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Review

Telomeres and telomerase as targets for cancer therapy

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Abstract. Telomeres are protective structures located at the ends of all eukaryotic chromosomes. Telomere shortening upon cell division restricts the proliferative capacity of most normal human cells due to the lack of telomerase, an enzyme synthesizing telomeric DNA *de novo*. Since most tumor cells are reliant on the activity of telomerase to maintain the stability of predominantly short individual telomeres, inhibition of this enzyme presents an attractive approach for a mechanism-based anticancer therapy. Here, we review advances and obstacles in targeting telomerase and telomeres and discuss potential applications of such approaches for the clinic.

Keywords. Telomerase, telomere, reverse transcriptase, cancer therapy.

Introduction

Telomeres are DNA-protein complexes that cap the end of linear eukaryotic chromosomes preventing them from degradation, recombination, fusions with other chromosomes and being mistaken for DNA double-strand breaks. Human telomeric DNA consists of repeated units of TTAGGG/AATCCC double-stranded sequences ending in a single-stranded G-rich 3′ overhang, that contributes to a higher-order terminal loop structure, the t loop [1]. This specialized structure is built and stabilized by proteins like telomeric-repeat-binding factors TRF1 and TRF2, and protection of telomeres-1 (POT-1), which bind specifically to telomeric DNA. In humans, telomere length is in the range of 2–15 kb [2]. A loss of telomeric DNA is found with each cell division due to the end-replication problem [3], nucleolytic processing of the 5′ strand [4] and oxidative damage [5]. Since short telomeres drive eukaryotic cells into replicative senescence, the maintenance of functional telomeres is crucial for continued proliferation. Almost all eukaryotic cells depend on the enzyme telomerase, a reverse transcriptase, for the *de novo* synthesis of telomeres [reviewed in ref. 6].

The ribonucleoprotein complex telomerase uses an RNA component TR as a template for the production of telomeric repeats via the catalytic subunit TERT [7]. Telomerase activity in most human cells is down-regulated during embryogenesis leading to successive telomere shortening, which ultimately limits their proliferative capacity [mortality stage 1 (M1) or replicative senescence]. The growth arrest in M1 is mediated by DNA damage signaling of a few short telomeres and can be bypassed by inactivation of cell cycle checkpoint genes like p53, resulting in continued proliferation eventually leading to critically short telomeres and massive cell death [mortality stage 2 (M2) or crisis]. Very infrequently, single cells can escape M2 by maintenance of their telomeres, becoming immortal cancer cells; this is realized in 90 % of all human tumor cells by reactivation of telomerase [8] (Fig. 1). Although mortality stages M1 and M2 are

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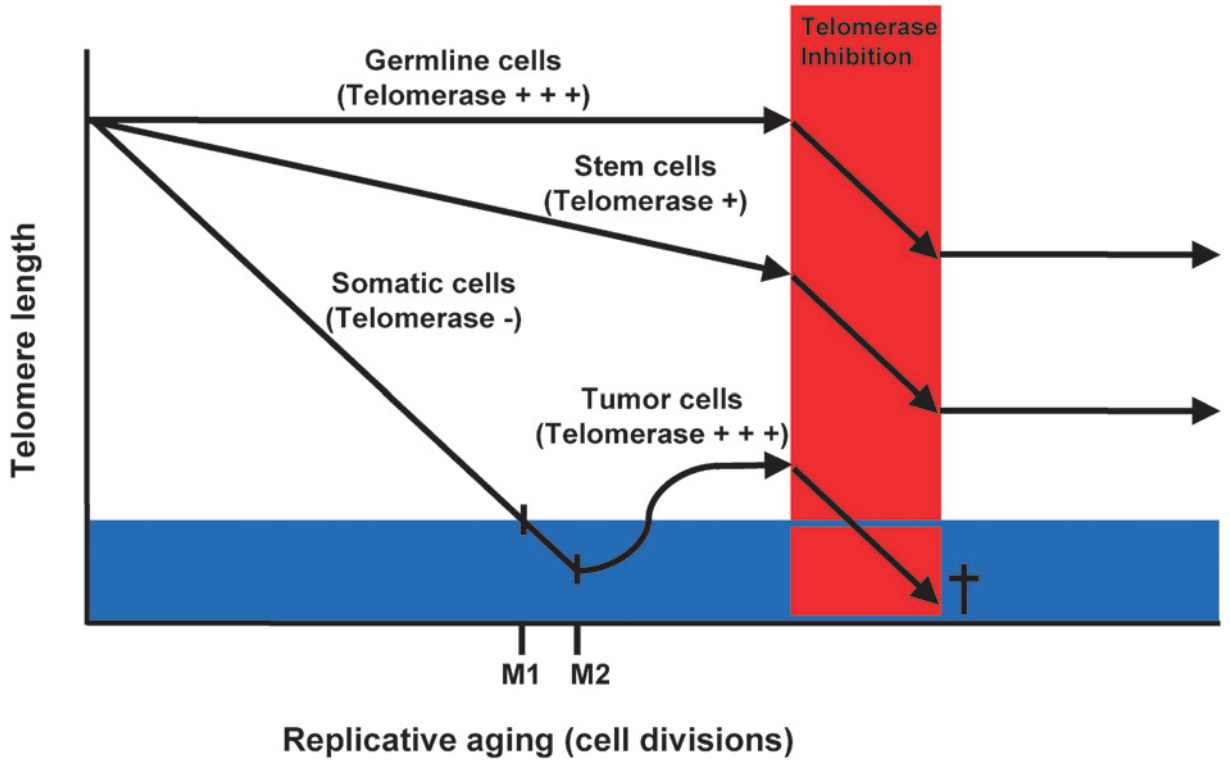

