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Review

Pharmacological intervention strategies for affecting telomerase activity: Future prospects to treat cancer and degenerative disease

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Abstract

Telomerase enzyme is a ribonucleoprotein maintaining the length of the telomeres by adding G-rich repeats to the end of the eukaryotic chromosomes. Normal human somatic cells, cultured *in vitro*, have a strictly limited proliferative potential undergoing senescence after about 50–70 population doublings. In contrast, most of the tumor cells have unlimited replicative potential. Although the mechanisms of immortalization are not understood completely at a genetic level, the key role of the telomere/telomerase system in the process is clear. The DNA replication machinery is not able to replicate fully the DNA at the very end of the chromosomes; therefore, about 50–200 nucleotides are lost during each of the replication cycles resulting in a gradual decrease of telomere length. Critically short telomere induces senescence, subsequent crisis and cell death. In tumor cells, however, the telomerase enzyme prevents the formation of critically short telomeres, adding GGTTAG repeats to the 3′ end of the chromosomes immortalizing the cells. Immortality is one of the hallmarks of cancer. Besides the catalytic activity dependent telomere maintenance, catalytic activity-independent effects of telomerase may also be involved in the regulation of cell cycle.

The telomere/telomerase system offers two possibilities to intervene the proliferative activity of the cell: (1) inhibition the telomere maintenance by inhibiting the telomerase activity; (2) activating the residual telomerase enzyme or inducing telomerase expression. Whilst the former approach could abolish the limitless replicative potential of malignant cells, the activation of telomerase might be utilized for treating degenerative diseases.

Here, we review the current status of telomerase therapeutics, summarizing the activities of those pharmacological agents which either inhibit or activate the enzyme. We also discuss the future opportunities and challenges of research on pharmacological intervention of telomerase activity.

Keywords: telomerase; telomerase inhibitor; telomerase activator; cancer therapy

1. Introduction

Telomerase is a ribonucleoprotein that elongates the telomeres in eukaryotic cells [1]. It catalyzes the synthesis of telomeric repeats (5′-GGTTAG-3′ in vertebrates), using its RNA component as template [1]. Telomeres have been implicated in protecting chromosomes from exonucleolytic degradation, chromosome-to-chromosome fusion and preventing other forms of aberrant recombination [2–4]. Because cellular DNA polymerases could not replicate the 5′ end of the linear eukaryotic DNA molecules the length of telomere decreases by 50–200 nucleotide/cell division [5]. Shortening the telomere may control the proliferative capacity of normal somatic cells. The activation of a telomere maintenance mechanism, as activation of telomerase enzyme, is indispensable

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for cancer cell immortalization. Indeed, telomerase activity has been detected in 85–90% of human tumors and tumor derived cell lines indicating the significance of telomerase activity in the immortalization of cancer cells [5–7] and offering a specific target for cancer therapy [8]. Cancer cell immortality, the limitless replicative potential, is one of the hallmarks of cancer [9]. It should be noted that some cancers maintain telomere length by one or more mechanisms that do not involve telomerase [10], designated as alternative lengthening of telomeres (ALT). ALT cells are characterized by instability at a specific minisatellite locus and by high rates of telomeric recombination exchange [11,12]. The detailed description of ALT is beyond the scope of this review. Since only about 10% of the cancers maintain their telomere by ALT the development of telomerase inhibitors as anti-cancer agents is feasible and reasonable.

1.1. Structure of the telomerase holoenzyme

The catalytically active telomerase enzyme is composed of chemically different subunits, proteins and RNA [2]. One of the protein components, human telomerase reverse transcriptase (hTERT; 127 kD), contains conserved catalytic reverse transcriptase motifs [13]. The amino-terminal moiety of the hTERT protein is essential for the nucleolar localization, and multimerisation. The COOH-terminal region is involved in the processivity of the enzyme and is indispensable for *in vivo* activity [14]. The central region contains the motifs characteristic of reverse transcriptase proteins and a conserved RNA-binding domain required for specific binding of hTR by the hTERT subunit [15]. Protein parts corresponding to novel functions, like anti-apoptotic activity or nuclease activity [16] serving a probable proofreading role, are almost unknown. The human telomerase RNA (hTR) extends on 451 nucleotides and contains the 11-nucleotide long template sequence for telomeric repeat synthesis. Ten conserved helical regions were proposed in vertebrate telomerase RNA including four distinct structural domains: the pseudoknot domain, the CR4–CR5 domain, the Box H/ACA domain, and the CR7 domain [17].

It was shown earlier [18] that dyskerin (57 kD) interacts with hTR; however, it was proved very recently that the presence of dyskerin is essential for the catalytic activity of the enzyme [19]. There is considerable evidence from *in vitro* reconstitution studies that telomerase exists as a dimer [20,21]; therefore, it was proposed that the catalytically active human telomerase composed of two molecules each of hTR, hTERT and dyskerin (about 670 kD).

1.2. Telomeres and telomere binding proteins

The special sequence characteristics and organization of telomeric DNA are different from the rest of the chromosome; consequently, most of the telomere-associated proteins are also specific ones, involved in the stabilization and length regulation of the telomere complex. Telomeric proteins play important roles in regulating telomere length, integrity and function. Main factor in telomere length regulation is the Telomeric Repeat binding Factor 1 (TRF1). The crude model of its action is the following: long telomeres recruit a large number of TRF1 proteins, which inhibit telomerase in adding more repeats, whereas, short telomere with less TRF1 has a higher chance to be elongated. Binding of TRF1 to the telomeres can be inhibited by Tankyrase 1 (TRF1-interacting ankyrin-related poly-(ADP-ribose)-polymerase 1) and Tankyrase 2. ADP-ribosylation of TRF1 by tankyrases enables telomerase to access the telomeres and elongate them [22]. Telomeric Repeat binding Factor 2 (TRF2) stabilizes the specific two-loop structure formed by the sequestered 3′ overhang. Inhibition of TRF2 results in the loss of the single-stranded termini followed by the induction of DNA damage pathway, finally leading to apoptosis [23]. TRF2 associates with several proteins involved in DNA damage and repair responses, notably RAD50/MER11/NBS1 complex, a key component of the homologous recombination and non-homologous end-joining pathways, the ERCC1/XPF nucleotide excision repair endonuclease, as well as ATM kinase, the WRN (Werner protein) and BLM (Bloom protein) helicases (reviewed in [24]).

The chemically different components of active telomerase enzyme can be attacked by chemically diverse molecules, including various small molecules, nucleotides and oligonucleotides. The pharmacological inhibition and activation of telomerase became a well defined, separate direction of research for treatment of cancer and degenerative diseases. In this review, we summarized those pharmacologically active compounds which either inhibit or activate the telomerase enzyme. We did not include the possible interventions to the gene expression of telomerase components, which approach may also have significance in the treatment of various diseases. We also did not include the G-quadruplex stabilizing agents, with telomere maintenance inhibitory activity, because it is discussed in detail elsewhere in this issue. Nevertheless, we propose the reader to consult with publications dealing with G-quadruplex stabilizing strategies, for a comparison [e.g. [25–27]]. The presentation of telomerase inhibitors, activators and discussions on their special features and mechanisms cannot be complete; therefore, the reader should refer other, recently published, excellent reviews of this field [e.g. [28–32]] offering further useful information.

1.3. Telomerase assays

The search for telomerase inhibitors and activators raises the question: how the inhibition or activation can be measured? This is not an easy task, considering the special characteristic features of telomerase, including its low copy number [19], consequently its low activity, even in tumor cells. The most direct way is to measure the inhibition on isolated telomerase enzyme. Several methods were published for determination of telomerase activity, and several kits are also available for measurement. However, linear, reproducible assays, suitable for enzyme-kinetic studies are very few.

