosine 5'-triphosphate
CdG-TP	carbocyclic 2’-deoxyguanosine 5’-triphosphate
CDV	cidofovir
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMV	cytomegalovirus
CR	conserved region

CTCF	CCCTC binding factor
(*R*)-6-cyprPMPDAP	(*R*)-2-amino-6-(cyclopropylamino)-9-[2-(phosphonomethoxy)propyl]purine
d4TTP	2',3'-didehydro-2',3'-dideoxythymidine 5'-triphosphate
DAT	dissociate activities of telomerase (domain essential for another cellular function of telomerase)
ddC	2',3'-dideoxycytidine
deazadATP	7-deaza-2'-deoxyadenosine 5'-triphosphate
deazadGTP	7-deaza-2'-deoxyguanosine 5'-triphosphate
(*S*)-DHPA	(*S*)-9-(2,3-dihydroxypropyl)adenine
DKC	dyskeratosis congenita
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-IS	DNA-internal standard
DNMT	DNA methyl transferase
dNTP	2'-deoxynucleoside 5'-triphosphate
ds	double-stranded
DS primer	duplex scorpion primer
DSB	double-stranded break
DS/TP-TRAP	duplex scorpion/two primer-TRAP
ELIPA	enzymatic luminometric PP_i assay
ERE	estrogen responsive element
FaraTTP	2'-fluoro-2'-deoxy-β-arabinofuranosylthymine 5'-triphosphate
FISH	fluorescence *in situ* hybridization
Flow-FISH	quantitative FISH analyzed with flow cytometry
FMAU-TP	2'-fluoro-2'-deoxy-5-methyl-arabinofuranosyluracil 5'-triphosphate
F-PymRf	5-fluoro-zebularine
G_0	the gap 0 cell cycle phase
G_1	the gap 1 cell cycle phase
G6PDH	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase

GIC_{50}	concentration which causes 50% growth inhibition
H3K9	lysine at position 9 on histone H3
H4K20	lysine at position 20 on histone H4
HAT	histone acetyltransferase
HBV	hepatitis B virus
HDAC	histone deacetylase
HIV	human immunodeficiency virus
hnRNP	heterogenous nucleolar ribonucleoprotein
HPA-TRAP	hybridization protection assay-TRAP
HPLC	high-pressure liquid chromatography
(*S*)-HPMPA	(*S*)-9-[(3-hydroxy-2-phosphonomethoxy)propyl]adenine
(*S*)-HPMPazaC	(*S*)-1-[(3-hydroxy-2-phosphonomethoxy)propyl]-5-azacytosine
(*R*)-HPMPG	(*R*)-9-[(3-hydroxy-2-phosphonomethoxy)propyl]guanine
HPV	human papillomavirus
HR	homologous recombination
Hsp	heat shock protein
HSV	herpes simplex virus
hTERT	human telomerase reverse transcriptase
hTP1	human telomerase-associated protein 1
hTR	RNA component of human telomerase
IC_{50}	concentration which causes 50% telomerase inhibition
IFN-α	interferon α
ITAS	internal standard
K9/20	lysine 9/20
kb	kilo base pairs
LHA	luminometric hybridization assay
Lys	lysine
M0/1/2	mortality stage 0/1/2
MAP kinase	mitogen-activated protein kinase
MDS	myelodysplastic syndrome
6-Me_2PMEDAP	2-amino-6-(dimethylamino)-9-[2-

	(phosphonomethoxy)ethyl]purine
MSV	murine sarcoma virus
NBS1	Nijmegen breakage syndrome 1
NDP kinase	nucleoside diphosphate kinase
NER	nucleotide excision repair
NHEJ	non-homologous DNA end-joining
NMP kinase	nucleoside monophosphate kinase
NSAID	nonsteroidal anti-inflammatory drug
nt	nucleotide
p53	p53 protein
PARP	poly(ADP-ribose) polymerase
PBGD	porphobilinogen deaminase
PCR	polymerase chain reaction
PKC	protein kinase C
PLA	phospholipase A2
PMEA	9-[2-(phosphonomethoxy)ethyl]adenine
PMEC	1-[2-(phosphonomethoxy)ethyl]cytosine
PMEDAP	2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine
PMEG	9-[2-(phosphonomethoxy)ethyl]guanine
PMEO-DAPy	2,4-diamino-6-[2-(phosphonomethoxy)ethoxy]pyrimidine
PMET	1-[2-(phosphonomethoxy)ethyl]thymine
PML bodies	promyelocytic leukaemia bodies
(*R*)-PMPA	(*R*)-9-[2-(phosphonomethoxy)propyl]adenine
(*S*)-PMPA	(*S*)-9-[2-(phosphonomethoxy)propyl]adenine
(*R*)-PMPDAP	(*R*)-2,6-diamino-9-[2-(phosphonomethoxy)propyl]purine
(*R*)-PMPG	(*R*)-9-[2-(phosphonomethoxy)propyl]guanine
(*S*)-PMPG	(*S*)-9-[2-(phosphonomethoxy)propyl]guanine
PNA	peptide nucleic acid
POT	protection of telomere
PP2A	protein phosphatase 2A
pRb	retinoblastoma protein
Q-FISH	quantitative FISH

RMN complex	RAD50/MRE11/NBS1 complex
RNA	ribonucleic acid
RNAi	RNA interference
RNP	ribonucleoprotein
RPII	RNA polymerase II
RT	reverse transcriptase
RTI	reverse transcriptase inhibitor
RTL	relative telomere length
RT-PCR	reverse transcription polymerase chain reaction
RTQ-TRAP	real-time quantitative TRAP
S-phase	the synthesis phase of the cell cycle
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
siRNA	small interfering RNA
SIV	simian immunodeficiency virus
snoRNA	small nucleolar RNA
snoRNP	small nucleolar ribonucleoprotein
SPA-TRAP	scintillation proximity assay
ss	single-stranded
STELA	single telomere length analysis
TALA	telomere amount and length assay
TBP	TATA box-binding protein
TDG-TP	6-thio-7-deaza-2'-deoxyguanosine 5'-triphosphate
TDV	tenofovir
thiodGTP	6-thio-2'-deoxyguanosine 5'-triphosphate
TIN2	TRF1-interacting nuclear factor 2
TMA/HPA	transcription-mediated amplification and hybridization protection assay
TNF	tumor necrosis factor
TPE	telomere position effect
TP-TRAP	two primer-TRAP
TRAIL	TNF-related apoptosis-inducing ligand

TRAP	telomeric repeat amplification protocol
TRF	telomere restriction fragment
TRF1/2	telomeric repeat binding factor 1/2
TS	telomerase substrate (nontelomeric oligonucleotide)
VV	vaccinia virus
WRN	Werner protein
WT1	Wilms' tumor 1 protein

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