Figure 1. Telomere dynamics and telomerase inhibition. Telomerase is down-regulated during embryonic development (−), which leads to telomere shortening with successive cell divisions in most somatic cells. Continued proliferation is associated with telomere dysfunction (blue bar) and growth arrest. Bypassing telomere-dependent growth barriers – termed mortality stages M1 and M2 – and reactivation of telomerase (+++) allow immortalization in 90% of all tumor cells. Adult stem cells from highly proliferative tissues undergo telomere erosion despite detectable levels of telomerase activity (+). Telomerase inhibition over an adequate period of time (red bar) could selectively kill tumor cells and spare other telomerase-positive cells like stem cells, since tumor cells exhibit predominantly shorter telomeres.

considered as tumor suppressor mechanisms, critically short telomeres may also promote genetic instability in a distinct genetic context [9].

The fact that the vast majority of tumor cells exhibit telomerase activity in order to bypass the telomere checkpoint and to obtain unlimited growth potential makes this mechanism an attractive target for selective cancer therapy. Numerous approaches have been described during the last 10 years on how to exploit this hallmark of cancer cells for therapeutic purposes (Fig. 2). In principle, they can be divided into the following major categories: (i) Direct or indirect inhibition of the enzyme activity and consecutive telomere decapping, (ii) immunotherapy using hTERT as tumor-associated antigen and (iii) gene therapy with telomerase promoter driven suicide genes.

Targeting hTERT

Since the rate-limiting compound of telomerase activity is hTERT and its expression is basically restricted to tumor cells, it is the ideal target for telomerase-based therapies. The rationale for targeting telomerase was supported in 1999 by two independent studies demonstrating that over-expressing dominant-negative (DN) mutants of hTERT resulted in telomerase inhibition, concomitant telomere shortening and subsequent growth arrest and apoptosis in various tumor cell lines [10, 11]. However, these studies directly face a major difficulty of such approaches, namely a lag phase between the initiation of telomerase inhibition and an impact on the proliferative capacity correlated with the initial telomere length of the investigated cell lines [12, 13]. Therefore, tumor cells with long telomeres will keep growing upon telomerase inhibition until substantial telomere erosion will has occured. This side effect limits the application of telomerase inhibitors in situations of high tumor mass in a patient.