Telomerase can be assayed by direct measurement of the telomerase products (conventional telomerase assay) using

α^{32}P-labeled deoxynucleoside-triphosphate as monomer substrates [1,19]. In this method the oligonucleotide substrate can have the natural telomere sequence serving as an excellent substrate for elongation. The 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 polymerase chain reaction (PCR) was applied to amplify the telomerase products for telomerase activity measurement, first time by Kim et al. [6], termed Telomerase Repeat Amplification Protocol (TRAP). The new method, although was time consuming and its linearity was questionable, revolutionized the telomerase assay. This protocol was followed by other PCR based assay-methods ([33–38] and [reviewed in [39]]); some of them were sensitive, reproducible and linear, thus, suitable to complete detailed inhibitory studies on telomerase enzyme. Real-time telomeric repeat amplification protocols were also developed suitable to identify and characterize telomerase inhibitors [40,41].

Using PCR based assays for studying telomerase inhibitors one must keep in mind that the telomerase is a special DNA polymerase. Thus, there is a chance that the compound, considered to be a telomerase inhibitor, may also inhibit the amplification process catalyzed by a DNA polymerase; therefore appropriate controls are required.

Another issue which must be considered is the source of the telomerase enzyme for inhibitory studies. Crude cell lysates [6] prepared from laboratory tumor cell lines (HeLa, HL-60) are good sources of the enzyme, with reproducible activity and good stability. Partial purification of human telomerase from the crude cell extract [35,36] could yield an enzyme with decreased concentration of endogenous inhibitors, therefore more suitable for detailed kinetic studies.

The characterization of a telomerase inhibitor or activator, *in vitro*, may include the measurement of the effects of inhibitors on tumor cell lines. To evaluate the action of the drug on cell culture, two widely used methods are applied:

1. Short term treatment for 24–48 h then analyzing biochemical and morphological changes including determination of telomerase activity in the cell extract.
2. Long term treatment and determination of the telomere length after 20–60 days of treatment.

Both approaches could provide useful information about the inhibitor, however, the long term treatment, resulting in measurable telomere shortening, rather indicates an activity specific for telomerase catalytic-activity. Such an example is the action of BIBR 1532. This compound did not produce any cytotoxic effect in a 7-day assay, but induced significant telomere shortening in a long term protocol [42]. A specific telomerase inhibitor will exert its effect by progressively decreasing the telomere length and the shortened telomere induces crisis then senescence or cell death [43]. Since about 50–200 nucleotides are lost per cell division, it is obvious that for loosing several thousand nucleotides many cell divisions must be completed. The senescence, induced by the shortened telomere, may occur after a long lag period. In contrast to the expected delay, found previously and also predicted [42–44], intriguing results were obtained recently: depletion of telomerase will induce rapid decrease in cell proliferation and cell death, without apparent decrease of telomere length and independently of wild type p53 [45–50]. These results indicate that the decrease of telomerase enzyme level, either through hTERT or hTR diminution, cause changes in global gene expression, leading subsequently to a rapid cellular response [47], in contrast to approaches which result in inhibition of telomerase activity and a telomere length dependent delayed effect. The enzyme activity-independent functions of telomerase can be mediated by its action in the regulation of anti-apoptotic and growth controlling genes [51,52]. It was observed in telomerase depleted cells that genes involved in the cell cycle progression, including Cyclin G2 and Cdc27, are rapidly down-regulated [47]. This observation is a strong evidence for the extra-telomeric effect of telomerase modifying our pervious picture about the possible application and effect of telomerase inhibition in anti-cancer treatment. It seems that the inhibition of the catalytic activity of telomerase enzyme and depletion of telomerase ribonucleoprotein from the cell generate different cellular responses.

2. Telomerase in clinics

Proliferation of telomerase negative cells results in progressive telomere shortening. When telomeres reach a critical length, proliferation will be irreversibly arrested in G1 phase of the cell cycle. *In vitro* cultured cells escape the initial growth arrest and divide until they enter crisis, when telomeres become extremely short, and apoptosis occurs. The onset of replicative senescence or apoptosis is initiated by telomere uncapping due to DNA damage or critical telomere shortening [53,54]. p53 is necessary to carry out both apoptotic and senescent pathways [55]. Cellular senescence is thought to serve as a protecting mechanism against cancer promoted by immortalization, but subsequent telomere dysfunction will be involved in tumorigenesis late in life. Unlimited and uncontrolled cell proliferation as well as inadequate function loss due to premature and accelerated cell senescence will have phenotypical effects appearing as diseases in everyday clinical practice.

Telomerase is activated in more than 85% of malignant tumors [6]. In contrast, telomerase activity is usually not detectable in normal somatic tissues. Already in the first years of telomerase research, the enzyme has been announced to be a universal cancer target, and the solution for treatment of malignant proliferative diseases seemed to be just as simple as finding the appropriate inhibitor. During the last decades a growing number of anti-telomerase strategies have emerged with more or less success. To induce cellular senescence has become an attractive therapeutic concept, since it excludes cells from the active proliferating pool. Concerns were raised about several aspects including possible reversibility, feeder-like growths that support the viability of the neighboring cells,

and escape from immuno-surveillance [56]. Apoptosis, similar as seen with conventional anti-cancer therapeutics, eliminates the neoplastic cells immediately and irreversibly. The advantage and reason, why more and more chemotherapeutics target the induction of senescence, is that these pathways seem to differ from those of the apoptosis, thus, they might be of use when conventional apoptosis inducing agents fail to act.

Growing sensitivity of telomerase assays extended the research to non-neoplastic cells. Besides the increased telomerase activity found in cancer cells, recent data support an essential function of the enzyme in normal cells with fine tuned regulation [57]. Although telomere shortening and onset of senescence termed to be the traditional fate of primary proliferating cells, it seems that deficiencies in telomere maintenance mechanisms will install an early senescent phenotype with the loss of function at cellular and systemic level [58]. Diseases due to eroded telomeres might be a consequence of primary telomere or telomerase dysfunction, for example hTERT or hTR mutation, or the result of premature telomere shortening as a secondary effect due to the disease mechanism (Table 1).

Dyskeratosis congenita (DC) was the first primary telomere maintenance disorder identified in human [18]. It is characterized by the mucocutaneous triad of skin hyperpigmentation, oral leukoplakia and nail dystrophy as well as progressive aplastic anaemia. Autosomal dominant cases result from mutations in the H/ACA domain of hTR [59], while the X-linked forms result from mutations in dyskerin which impair telomerase assembly. Both of these mutations lead to defective telomere maintenance in stem cells with proliferative activity. Recently a null mutation in the reverse transcriptase domain of hTERT was associated with the autosomal dominant form, which underlines the significance of telomerase function in this disease [60]. New insights in the pathogenesis of *adult-onset pulmonary fibrosis* showed that certain familiar cases can be as well associated with hTERT mutations, and shortened telomeres [61]. Mutations in telomeric proteins, leading to chromosomal instability, are characterized by *premature ageing syndromes.* It is likely that the presence of critically short telomeres is an important part of the disease presentation in cases of *ataxia telangiectasia* (ATM), *Werner syndrome* (WRN), *Bloom syndrome* (BLM), *Nijmegen breakage syndrome* (NBS) and *ataxia telangiectasia-like disorder* (MRE11) [24,62].