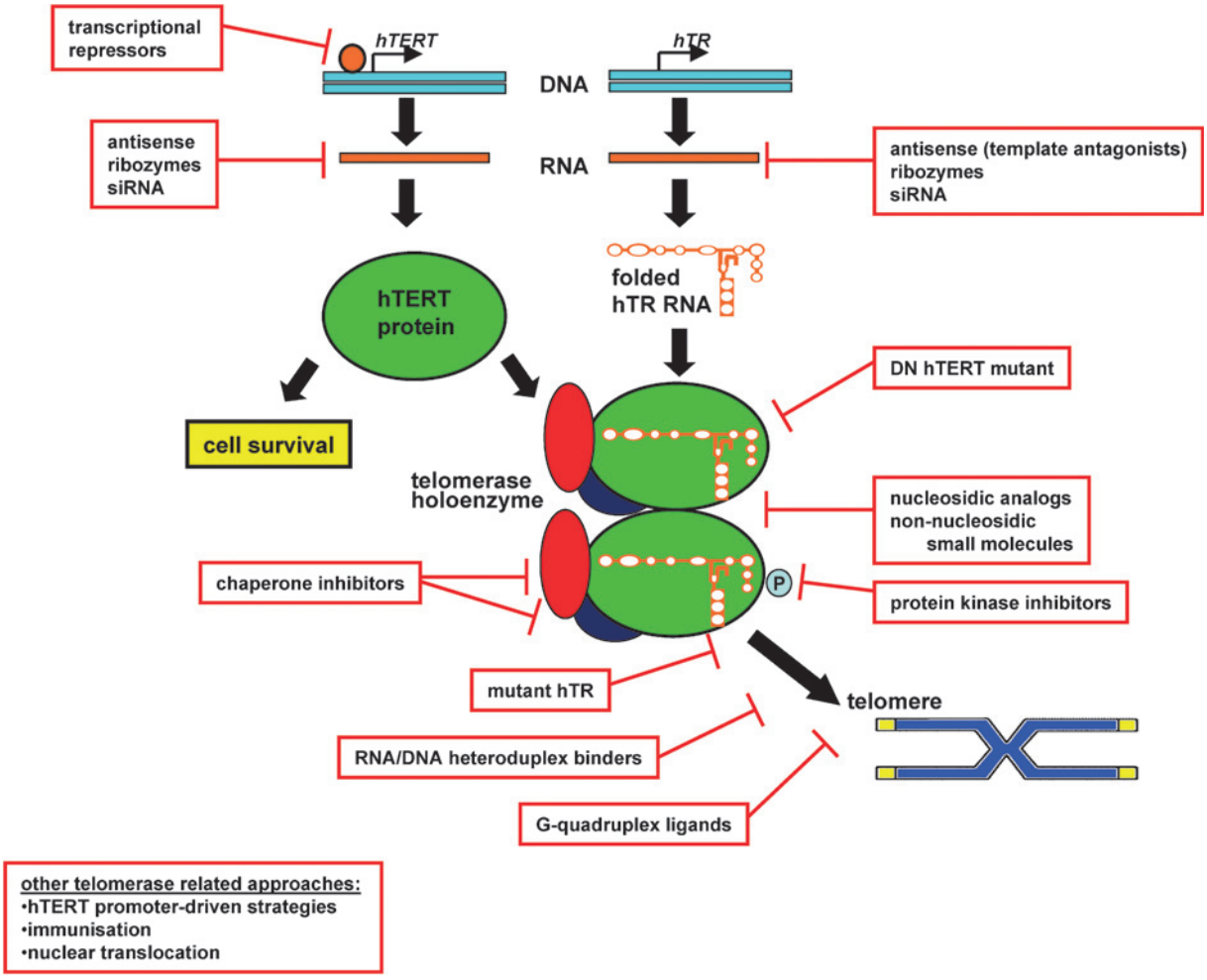

Figure 2. Targeting telomerase. Various strategies to achieve the common purpose of telomerase inhibition include repression of telomerase genes, posttranslational modifications of the telomerase proteins, direct ablation of enzymatic activity and blocking of the accesibility of telomerase substrate, the telomere. Note the potential dual role of hTERT in telomere maintenance and cell survival, making hTERT a particularly interesting target for telomerase-based cancer therapies. For details see text.

Nucleoside analogs

Tested originally in the ciliate *Tetrahymena,* nucleoside analogues like 3′-azido-2′,3′-dideoxythymidine (AZT) showed less than an efficient and specific inhibition of telomerase [14]. Other reverse transcriptase inhibitors such as L-dTTP and L-dGTP also have low specificities for telomerase [15], in contrast to 6-thio-7-deaza-2′-deoxyguanosine 5′-triphosphate (TDG-TP), which is not only more specific but also has a low IC_{50} (0.06 μM) [16].

Although other nucleoside triphospate analogs like arabinofuranyl-guanosine (Ara-G), dideoxynosine (ddI) and dideoxyguanosine (ddGTP) inhibit telomerase and induce telomere shortening as well [17], *in vivo* studies supporting the efficiency of nucleoside analogs are still missing.

Non-nucleosidic catalytic inhibitors

A large-scale screen of a chemical library identified the highly selective isothiazolone-derived telomerase inhibitor 2-[3-(trifluoromethyl)phenyl]isothiazolin-3-one (TMPI), which most likely acts at a cysteine residue (IC_{50} 1 μM) [18].

The quinone antibiotic beta-rubromycin inhibited activity of human telomerase with an IC_{50} of 3 μM, in addition to showing activities on retroviral reverse transcriptase, mammalian DNA polymerases and terminal deoxynucleotidyl transferase, classifying it as a more diversified DNA polymerase inhibitor [19, 20].

At first more promising was the discovery of a synthetic, non-nucleosidic compound (2-((E)-3-naphtalen-2-yl-but-2-enolylamino)-benzoic acid) (BIBR1532), a potent and highly selective telomerase inhibitor capable of inducing telomere shortening and senescence in human cancer cells [21]. BIBR1532

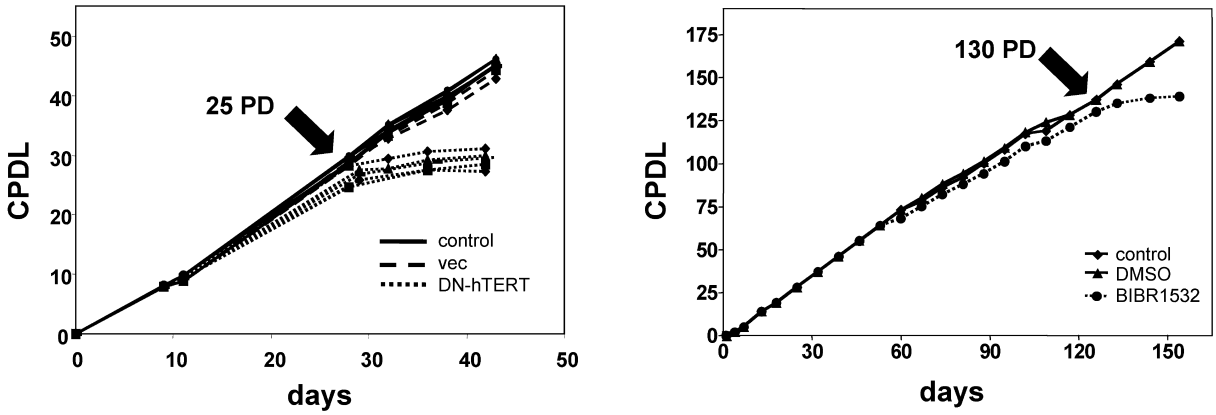

Figure 3. DN-hTERT versus pharmacological telomerase inhibition. The gold standard for telomerase inhibition is still a dominant-negative mutant of the telomerase catalytic subunit hTERT (DN-hTERT). Over-expressing DN-hTERT leads to complete ablation of telomerase activity and growth inhibitory effects, e.g. after only 25 population doublings (PD●) in clones of the lung cancer cell line NCI-H460 [12]. Pharmacological telomerase inhibitors like the non-nucleosidic compound BIBR1532 often reveal a more pronounced lag phase [21] despite high specificity for telomerase and low IC_{50} values, mainly due to a reduced bioavailability of such compounds and incomplete telomerase inhibition. An effect on the growth of cells similar to DN-hTERT in the NCI-H460 line treated with 10 μM BIBR1532 did not occur until 130 PDs. CPDL, cumulative PD level.

seems to interfere with the processivity of the telomerase enzyme, in the manner of a mixed-type non-competitive inhibitor with a proposed drug-binding site distinct from the sites for deoxyribonucleotides and the DNA primer. Conformational changes in the telomerases catalytic center make BIBR1532 mechanistically similar to non-nucleosidic inhibitors of HIV1 reverse transcriptase [22].