Good examples for cases, where telomere attrition is a secondary effect, could be AIDS or X-linked Lymphoproliferative Syndrome (XLT) [63]: in these cases telomere erosion is caused by excessive T-cell proliferation. In case of HIV infection, continuous antigen exposure will lead to this accelerated telomere loss [64], while proliferation of lymphocytes in patients with XLP is due to a defective protein named SLAM Associated Protein (SAP) that should modulate T-cell receptor signals leading to activation-induced cell death [65]. Less apparent but maybe more important in view of epidemiologic data seen in Western-type societies are the cardiovascular diseases that have been recently linked with telomere-dependent senescence [reviewed in [66]]. Atherosclerosis is a complex process: inflammatory and autoimmune elements are as well involved in the pathogenesis. The accelerated cell turnover and the stress induced telomerase activation causing hyperplastic cell growth at early stages of atherosclerosis contribute to the exhaustion of telomeres [67]. Senescent cells were found in atherosclerotic plaques [68] and shortened telomeres in coronary endothelial cells in patients with coronary artery disease [69].

Telomerase is a molecular target seducing experimental pharmacologists and clinicians since its discovery in the 1980s. The solution would be of course a method to regulate telomerase in a time and site specific manner. Taking our recent knowledge about telomerase regulation, we can postulate that this lies far beyond our possibilities. The challenge, however, is big: basic research as well as pharmaceutical industry are seeking for a way to have the control over lifespan of living cells.

3. Pharmacological inhibition of telomerase

3.1. Inhibition by nucleotides and nucleoside-type reverse transcriptase inhibitors

Telomerase is a highly specialized reverse transcriptase (RT), utilizing its endogenous template to synthesize telomeric repeats to the 3′ ends of the linear eukaryotic chromosomes. Based on sequence comparisons, it was suggested that the polymerase domains of RTs are evolutionary related to the telomerase catalytic subunit of the host cells [13]. Therefore, it

Table 1
Diseases with impaired telomere/telomerase system

	Mutation/defect component or cause		Disease
‘‘Deficient’’ telomerase function			
Primary dysfunction in telomeres or telomerase	Telomerase subunits	hTR	Dyskeratosis congenita Aplastic anaemia
		hTERT	Dyskeratosis congenital Pulmonary fibrosis
	Telomere associated proteins	RAD50/MER11/NBS1 ATM WRN BLM	Premature ageing syndromes
Telomere dysfunction is a *secondary* effect	Excessive proliferation Environmental stress		e.g. AIDS, XLP Atherosclerosis
‘‘Excessive’’ telomerase function	hTERT overexpression		Cancer

was an obvious idea to utilize RT inhibitors for the inhibition of telomerase activity. Since a number of reverse transcriptase inhibitors were developed from the mid-eighties for the treatment of HIV infections, these compounds were readily available. A further advantage of the RT inhibitors, used as antivirals, was their known toxicity and pharmacology.

Strahl and Blackburn [70] analyzed the telomere maintenance in some immortalized human cell lines including B-cell line JY616 and T-cell line Jurkat E6-1, and determined whether RT inhibitors could perturb telomere length and growth rates of these cells in culture. Dideoxyguanosine (ddG) caused telomere shortening over several weeks of passaging, after which the telomeres stabilized and remained short. However, the prolonged passaging in ddG caused no observable effects on cell population doubling rates and morphology. Azidothymidine showed similar effects but not in all of the studied cell lines. They showed by conventional telomerase assay that the RT inhibitors (ddGTP and AZT-TP) exerts its telomere-shortening effects by binding to and competing for the nucleoside triphosphate-binding site of telomerase rather than by being incorporated and causing chain termination; since no evidence was found for incorporation of chain-terminating ddG residues *in vitro*. This publication initiated a series of telomerase inhibitory studies using nucleoside RT inhibitors or related nucleotide analogues as anti-telomerase agents (Table 2 and Fig. 1).

The effect of AZT treatment on T-cell leukemia virus I-infected cell indicates the success of nucleoside RT inhibitors as anti-telomerase compounds [81]. The AZT treatment results in inhibition of telomerase activity, telomere shortening and increased p14(ARF) expression. The known pharmacology of AZT made possible to complete a human study in adult T-cell leukemia/lymphoma (ATL) patients. While ATL patients carrying a wild type p53 enter remission following treatment with AZT, those with mutated p53 did not respond, and patients' disease relapse was associated with the selection of a tumor clone carrying mutated inactive p53.

3.2. Inhibition by non-nucleoside small molecules

Another class of telomerase inhibitors is a group of small molecules with non-nucleoside structures. This group includes chemically diverse molecules acting mostly on the RT-protein subunit of telomerase, hTERT. Some of these agents, regarding their mode of action, resemble to the non-nucleoside reverse transcriptase inhibitors used in the treatment of HIV infections. The non-nucleoside reverse transcriptase inhibitors are able to interact specifically with RT [83]; a similar mode of action against telomerase protein, hTERT, is biochemically feasible, since the RTs and telomerases share similar protein domains [13].

It was reported that some quinoline antibiotics inhibit cell proliferation *in vitro* [84] by unknown mechanism. This result prompted a study to determine the anti-telomerase activity of these agents [85]. Ofloxacin and levofloxacin moderately inhibited the telomerase in cell extract as determined by a semi-quantitative TRAP assay.

MKT077, a toxic rhodacyanine dye analogue, which preferentially accumulates in tumor cell mitochondria and inhibits telomerase [86], was used as a lead structure for the development of a more potent telomerase inhibitor, designated as FJ5002 [87]. The IC_{50} of FJ5002 was $\sim$2 μM. Long term cultivation of U937, a human leukemia cell line, with subacute concentrations of FJ5002 resulted in population-doubling-dependent changes characterized by progressive telomerase erosion (from $\sim$10 to $\sim$4 kb) inducing senescence and crisis like features.

More than 16,000 synthetic compounds were screened using telomere repeat amplification protocol (TRAP) to identify telomerase inhibitors [88]. Six active compounds were found,

Table 2
Reverse transcriptase inhibitors and nucleoside/nucleotide analogues as telomerase inhibitors

Compounds	Reference	Notes
AZT, carbovir ddC	[71]	AZT and carbovir but not ddC induced senescence-like process in cultures of immortal mouse fibroblasts.
7-Deaza-dGTP, 7-deaza-ATP	[72]	Potent inhibitors of telomerase ($IC_{50} = 8-11$ μM) in cell free assay, incorporates into telomeric DNA.
AZT	[73]	Irreversible telomer shortening was induced by high concentration of AZT.
ddGTP, 6-thiodGTP, carbovir-TP, D-carbocyclic-2′-deoxyguanosine	[74]	Inhibitors of telomerase; the clinical effects of these nucleotides may be partly attributed to their telomerase inhibitory activity.
AZddG, C.OXT-G (carbocyclic oxetanocin GTP)	[75]	Potent inhibitors of telomerase in HL-60 cells, inducing telomere shortening.
AZT	[76]	AZT induced apoptosis in parathyroid cancer cells, inhibiting telomerase activity.
Acyclovir, ganciclovir, penciclovir	[77]	Antiviral acyclic nucleosides were found to be potent inhibitors of telomerase (patent).
AZT	[78]	MCF-7 cancer cells were treated with AZT for extended period. When treatment was halted then restarted, shortening of telomeres occurred much more rapidly.
Diphosphates of acyclic nucleoside phosphonates	[79]	*In vitro* studies with cell extract showed that the guanosine derivatives are the most active agents.
3′-azido-2′,3′-dideoxy-2-aminopurins	[80]	*In vitro* telomerase assays indicate that the 2-amino groups of these compounds significantly increase their inhibitory potential.
AZT	[81]	Enduring AZT treatment of T-cell leukemia virus I-infected cells, *in vitro* and *in vivo* in ATL patient, results in inhibition of telomerase activity and progressive telomere shortening.
AZT	[82]	Radiosensitization effect of AZT is shown on human malignant glioma cells.