Despite a high specificity for telomerase and a low IC_{50} of 0.093 μM, the lag phase was even more pronounced than in the genetic approach described above. While DN-hTERT-over-expressing clones of the lung cancer cell line NCI H460 typically stopped dividing after 25 population doublings (PDs), a similar effect on the growth of cells of the same line treated with 10 μM BIBR1532 did not occur until 130 PDs [12] (Fig. 3). It is likely that both approaches interfere with the enzymatic activity of telomerase, eventually resulting in telomere dysfunction. The difference in the timing of the effect may be found in the extent of telomerase inhibition. Over-expression of DN-hTERT is accompanied by a complete ablation of the enzyme activity, which is a prerequisite for a grave disturbance of telomere function with a direct consequence on the growth capacity of the cells. Minimal telomerase activity possibly remaining in the BIBR1532-treated cells could be sufficient to repair the damage on telomeres, which subsequently does not have such a significant effect on proliferation. Data of our own laboratory suggest that the degree of telomere dysfunction is decisive for the efficiency of telomerase inhibition. The number of decapped chromosome ends and end-to-end fusions per metaphase, as a measure of telomere dysfunction, is twofold higher in the genetic compared to the pharmacological approach [12]. Interestingly, when using the compound at higher concentrations ranging from 30 to 80 μM, a direct cytotoxic effect was observed in malignant cells of the hematopoietic system, which appears to derive from direct damage to the structure of individual telomeres, in contrast to normal hematopoietic progenitor cells, which were not affected [23]. In general, a selective decapping of chromosomes in tumor cells with concomitant cell death is a highly investigated strategy bypassing extensive lag phases in the course of conventional telomerase inhibition.

A similar compound 2,3,7-trichloro-5-nitroquinoxaline (TNQX), like BIBR1532 a mixed-type non-competitive inhibitor, is a highly potent (IC_{50} = 1.4 μM) and selective antitelomerase agent, which caused progressive telomere erosion and induction of the senescence phenotype in the breast cancer MCF7 cell line [24]. A later survey found no evidence for such activities of related water-soluble benzoheterocycle triosmium clusters in the same cell line, suggesting problems for their cellular uptake, and even resulting in an acute cytotoxicity [25].

Targeting hTERT mRNA

Newer genetic approaches introduce small interfering RNAs (siRNAs) complementary to sequences present in the hTERT mRNA in various cell lines in order to deplete hTERT expression and telomerase activity [26–30]. Besides telomere shortening and inhibition of cell proliferation, down-regulation of hTERT expression by siRNA attenuated the tumor growth in a xenograft model [30]. Interestingly, depletion of hTERT by siRNA in cervical cancer cells facilitated

the induction of apoptotic cell death by chemotherapeutic agents via the activation of Bax protein, which in turn could be abolished by Bax knockout [31]. Moreover, such hTERT siRNA treatment of carcinoma cells increased the sensitivity not only to chemotherapeutic agents but also to ionizing radiation [32]. Similar to siRNA approaches, antisense-based treatments of tumor cells using phosphorothioate anti-hTERT oligomers can cause a rapid loss of tumor cell viability and induce apoptosis independent of telomerase enzymatic function [33–37]. For a review, we refer the reader to Folini and Zaffaroni [38].

A third attempt to disrupt hTERT mRNA expression is the use of hammerhead ribozymes, small catalytic RNA motifs that catalyze self-cleavage reactions. Targeting the 5′ end of hTERT mRNA by such means inhibited telomerase activity in endometrial cancer cell lines [39]. Other anti-hTERT hammerhead ribozymes have pro-apoptotic effects on various tumor cell lines, again mostly independent of telomere shortening [40–42]. In line with the hTERT siRNA studies, such hTERT-specific hammerhead ribozymes could chemosensitize cancer cells [40], highlighting the advantages of a combined treatment using conventional chemotherapy and anti-telomerase strategies.

In general and in contrast to DN mutant hTERT, inhibited hTERT protein expression related to its mRNA disruption seems to prevent potential hTERT downstream pathways apart from functions in telomere maintenance. This novel dual role of hTERT provides an opportunity in attacking two essential cancer properties, namely unrestricted proliferation and protection from apoptosis by targeting only one molecule.

hTERT phosphorylation inhibitors

Telomerase activity can be modulated by a variety of protein kinases. In such a way, protein kinase C (PKC) increases telomerase activity via hTERT phosphorylation [43]. PKC inhibitors like bis-indolylmaleimide I (BIS) and H-7 were shown to inhibit telomerase activity in nasopharyngeal [44, 45] and cervical [46] cancer cells. Recently, the exact mechanism by which PKC activates telomerase was elucidated, demonstrating the relevance of hTERT phosphorylation by PKC for telomerase holoenzyme integrity and function [47]. Interestingly, the same study provided evidence that disruption of PKC phosphorylation by BIS significantly increases the chemosensitivity of tumor cells to cisplatin.

Protein kinase B (Akt) also phosphorylates hTERT which in turn enhances telomerase activity [48]. Overall, inhibiting protein kinases does not seem to be the most specific way to suppress telomerase activity due to the broad spectrum of molecules targeted by those enzymes.

Inhibiting hTERT transcription

Since telomerase activity seems to be controlled mainly by regulation of hTERT transcription, a complex network of transcription factors is required [for a review see ref. 49]. Several tumor suppressor/oncogene pathways are involved in natural hTERT repression, including the Mad1/c-Myc and the transfoming growth factor (TGF)-beta pathway, which could be exploited to inhibit cancer growth [50–52]. In this regard, recent studies demonstrated that natural products like gambogic acid and genistein are able to repress c-Myc, a known activator of hTERT transcription, which ultimately results in a reduction of telomerase activity in cancer cells [53, 54]. Interestingly, genistein also showed a down-regulation of Akt activation and thereby hTERT phosphorylation, indicating a double effect on telomerase activity [54].

Ceramides have been demonstrated to have a repression effect on other positive regulators of hTERT transcription, namely Sp1/Sp3 [55].

In addition, arsenic is thought to inhibit hTERT transcription via c-Myc and Sp1 repression, but it has carcinogenic properties as well [56, 57].

Inhibiting hTERT nuclear translocation

Telomerase activity can also be regulated by the translocation of hTERT between the cytoplasm and the nucleus [58], exhibiting another point for attacking telomerase-positive tumor cells. Tumor necrosis factor alpha (TNF-alpha) modulated telomerase activity by inducing translocation of hTERT protein from the cytoplasm to the nucleus by direct interaction with NF-kappaB p65. This TNF-alpha-induced hTERT nuclear translocation could in turn be blocked by specific inhibitors of the NF-kappaB pathway [59]. Since NF-kappaB is a key actor in tumorigenesis, targeting it should be effective in the prevention and treatment of cancer.

Targeting hTR

The telomerase core components hTERT and hTR are essential and sufficient to reconstitute telomerase activity [60]. In contrast to hTERT, hTR is constitutively expressed in most cells, but does not seem to have a function in telomerase-negative cells, rendering it a valuable target for telomerase-inhibition.

Antisense (template and non-template specific)

Antisense oligonucleotides could either target the hTR template region to inhibit directly the enzymatic activity of telomerase or hTR non-template regions to disrupt the assembly of the telomerase holoenzyme. The hTR template region must be exposed since it interacts directly with the telomeric DNA which makes it the perfect target for such oligonucleotides. In fact, using a long antisense-RNA-targeting template and non-template regions proved the identity of hTR as the RNA component of telomerase that caused telomere shortening and cell death in HeLa cells [61]. A vector derived from this hTR antisense construct was transfected into human malignant glioma cells where it inhibited telomerase activity and subsequently induced either apoptosis or differentiation [62]. Although initial antisense approaches like the ones mentioned above functioned in principal they also revealed major weaknesses of the systems used in terms of impaired cellular uptake, stability and bioavailability [63]. Further development coud be achieved by modifications of the oligonucleotides, increasing their stability and bioavailability. Among the compounds stablized in this way are peptide nucleic acids (PNAs) carrying a pseudopeptide backbone consisting of N-(2-aminoethyl) glycine instead of the sugar phosphates in the DNA, which bind RNA with high affinity [reviewed in refs. 64 and 65). While targeting very specific regions in hTR by PNAs, telomerase activity could be inibited with IC_{50} values in the pico- to nanomolar range [66]. High-affinity recognition by overlapping PNAs was performed to identify nucleotides within the RNA active site of telomerase that are determinants for inhibitor recognition [67].

In general, the cellular uptake of PNAs is inefficient and could be enhanced by electroporation [68], lipofection [69, 70], the use of PNA-cationic peptide conjugates [71] or photochemical internalization [72], all of which eventually improve the extent of telomerase inhibition in various cell lines, in part resulting in telomere shortening and reduced cell survival [68, 70, 72].

In contrast to high selectivity of inhibition by PNAs, phosphorothioate (PS) oligomers inhibit telomerase in a non-sequence-selective fashion, binding to the primer binding site of hTERT but poorly to hTR [73]. In general, the inhibition for PNAs is considerably more efficient than inhibition by analogous PS oligomers [66]. Nevertheless, an inhibitory effect on telomerase activity and cell growth in a colorectal cancer cell line was demonstrated using PS oligomers [74].

Despite low binding affinity relative to PNAs 2′-*O*-methyl-RNA (2′-O-MeRNA) RNAs with methyl-substituted ribose, show potent telomerase inhibition in human cancer cell lines with concomitant telomere shortening leading to apoptosis [75, 76].

Telomerase inhibition at nanomolar concentrations was achieved using RNAs with methoxyethyl-substituted ribose [2′-*O*-(2-methoxyethyl)-RNA] in DU145 prostate cancer cells [77]. The pharmacokinetic properties of these molecules were enhanced by PS linkers and the use of corresponding RNA/DNA hybrids.

2′,5′-Oligoadenylate (2–5A) antisense oligomers were also shown to be potent inhibitors of telomerase [78, 79]. 2–5A oligomers are supposed to recruit RNase L which in turn cleaves the RNA template [80]. Treatment of prostate, bladder and glioma cancer cells with appropriate 2–5A antisense telomerase RNAs exhibited promising results *in vitro* and *in vivo*, similar to the significant suppression of tumor growth through induction of apoptosis in nude mice models [81–84]. Synergistic effects were described for treatment of malignant glioma cells with 2–5A oligomers in combination with cisplatin [85].

Interstingly, the short-term pro-apoptotic effect of 2–5A antisense oligomers seems to be independent of telomere shortening and might be activated by caspase family members [86].