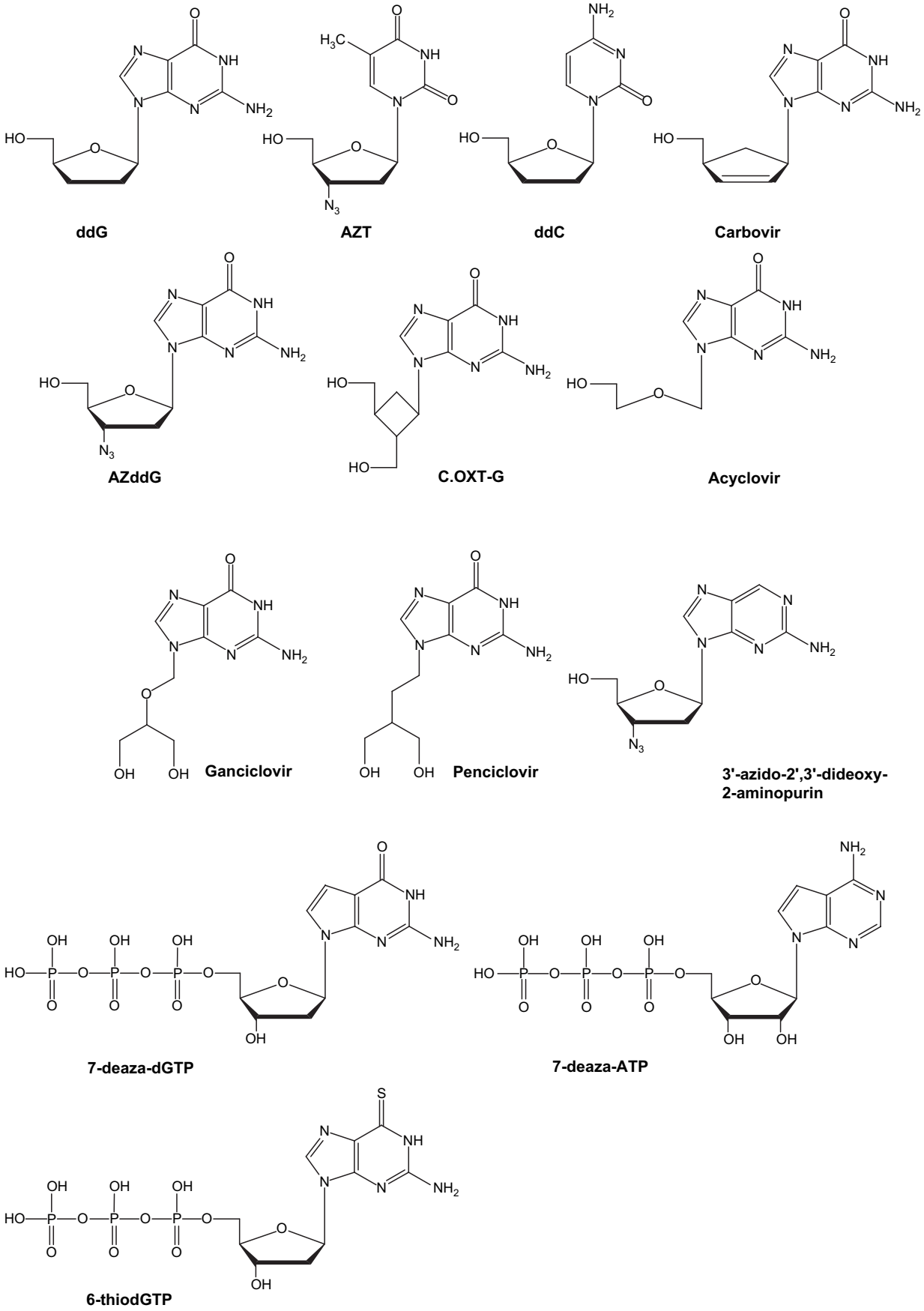

Fig. 1. Nucleoside-type telomerase inhibitors.

including four isothiazolone derivatives and two unrelated compounds. The most potent inhibitor was 2-[3-(trifluoromethyl)phenyl]isothiazolin-3-one (TMPI); 50% inhibition was achieved at a concentration of 1.0 μM. It was a noncompetitive inhibitor with respect to the oligonucleotide substrate and mixed inhibitor to dNTPs. It showed a remarkable selectivity not affecting the DNA polymerase α and β as well as HIV reverse transcriptase. Glutathion or dithiotreitol quenched its inhibitory activity, suggesting that TMPI inhibits telomerase by acting at a cystein residue.

Since rubromycins and some of its analogues were demonstrated to be potent inhibitors of retroviral RTs [89], a logical extension of these findings were the inhibitory study performed on telomerase with rubromycin and related compounds [90]. The β and γ rubromycins and purpuromycin appeared to be potent telomerase inhibitors, with 50% inhibitory concentrations of about 3 μM. Opening the spiroketal system of β-rubromycin converting it to α-analogue a substantial decrease of its telomerase inhibitory potency was observed, indicating the essential role of the spiroketal system in telomerase inhibition. 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. Based on these results, it was concluded that β-rubromycin appears to be a lead structure for the development of selective and potent inhibitors of human telomerase.

Promising activity of a non-nucleosidic telomerase inhibitor, BIBR1532, was reported recently. BIBR1532 is a simple synthetic molecule: 2-[(E)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid, selective for telomerase, with an *in vitro* IC_{50} of 93 nM [42]. Treatment of cancer cells with BIBR1532 leads to progressive telomere shortening, with no acute cytotoxicity. 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*. HT1080 cells were pre-treated with BIBR1532 inoculated in nude mice, observing a decreased tumorigenic potential of the pre-treated tumor cells. BIBR1532 proved to be active against primary cells from patients with acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) in a short term culture assay [91] and decreased the telomere length of germ cell tumor cell line, 2102 EP [92]. Treatment of drug-resistant leukemia and breast cancer cells with BIBR1532 sensitized the tumor cells to chemotherapeutic agents [93]. BIBR1532 is a mixed type of inhibitor, its binding site distinct from the sites for deoxyribonucleotides and for DNA primer, respectively [94]; therefore, its mode of action may be considered as a very similar one to the non-nucleosidic inhibitors developed against HIV-1 reverse transcriptase. Somewhat contradictory results were published concerning the *in vitro* IC_{50} of BIBR1532 from another laboratory, 5 μM (versus 93 nM) [95]. The discrepancy may be explained by the different assay systems and/or the source of the telomerase enzyme.

Helenalin, a natural sesquiterpene lactone was found to be an inhibitor of telomerase in cell extract *in vitro*, and in cell culture [96]. The mode of action of this, otherwise cytotoxic, agent is not clear.

Intriguing result is that polyunsaturated fatty acids, with *cis*-double bond, inhibit the telomerase enzyme by interacting with the protein directly and they also inhibit the gene expression of hTERT [97].

An aminosteroid phospholipase C inhibitor with alkylating activity, U-73122, was proved to be a potent and selective inhibitor of isolated telomerase enzyme with an IC_{50} of 0.2 μM [98]. Furthermore, it was demonstrated that U-73122 inhibits telomerase in hematopoietic cancer cells.