Another group of oligomeric telomerase inhibitors contain N3′-P5′ phosphoramidate (NP) linkages and a variety of 2′-deoxy, 2′-hydroxy, 2′-methoxy, 2′-ribofluoro and 2′-arabino-fluoro substituents in the ribose rings [87]. These compounds demonstrated sequence-specific and dose-dependent activity, with IC_{50} values in the sub-nanomolar concentration range [87]. A further improvement is provided by sulfur containing N3′-P5′ thio-phosphoramidates (NPS) which combine the advantages of PS and NP oligonucleotides [88]. Much attention has been paid to the optimized 13-mer NPS GRN163 complementary to a sequence partially overlapping the hTR template and (like all NPS oligomers) working rather in the mode of a template antagonist than through the classic antisense mechanism including RNase-H activation [89]. GRN163 exhibited telomerase inhibition at nanomolar concentrations, gradual telomere shortening, followed by cellular senescence and/or apoptosis in tumor cells, dependent on the initial telomere length [89, 90]. The efficiency of GRN163 in several xenograft models was also higher when cells with shorter telomeres, like DU145 prostate cancer cells, were used [74, 89, 91]. As with other oligonucleotides, the cellular uptake of such a compound is a major obstacle and is facilitated by the use of lipid carriers applied together with the compound [88, 89]. In this regard, a lipid modification of GRN163 (GRN163L), where a palmitoyl group is directly attached to the thio-phosphoramidate, was shown to enhance the potency of telomerase inhib-

ition and biodistribution [92–94]. Xenograft models of human lung and liver cancer substantiate the efficacy of GRN163L at pharmacological doses, smooth the way for this compound into the clinic [93, 95]. GRN163L has recently received clearance by the US Food and Drug Administration to enter human phase I/II clinical testing for chronic lymphocytic leukemia.

Ribozymes

Hammerhead ribozymes cleaving the hTR template were able to inhibit telomerase activity in cell extracts [96], endometrial carcinoma and melanoma cells [97, 98]. More recently, the efficacy of telomerase inhibition in terms of telomere shortening and a lower proliferation rate with hTR-directed ribozymes was shown in human breast cancer cells [99]. Interestingly, ribozyme-mediated suppression of telomerase RNA in a murine melanoma model revealed that tumor invasion and metastatic potential were reduced, suggesting that telomerase controls the expression of several glycolytic pathway genes [100].

RNA/DNA heteroduplex

The DNA synthesis by telomerase requires the transient formation of a duplex consisting of the hTR RNA and telomeric DNA, which could be theoretically disturbed by agents binding specifically to this heteroduplex. Several compounds of this type showed promising lead activity in the low micromolar range [101, 102]. However, it remains unclear how RNA/DNA duplexes involved in other cellular processes would be affected by such approaches. Nevertheless, the susceptibility of this heteroduplex was recently demonstrated by the identification of hPif1, a human helicase that inhibits telomerase activity. Ectopic expression of hPif1 caused telomere shortening in HT1080 cells, likely by unwinding the DNA/RNA duplex [103].

Mutant hTR template/siRNA

In *Tetrahymena* and yeast, mutations in the template region of telomerase RNA resulted in the synthesis of mutant telomeres and in impaired cell growth and survival [104, 105]. Over-expression of mutant hTR in immortal human cells led, furthermore, to a reduction in plating efficiency and growth rate and an increase in the number of senescent cells in colony-forming assays [106]. More recently, a mutant hTR was reported to increase the sensitivity to antitumor agents in cancer cells with different initial telomere lengths and mechanisms of telomere maintenance and without requiring overall telomere shortening [107].

siRNAs targeting hTR are in principle able to inhibit telomerase activity [108]. Recently, lentiviral co-transduction of a mutant hTR and an siRNA directed against wild-type hTR – each separately providing pro-apoptotic effects – was shown to result in a fast and synergistic killing of cancer cells independent of p53 and telomere shortening [109]. The same group investigated the cellular and gene expression responses in line with siRNA-mediated telomerase RNA knockdown in cancer cells indicating a novel response pathway, which includes suppression of specific genes implicated in angiogenesis and metastasis, and which is distinct from the expression profile changes induced by telomere-uncapping mutant template telomerase RNA [110]. These studies add evidence for functions of telomerase in tumor growth and progression besides telomere maintenance and suggest a form of 'telomerase-addiction' of cancer cells. Nevertheless, the efficacy of approaches combining mutant hTR and siRNA seem to depend on a catalytically active hTERT that is able to act on telomeres, as the use of different hTERT variants in such experiments demonstrated [111].

Targeting additional telomerase components

The telomerase holoenzyme requires besides the catalytic subunit hTERT and the RNA template hTR many other factors for its assembly, activation, stabilization and regulation which could in theory be targeted to inhibit telomerase activity [reviewed in ref. 112]. *In vivo* and *in vitro* experiments with antisense oligonucleotides against each of six telomerase components demonstrated a decrease of telomerase activity, providing a rationale for these approaches [113].

Telomerase-associated protein 1

The function of the telomerase-associated protein 1 (TP1) remains unclear although it is a main component of the holoenzyme. However, treatment of leukemic cells with poly(ADP-ribose) polymerase (PARP) inhibitors suppressed telomerase activity by down-regulating TP1 expression while leaving hTERT and hTR expression unaffected [114].

Chaperones

Blocking the interaction of hTERT with the molecular chaperones p23 and Hsp90 inhibits the assembly of active telomerase *in vitro* [115]. Since a wide range of oncogenic key proteins including c-Raf-1, ErbB2, mutant p53, c-Met and Akt/PKB require Hsp90, its inhibition should block multiple-mission-critical oncogenic pathways in the cancer cell, making Hsp90 an exciting new target for the treatment of cancer [for a review see ref. 116]. Two well-known Hsp90 inhib-

itors (geldanamycin and 17-allylamino,17-demethoxygeldanamycin (17-AAG)) demonstrated inhibition of telomerase activity in human melanoma cells and subsequent growth arrest. Interestingly, the sensitivity for 17-AAG was significantly increased in cell clones stably transduced with a hammerhead ribozyme targeting hTR [117]. Recent data from Hsp90 inhibitor experiments suggest that Hsp90 is needed for loading telomerase onto the telomere rather than for the assembly of telomerase [118]. Another study in which depletion of functional Hsp90 by siRNA caused dramatic telomere shortening followed by apoptosis in prostate cancer cells provided evidence of a novel mechanism directly contributing to telomere erosion while cells exhibit a high level of nitric oxide synthase (NOS)-dependent free radical production [119]. Currently, several geldanamycin- and non-geldanamycin-based compounds are in clinical development.

Gene therapy using telomerase promoter sequences

Since hTERT transcription is largely restricted to tumor cells, the hTERT promoter is a perfect instrument to guide the expression of therapeutic genes to these cells.

Proof of principle was given by two initial studies using hTERT promoter-driven vectors for caspase-8 and Bax gene expression, which in each case elicited tumor-specific apoptosis *in vitro* and suppressed tumor growth in nude mice [120, 121]. Another study demonstrated that hTERT- and hTR-promoter-driven expression of diphtheria toxin A chain (DT-A) killed bladder and hepatocellular cancer cells while sparing telomerase-negative cells [122].

A constitutively active caspase-6 (rev-caspase-6) should induce apoptosis independently of the initiator caspases. An hTERT/rev-caspase-6 construct induced apoptosis in malignant glioma cells, but not in telomerase-negative cells. In addition, the growth of tumors in nude mice was significantly suppressed by the treatment with this construct [123].

The herpes simplex thymidine kinase (HSTK) could also be expressed under the control of the hTERT promoter specifically in various tumor cells, sensitizing them to the pro-drug ganciclovir. Moreover, this vector caused tumor regression and survival upon ganciclovir treatment in a xenograft model while preventing the hepatotoxicity encountered with constitutive promoters [124].

Further analogous studies successfully used the hTERT promoter to express the Fas-associated protein with death domain (FAAD) [125], the TNF-related apoptosis-inducing ligand (TRAIL) [126] and the bacterial nitroreductase which sensitizes human cancer cells to the pro-drug CB1954 [127].

Although these results are promising since they demonstrated the feasibility of hTERT-promoter-driven suicide strategies, they are hard to translate into the clinic due to low transduction rates of the used vector systems in cancer cells. This restriction can be overcome by using replicating viruses which allow rapid lysis, spreading of infection and oncolysis throughout the tumor while leaving normal cells unaffected [reviewed in ref. 128]. Several studies used conditionally replicating adenoviruses (hTERT-Ad) expressing E1 genes under control of the hTERT promoter which presented selective replication, oncolysis and spreading in telomerase-positive cancer cells in addition to growth-inhibitory effects in xenograft models [129–132]. The oncolytic effects of such viruses could be enhanced by modification of their tropism [133] or their combinatorial application with chemotherapeutic agents [134] and histone deacetylase inhibitors [135]. A higher specificity and broad anticancer spectrum was demonstrated by the dual promoter-controlled oncolytic adenovirus CG5757 which features the promoters of hTERT and human E2F1 genes, to drive E1 gene expression. The E2F1 promoter is active in tumor cells that have a defective retinoblastoma (Rb) pathway, as in 85 % of all tumor types which should complement hTERT promoter activity in the 90 % telomerase-positive tumor cells [136].

Telomerase immunotherapy

Immunotherapy attempts to stimulate the immune system to attack cancer cells via its exposure to an antigen highly specific for the cancer cells. After hTERT was recognized as a widely expressed tumor-associated antigen (TAA) capable of triggering anti-tumor cytotoxic T lymphocyte (CTL) responses [137, 138], it became evident that the telomerase catalytic subunit would be a valuable target for this type of therapy. Mouse model systems using TERT RNA-transfected dendritic cells (DCs) have confirmed these *in vitro* observations by the induction of immunity against tumors of unrelated origin [139]. It is promising that there are no signs of autoimmunity expressed by hTERT-specific CTLs not lysing either telomerase-positive CD34+ hematopoietic progenitor cells or activated T lymphocytes *in vitro* [137, 138, 140], making hTERT a candidate for a 'universal cancer vaccine' [141]. Since these successful preliminary studies, rapid progress has been made in the development of telomerase-dependent immunotherapy towards clinical application. A phase I clinical

trial demonstrated the immunological feasibility of vaccinating patients against telomerase. Here, hTERT-specific T lymphocytes were induced in four of seven patients with advanced breast or prostate carcinoma after vaccination with dendritic cells pulsed with hTERT peptide, resulting in partial tumor regression in one patient [142]. Another clinical trial was directed against hTERT mRNA-transfected dendritic cells in patients with metastatic prostate cancer [143]. A trial investigating vaccination with hTERT peptides in patients with non-small cell lung cancer demonstrated immune responses in 12 of 24 evaluable patients during the primary regimen, with a complete tumor response observed in 1 patient [144]. After demonstration of the safety, tolerability and clinical response to telomerase vaccinations, clinical phase II and III trials are currently underway to evaluate the potential of immunotherapy exploiting hTERT as a universal cancer antigen.

Targeting the telomere

Telomerase inhibition can be achieved not only by affecting the holoenzyme but also by sequestration of its substrate, the telomere itself. Admittedly, there is a major concern targeting telomeres, since – unlike telomerase – they are present in normal and cancer cells, hence the risk of cytotoxicity in the course of such approaches. Nevertheless, blocking the access of telomerase to the telomere by altering its structure has been well investigated, since it has the potential for more rapid growth-inhibitory effects in tumor cells than 'classic' telomerase inhibition [145, 146].