A unique approach was reported very recently to inhibit the telomerase: using intercalating compounds which targets specifically the RNA/DNA heteroduplex, formed by the interaction of telomerase RNA and 3′ ends of the chromosomes [99].

Based on human epidemiological observations and animal studies the telomerase inhibiting activity of tea catechins was studied. It was demonstrated that epigallocatechin gallate (EGCG), a major tea catechin strongly and directly inhibit the telomerase enzyme [100]. It was also demonstrated that the tea polyphenols, EGCG and others, undergo structural rearrangements at physiologically permissible conditions that result in remarkably increased telomerase inhibitory activities [101]. The chemical structures of the most important non-nucleoside telomerase inhibitors are shown in Fig. 2.

3.3. Oligonucleotide inhibitors of telomerase activity

The successful cloning and characterization of the RNA component (hTR) of human telomerase [102] opened the avenue for rational design of oligonucleotides inhibiting the telomerase enzyme. The hTR transcript comprises 451 nucleotides and contains an 11-nucleotide long sequence (CUAACCCUAAC) serving as a template to synthesize the human telomere. The template region is functionally divided into two distinct domains. The 3′ CUAAC acts as an alignment domain for binding the 3′ overhang of the telomere, whilst the 5′ domain, CUAACC, acts as a template for elongation. The template region seems to be a feasible target for telomerase template antagonist (often referred as antisense) inhibition, since its accessibility is a prerequisite to perform its biological function. The secondary structure of telomerase RNA was solved verifying the excellent accessibility of the template region [103,104].

The application of antisense oligonucleotides to down-regulate mRNA levels had already a long history when the sequence of the hTR was described [102]. Thus, most of the techniques used in the antisense technology were known and widely utilized. It was relatively simple to adapt these techniques for targeting telomerase. The inhibition of telomerase by template antagonists was very attractive area of the antitumor research, since the target was well defined (11mer template sequence). Its accessibility and specificity was unquestionable; therefore large numbers of articles were published in this field. Besides the hTR, the mRNA of the hTERT offered another target for the attack by antisense oligonucleotides.

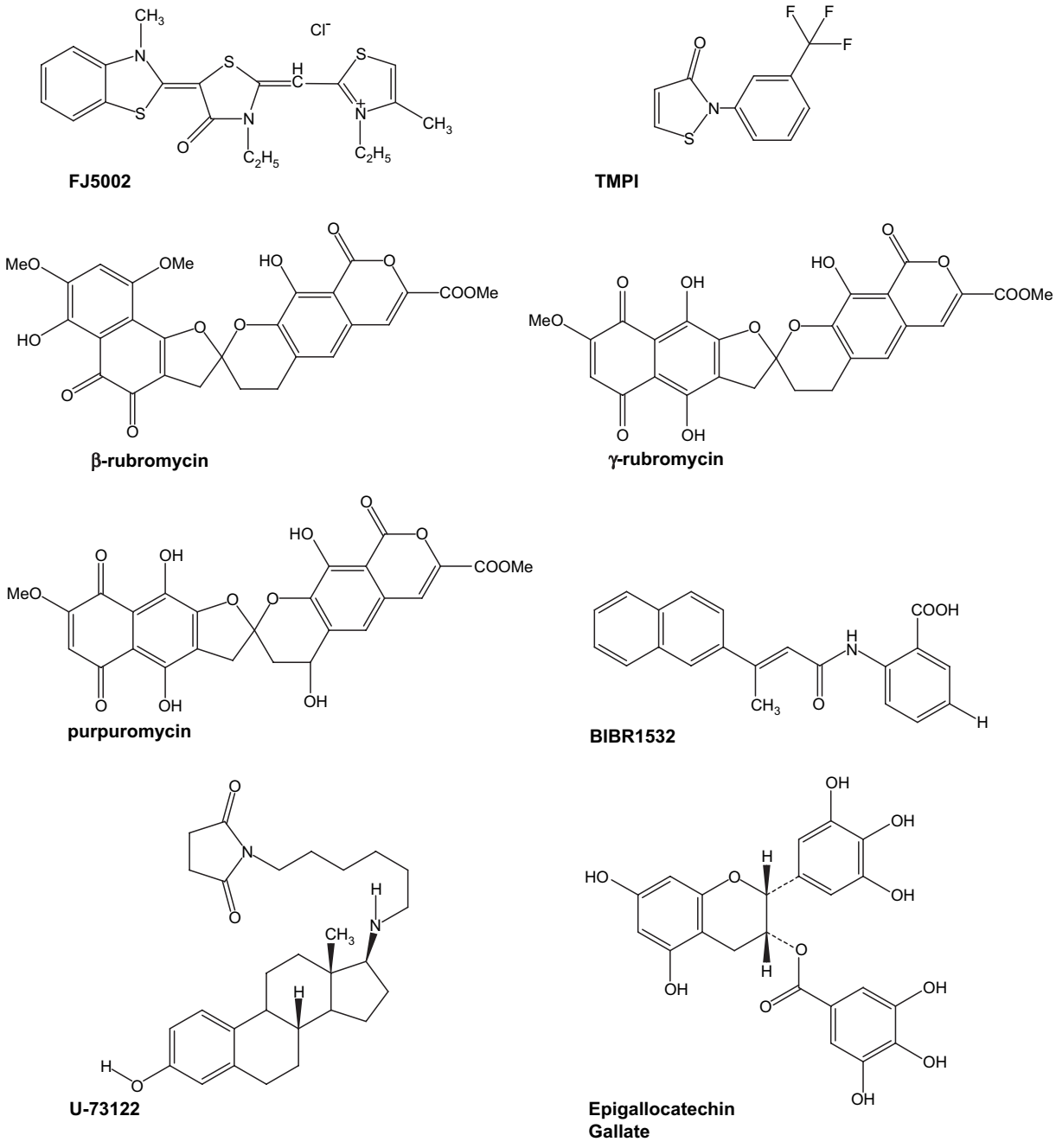

Fig. 2. Nonucleoside small molecular-weight telomerase inhibitors.

The emerging new technologies, the use of siRNAs, ribosymes and aptamers, further extended the seemingly limitless possibilities to inhibit the proliferation of cancer cells by oligonucleotides or nucleic acids targeting telomerase activity.

There are two main problems with use of oligonucleotides as therapeutic agents: (1) their poor uptake and (2) their low-stability in biological environment. The active research for more stable and cell penetrating oligonucleotides resulted in a number of chemically modified compounds which, in spite of their non-natural structural modifications, retained most of their biological activity. The detailed description of the chemical modifications of oligonucleotides, used for experimental therapeutic purposes, is out of the scope of this paper; please refer reviews on this field [105–108]; although, mentioning some of the specific cases the exact structures will be introduced herein, briefly.

Oligonucleotide inhibitors could decrease the enzymatic activity of telomerase attacking various sites with various modes of actions. Here, we classify the oligonucleotide inhibitors of the telomerase enzyme according to their mode of action and targets (Table 3). It must be noted that the various

Table 3
Classification of oligonucleotide telomerase inhibitors

1. Template antagonists and antisense oligonucleotides
(a) Against hTR
(b) Against hTERT mRNA
(c) Chimeric oligonucleotides interacting with hTR and protein subunit (hTERT)
2. Small interfering RNAs, ribozymes
(a) Against hTR
(b) Against hTERT mRNA

classes of inhibitory oligonucleotides have some overlapping features in their mode of actions; e.g. the inhibitory activity of siRNAs can also be considered as a specific antisense (template antagonist) inhibition. The two main targets, hTR and hTERT mRNA may be attacked simultaneously.

To increase the stability of oligonucleotides the internucleotide linkages are thiolated (Fig. 3), i.e., one of the non-bridging oxygen is replaced by sulfur [107]. This modification converts the oligonucleotide relatively resistant to nucleases; therefore, this modification is the most widely used one in the antisense technology. However, the phosphorothioate-modified oligonucleotides inhibit the telomerase by a non-sequence-specific manner [109]. Another thiolated oligonucleotide a homo-oligomer, $[(s^4dU)_{35}]$, a strong inhibitor of purified reverse transcriptase and HIV entry [110,111], proved to be a potent inhibitor of telomerase in a cell free assay [35]; unfortunately it could not be taken up by mammalian cells [111].

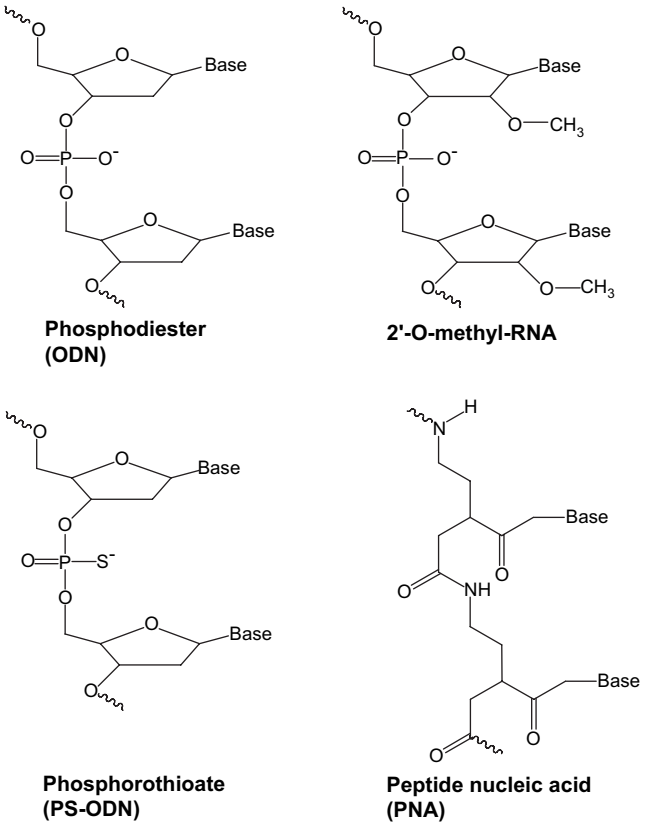

Fig. 3. The most common chemical modifications on the backbone of nucleic acids used in anti-telomerase strategies.

Although, the sequence non-specific inhibitory activity of phosphorothioate oligonucleotides is well documented [109], several publications were released using telomerase template antagonist oligonucleotides with phosphorothioate internucleotide linkages. The results of these publications are somewhat questionable, or at least, the proposed sequence specific mode of action may not be correct. The inhibitory activity of these oligonucleotides may be due to non-sequence specific interaction with hTERT.

A 19mer fully phosphorothioate oligonucleotide, targeted against the template area of hTR, showed a sequence specific telomerase inhibition in a short term treatment of HeLa cells, when FuGENE6 transfection agent was used [112]. The unmodified counterpart was completely inactive. A 15mer phosphorothioate oligonucleotide, also targeted against the template area of hTR, was active against gastric cancer cell lines in 10 μM concentration, as measured by the decreased telomerase activity and decreased cell viability [113]. The missense oligonucleotide was inactive. In another study [114], phosphorothioate 20mer antisense oligonucleotide was designed against the hTERT mRNA and examined in human laryngeal squamous cell carcinoma cells *in vitro*. It was found that short term antisense treatment can induce a decrease in hTERT mRNA expression and telomerase activity, as well as decreased cell viability and an increased rate of apoptosis.

The 2′-O-alkyl modifications of ribo-oligonucleotides are also widely used methods and represent a successful chemistry for antisense technology (Fig. 3). These modifications were also adapted to various anti-telomerase strategies.

Pitts and Corey showed for the first time that 2′-O-methyl-RNA oligonucleotides directed to the template region of hTR could be potent inhibitors of telomerase and active against human prostate-tumor derived cell line [115,116]. Their most successful 2′-O-methyl modified telomerase template antagonist ribonucleotide was a 13mer, carried phosphorothioate internucleotide linkages at both 3′ and 5′ end in order to protect the molecule against the degradation by exonucleases; its sequence was CAGUUAGGGUUAG (the underlined nucleotides had phosphorothioate linkages). This oligonucleotide blocked 95% of the telomerase activity after 1 day of treatment. The potent inhibitory activity of this short oligonucleotide is somewhat surprising; it may be explained by the 2′-O-methyl modification, which increase the affinity of the oligomer (T_m) to the complementary sequence [106,107] and the cellular uptake may also be positively affected. It must be noted, the presence of the transfection reagent, FuGENE6, was absolutely required for successful transfection and telomerase inhibition.

Fully phosphorothioate-modified 2-O-methyl-ribo-oligonucleotide targeting a splicing site within hTERT pre-mRNA induced almost complete inhibition of telomerase activity as a consequence of a significant reduction of mRNA level, an early decline of DU145 cell growth, without detectable telomere shortening [117]. Conversely, exposure of DU145 cells to an oligonucleotide with the same chemical modifications targeting the template region of hTR failed to interfere with cell proliferation in spite of the almost complete abrogation

of telomerase activity. These results verify evidences on the activity-independent, extra-telomeric effects by which, hTERT maintains tumor cell survival and proliferation [48].

Another 2′ modified ribo-oligonucleotide, the 2′-O-(2-methoxyethyl) derivative, was studied as telomerase inhibitor. A surprising advantage of these oligomers is that they could diffuse across cell membranes without the need for cationic carrier lipid [118]. Antisense oligonucleotides, complementary to the hTR template region with 2′-O-(2-methoxyethyl) modification, proved to be highly potent inhibitors of telomerase in cell free system ($IC_{50} = 5-10$ nM) and inhibited the proliferation of prostate cancer cells [119,120].

Peptide nucleic acids (PNAs), analogues of DNA or RNA (Fig. 3) which bind to complementary nucleic acids with very high affinity [121] were evaluated as telomerase template antagonists [122–124]. In cell free system some of the selected sequences showed extremely potent [$IC_{50} < 1$ nM] inhibitory activity, however, their cellular uptake was very poor, required electroporation or duplexed with DNA. In these PNA/DNA partial duplex molecules, the DNA portion allows for efficient transfection by means of cationic lipids [125].

2′–5′ Oligoadenylate is the activator of RNase L, a single-strand specific endoribonuclease [126]. Antisense oligonucleotides with covalently attached 2-5A tail could activate RNase L, at the target site, enhancing the efficacy of the antisense treatment. This approach was utilized to inhibit telomerase. 2-5A carrying oligonucleotides, complementary to the hTR template region, were active *in vitro* and *in vivo* in nude mice, against intracranial malignant glioma [127,128].

Telomerase inhibitory oligonucleotides may be designed interacting with the RNA subunit of the enzyme as well as with the protein component. Matthes and Lehmann [109], and independently our laboratory [36] showed first that chemically different thiolated oligonucleotides could inhibit the telomerase by sequence independent manner. This discovery was extended when telomerase template antagonists were designed carrying phosphorothioate modified sequence or $(4\text{-thio-dU})_n$ [129,130] at their 5′ end. The antisense stretches of these inhibitors interact the hTR and the protruding 5′ thiolated part could interact with the protein subunit, presumably with the putative primer binding site. Tarkanyi et al. [130] reported that the $(s^4dU)_8$AGTTAGGGTTAGA chimeric oligonucleotide inhibits the isolated telomerase with an $IC_{50} = 38$ nM, which is about five times lower than the IC_{50} for the AGTTAGGGTTAGA control, measured under the same conditions. It was also proved that the chimeric oligonucleotide inhibitor is taken up by the cells.

An extremely successful template antagonist oligonucleotide, the GRN163, and its lipid-modified counterpart GRN163L were developed recently [131,132]. GRN163 is a 13mer 2′-deoxyoligonucleotide N3′ → P5′ thio-phosphoramidite complementary to hTR. The lipid-modified version, (GRN163L) carries a palmitate at its 5′ end attached covalently with a linker. This modification increases the cellular uptake of the oligonucleotide and the lipid moiety could interact with the protein subunit of the enzyme enhancing the telomerase inhibitory activity. GRN163L was seven times more active in tissue culture experiment concerning the IC_{50}s than the parent molecule, GRN163. GRN163L was active against human lung cancer cells, human breast cancer cells and human hepatoma cells *in vitro* and *in vivo* (xenograft mice experiment) [133–135].

A highly specific, potent and natural way to down-regulate gene expression is the application of RNA interference. The utilization of siRNAs to inhibit telomerase and to treat cancer is an attractive challenge, and the number of publications with promising results is increasing. There are two targets for siRNAs to inhibit telomerase expression: (1) hTERT mRNA [136,137] and (2) hTR [46,47,138]. Both direction shows excellent progress, and could rich the clinical stage in the near future.

4. Telomerase reconstruction

The idea of telomerase reconstruction is a relatively new trend since first inherited diseases dyskeratosis congenita and aplastic anaemia, affecting the function of telomerase, have been discovered [139]. Speaking about telomerase reconstruction, we have to distinguish among the following strategies: (1) classical gene therapy, i.e., transfection with exogenous telomerase (hTERT or hTR) encoding sequences, (2) methods to re-express silenced telomerase, and (3) using molecules that enhance the low, residual telomerase activity, interacting directly with the enzyme complex or affecting its activity by natural posttranscriptional modifications.

4.1. Ectopic hTERT/hTR

The number of experimental trials using ectopic telomerase expression for cell therapy and tissue engineering is continuously increasing. Manifestation of chronic diseases, with independent etiology, has been associated with shorter telomeres, as a solution the use of ‘‘telomerized’’ tissues was proposed. Gene therapy has been evaluated for age-related macular degeneration [140], neural progenitor cells to replace dying postmitotic neurons [141], chronic ischaemic wounds, by transfecting dermal fibroblasts [142], or blood vessels using smooth muscles and endothelial cells [143]. Besides *in vivo* application *in vitro* telomere/telomerase level optimalization could be desirable in cases of stem cell transplantation, since donor cells with short telomeres might have a decreased value in means of bone marrow repopulation [144]. Moreover, experiments on telomerase knock out mice showed that telomere dysfunction shown in mesenchymal progenitor cells, reduced the capacity of bone marrow stromal cells to maintain the haemopoietic stem cells population [145], which indicates that stromal cell compartment might represent therapeutic targets for gene therapy.

It is theoretically reasonable that the presence of unregulated telomerase activity, due to ectopic expression, could be favorable for carcinogenesis. Experimental proofs concerning tumorigenic side effects of ectopic telomerase expression, however, do not support the notion that introduction of hTERT alone could induce malignant transformation. hTERT overexpression failed to induce tumorigenic transformation of various cell types tested

in vitro [146–148], and *in vivo* [146,149]. We have to be aware the possible carcinogenic side effect of the gene therapy, depending on the transfecting vector, and the possible permissive property of telomerase for the process of malignant transformation.

Considering the possible malignant transformation in the presence of constitutively expressed telomerase, when fine regulation of telomerase may not be possible, transitional expression should be a favorable approach. Solutions for this application can be binary expression systems, like the Cre-loxP recombinase system used by Ugrin and Harrington [150].

4.2. Activation of endogenous hTERT expression

Telomerase promoter reprogramming, in order to reactivate the production of repressed hTERT, would be the most physiological way to a controlled elevation of telomerase activity. Differentiation induced telomerase repression is predominantly due to the transcriptional downregulation of hTERT expression, governed by complicated regulatory pathways [reviewed in [29]] and influenced by epigenetic mechanisms as well [151]. Histone deacetylase inhibitors were shown to reactivate telomerase in adult mesenchymal cells [152]. Raloxifene, a selective estrogen receptor modulator, could induce hTERT expression, and enhanced its activity by promoting phosphorylation trough Akt cascade, as well as association with NFκB [153].

4.3. Enhancing telomerase activity

Despite the fact that usage of telomerase activator molecules, interacting directly the telomerase enzyme, could be clinically the safest method to activate telomerase, their number is surprisingly modest. One of the limitations is that their therapeutic effect can be exerted only in tissues with the presents of residual telomerase activity. Recent findings support that stem cells of regenerative tissues belong to the possible targets fulfilling the above described conditions; they do contain low level of telomerase activity. Lymphocytes could also be ideal targets for the treatment with telomerase activators, since they have a low level telomerase activity when unstimulated, which increases during activation-induced clonal expansion, mainly by enzyme phosphorylation causing nuclear translocation [154]. Upon repeated stimulation, either as a consequence of the antigen challenge or simply because of the age of the host organism, T-cells progressively lose their ability to induce telomerase activity, resulting in replicative senescence in the highly differentiated memory T-cell population [reviewed in [155]]. Individuals with lymphocytes possessing low telomerase activity following antigen encounter, will thus experience an exhaustion of their memory cells and decline of their immune system. Molecules increasing telomerase activity could restore their proliferative capacity as well as some other functions. Augmentation of telomerase activity can be achieved by direct and indirect pharmacological intervention. The previous is represented by molecules interacting with the telomerase holoenzyme while latter comprises agents influencing telomerase activity by affecting gene expression at different levels.

One of the most promising trials for directly acting small-molecule telomerase activators was presented at the conference ‘‘The Biology of Ageing’’ (2006, Ventura, California). The telomerase activator molecules TAT0001 and TAT0002 (Geron®) were shown to improve proliferative response of $CD8^+$ T-cells, and augmented their interferoneγ production. Thus the cells had enhanced ability to inhibit viral replication when co-cultured with HIV infected $CD4^+$ cells. The company announced to be in the process to apply for the approvals to start Phase I/II clinical trials.

TA-65®, a telomerase activator agent derived from the Chinese *Astragalus* plant, is licensed to Telomerase Activation Sciences and Geron, has already been tested in a pivotal clinical study, and showed to improve immune function, eye sight, sexual function and skin characteristics. This product, told to be available on the market in 2007, is planned to be sold as a food supplement (see company’s website).

Although telomere or telomerase specific agents are rare, several agents are known to counteract senescence in an indirect but telomere/telomerase dependent way. Endothelial cells as well as vascular smooth muscle cells have a low telomerase activity. It has been demonstrated several years ago, that hTERT introduction into human endothelial cells can extend their lifespan [143]. Since application of gene therapy for the treatment of general atherosclerosis seems to be unrealizable for the moment, pharmacological agents augmenting telomerase activity could be applied advantageously. Mitochondrial dysfunction causing elevated production of mitochondrial reactive oxygen species has been assigned to be the major determinant of telomere-dependent senescence at the single-cell level that is responsible for cell-to-cell variation in replicative lifespan [156]. Antioxidants have been proven to delay the onset of vascular senescence in a telomerase dependent way. In an *in vitro* study the reactive oxygen species (ROS) were shown to decrease the level of nuclear hTERT protein and telomerase activity in endothelial cells, which was followed by the early onset of senescent phenotype, while incubation with the antioxidant *N*-acetylcysteine blocked this nuclear export of hTERT into the cytosol [157]. α-Tocopherol, another well known antioxidant, has also been shown to repress telomere shortening and retain telomerase activity in brain microvascular endotheliocytes [158].

More interesting findings reported about telomere-dependent delay of senescence following HMG-CoA reductase inhibitor therapy. Partially fitting into the above theory, statins seem to interfere with the redox balance of endothelial cells [157]. Another potential mechanism could be the elevated expression of TRF2, a telomere binding protein, which is thought to stabilize the telomeric structure at the t-loop [159]. A recent study, with 484 individuals participating in the West Scotland Primary Prevention Study (WOSCOPS), showed that treatment of patients with statin is associated with a reduction in the number of cardiovascular clinical events in individuals with increased risk indicated by short lymphocyte telomere length [160]. Besides synthetic molecules, natural products were tested as

well. Ginko Biloba extract was shown to delay the onset of senescence through activating telomerase via PI3k/Akt signaling pathway [161].

In the last decades successful therapies were developed for acute diseases, like infections and injuries increasing the relative prevalence of incurable chronic degenerative illnesses of cardiovascular and central nervous system. Therapies targeting these chronic diseases should include treatments for tissue regeneration, which process may be promoted by telomerase activating agents. Thus, there is an urgent need for telomerase activators, preferably small molecules, which may be included in the armament of therapy against degenerative diseases in the near future.

5. Future prospects and discussion

The telomerase became a primary target of the experimental tumor therapy, established by the basic research of the last 15 years. Herein, we showed a portion of the telomerase inhibitors and activators which may have future utility as telomerase therapeutics. However, translation of these results to clinical success is very slow.

Clinical trials were launched at the end of 2006 with GRN163L sponsored by Geron Corporation (California). Safety and dose studies (Phases I and II) are in progress with chronic lymphocytic leukemia patient (Identification number: NCT00124189). Another clinical trial against solid tumors was initiated very recently with GRN163L. It is too early to predict the final outcome of these clinical experiments, however, the results of tissue culture and animal studies are promising indicating the feasibility of the extension of these experiments to clinics.

AZT, mainly used in the treatment of HIV infection, showed a potent activity against T-cell leukemia/lymphoma. *In vitro* enduring treatment of HTLV-I-infected cells with AZT results in telomere attrition and reactivation of p53 expression leading to senescence of tumor cells. In good agreement with this *in vitro* performed experiment ATL patients treated with AZT responded to therapy when p53 was wild type in sequence [81]. AZT is an orally active nucleoside which besides its anti-retroviral activity may have utility in the future for the treatment of ATL, as a telomerase therapeutic. Growing evidence suggests, that p53 might be required for the signaling pathways of senescence as well as apoptosis induced by short telomeres. This suggests that telomerase inhibitors will be most effective in cells with intact p53 function [55].

Thus, the few clinical trials performed so far gave promising results, but there are still many gaps in our understanding. This is partly indicated by many contradictory data published in this field. However, we can be optimistic, because the chemical structure of telomerase inhibitory molecules is very diverse, and significant progress was achieved only by oligonucleotide therapeutics. Orally active small molecules, nucleotide reverse transcriptase inhibitors like AZT described above and siRNAs [162] are under development for telomerase based anti-cancer therapy. These, unexploited potential therapeutic approaches may provide surprising positive, clinically useful, results in the near future. The extra-telomeric effect [48] of hTERT is another important observation offering possibilities for therapeutic intervention. The polymerase independent regulatory features of hTERT are completely unexploited, and there are exciting new findings which may be utilized for development of cancer therapeutic agents. We cannot exclude anyhow that certain already used activity-inhibitors could act on other functions of telomerase besides telomere maintenance, interfering with other survival functions of the enzyme.

The development of telomerase activators, a relatively new field of telomerase therapeutics, may provide agents for the treatment of degenerative diseases. The new trend in medicine, application of stem cell therapy for almost all types of hypo-regenerative conditions, might bring activators soon into the spotlight.

There are seemingly contradictory observations reported about the effects of telomerase inhibitors on various tumor cell lines: some agents have a long term effect, decreasing the length of the telomere in the treated cells, and, in other cases, the inhibition of the telomerase exerts a prompt and very potent anti-proliferative activity. We found a possible explanation to these seemingly contradictory observations. When the effect is the specific inhibition of the DNA polymerase activity of the telomerase, not affecting any other extra-telomeric functions of the enzyme, long term treatment is required to induce telomere shortening, to lose the limitless replicative potential of tumor cells and inducing senescence or apoptosis. The BIRB1532 may exert its activity with such a mode of action [46]. However, when the cell is depleted of telomerase, or the inhibitor affects several regulatory functions of the enzyme, besides its polymerizing activity, a prompt and potent anti-proliferative effect may be observed. The rapid and significant reduction of telomerase activity by inhibition of hTERT production with antisense peptide nucleic acid or siRNA directed against the hTERT mRNA may have such a mode of action, resulting in a prompt growth inhibition of cancer cells [45,162].

We have to emphasize that the above described theory is not proved yet; however, looking over the literature published from leading laboratories, we found this explanation to be feasible. A recently published intriguing result opposes this attractive hypothesis suggesting that the telomere maintenance, rather than the enzyme itself is responsible for the proliferative potential of the cell [163]. More detailed analyses and specific experimental verification is required to solve the above described conflicting experimental results. Recently was discovered that the dyskerin is an essential component of the active telomerase [19]. This observation may initiate intensive research in the near future for inhibitors interacting directly with dyskerin or inhibiting the interaction of dyskerin with other component of the telomerase complex to diminish telomerase activity and induce telomere attrition. According to our best knowledge, such results were not published yet, however, in an earlier publication, dyskerin gene silencing in the MCF-7 human breast carcinoma cells reduced telomerase activity in good agreement with the fact that diskeryn is an essential component of the active telomerase [164].

Another issue which should be considered in respect to the clinical application of telomerase therapeutics is the cost of the drugs. This should not be a problem for small molecular inhibitors. However, the cost of oligonucleotides sometimes believed to be a limiting factor for their clinical use. This problem has also been solved; new large-scale oligonucleotide synthesizers, like OligoProcess Systems (General Electric Healthcare), are able to produce kilogram quantities of crude oligonucleotides for moderate prices in one run, with diverse chemical modifications.

Oligodeoxynucleotides with unmethylated deoxycytidyl-deoxyguanosine (CpG) dinucleotides mimic the immunostimulatory activity of bacterial DNA and are recognized by the Toll-like receptor 9 (TLR9) [165]. This phenomenon raised questions if therapeautic oligonucleotides would produce immunological side effects. It seems that new generation of ODNs with modified (not fully phosphorothioate) backbones are free of immune-stimulatory properties.

In summary, no telomerase therapeutics was approved yet for clinical use. However, based on the promising *in vitro* and *in vivo* results and successful clinical trials, it can be predicted that telomerase therapeutics will be approved and utilized soon in the combat against malignancies and degenerative diseases. The search for specific telomerase inhibitors and activators is a hot area pursued in research laboratories, universities and laboratories of pharmaceutical companies. This active research is justified, because the telomere/telomerase system is an extremely promising target offering possibilities to decrease or increase the viability of the cell for therapeutic purposes.

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