DNA sequences which are rich in guanine like the telomere are capable of forming four-stranded structures called G-quadruplexes. In a first study, it was shown that telomeric G-quadruplexes can be stabilized by K^+ which in turn inhibits telomere elongation by telomerase [147]. A small molecule (2,6-diamidoanthraquinone) was discovered that had similar G-quadruplex-stabilizing effects [148]. Since then a large number of quadruplex ligands of different compound classes have been developed potentially inhibiting telomere accessibility for telomerase. G-quadruplex-stabilizing agents include cationic porphyrins (TMPyP4) [149], perylenes (PIPER) [150], trisubstituted acridines (BRACO19) [151], bisacridines [152], pentacyclic acridines (RHPS4) [153], natural products (telomestatin) [154], ethidium derivatives [155], dibenzophenanthrolines [156], triazines (12459) [157], fluoroquinophenoxazines (QQ58) [158] and anionic porphyrins (NMM) [159].

In particular, telomestatin appears very promising due to its high selectivity toward quadruplexes compared to other nucleic acid conformations [154, 160]. Telomestatin induces apoptosis in various tumor cell lines and is more selective for cancer cells than for normal progenitor cells [160–165]. In addition, telomere shortening is observed in cells treated with telomestatin, but appears earlier than expected for simple telomerase inhibition [160, 166]. Telomestatin treatment of U937 cells in a xenograft mouse model displayed decreased tumor telomerase levels and reduced tumor volumes without any signs of toxicity [167]. Most recent publications indicate a mode of action of telomestatin by dissociation of telomere-binding proteins TRF2 and POT1 eventually inducing a rapid decrease of the telomeric (3′)-overhang and of the double-stranded telomeric repeats [168–170]. Finally, a total synthesis of telomestatin has been achieved in good accordance with the natural product [171].

BRACO-19, a member of the family of trisubstituted acridines produces growth inhibition and senescence in human tumor cell lines at subcytotoxic concentrations, after days rather than weeks [172]. A fast *in vivo* efficacy was seen for BRACO-19 against a uterus carcinoma xenograft model which produced growth inhibition of 96% compared with controls [173]. Nevertheless, the very poor permeability of BRACO-19 might be its main biopharmaceutical limitation. Further applications will require a suitable formulation to warrant adequate delivery across cellular barriers [174].

Targeting telomere-associated proteins

As mentioned above telomere-binding proteins like TRF2 play a fundamental role in the stability of the telomere. Over-expression of a DN-TRF2 in human cancer cell lines leads to rapid telomere loss and senescence or apoptosis [145, 146, 175].

The other important duplex telomeric DNA binding factor TRF1 inhibits telomere elongation by telomerase [reviewed in ref. 176]. The poly(ADP-ribose) polymerase tankyrase catalyzes poly(ADP-ribosyl)ation of TRF1 blocking the ability of TRF1 to bind to the telomere. This eventually results in the disruption of the t loop structure allowing telomerase to act on the telomere, rendering tankyrase itself an attractive target for cancer therapy. In fact, it was demonstrated recently that pharmacological targeting of tankyrase 1 enhances telomere shortening by means of a telomerase inhibitor and results in earlier crisis of human cancer cells [177]. Thus, telomerase inhibition in combination with PARP inhibition might represent a potential strategy to circumvent the problem of the lag phase by application of a telomerase inhibitor alone.

Indeed, several PARP inhibitors are currently in clinical trials, not only for cancer but also for other diseases such as stroke, myocardial ischemia, diabetes and central nervous system injury.

Conclusions

The impact of telomerase in tumor development and sustainment has been clearly established during the last decade, suggesting that targeting this enzyme is a valid strategy to combat cancer. However, translation of this knowledge into the clinic appears to be slow. Using genetic approaches, the complete inhibition of telomerase activity has been shown to induce a strong antiproliferative signal in various cancer cell lines and xenografts. However, a major obstacle with direct inhibition of telomerase activity is the problem of the delay in antiproliferation depending on the length of telomeres in a given tumor cell. From a clinical perspective, preselection of patients with very short telomeres and low tumor burden may be necessary to show any effect within a short time of treatment. Consequently, there is a need for measurement of the telomere length in a pathological specimen. Alternatively, telomerase inhibitors could be used as an adjuvant treatment in combination with surgery, radiation treatment and conventional chemotherapy. Another potential application could be postremission therapy in order to eliminate minimal residual disease. Thus, treatment may have to be administered continuously for weeks to months and the success of a telomerase inhibitor therapy requires compounds that are be sufficiently well tolerated, have a low toxicity profile and are easy to administer (ideally orally). It will be challenging to design clinical studies in which endpoints such as telomerase suppression and telomere shortening are monitored properly.

Unfortunately, the development of non-nucelosidic telomerase inhibitors has not yet generated compounds which are capable of inducing a complete inhibition of the enzyme activity in an *in vivo* situation over a longer time period. It is likely that even low or transient telomerase activity is sufficient to repair the damage on telomeres which subsequently does not have a significant effect on proliferation. However, it is interesting that an oligonucelotide-based therapeutic has moved from preclinical development into a phase I/II clinical trial in patients with chronic lymphocytic leukemia. This compound, GRN163L, is a telomerase RNA (hTR) template antagonist agent with a lipid palmitate moiety preventing hTR forming a complex with hTERT. It has been reported that this oligonucleotide has sufficient cellular uptake and biodistribution at even low concentrations *in vivo*, which is crucial for fulfilling the criteria of success mentioned above. Furthermore, it is not excluded that significant off-target effects may occur upon treatment by this approach. Nevertheless, the ongoing trial is a landmark in the development process of telomerase therapeutics, and it will be challenging to determine if the preclinical concepts hold true for the treatment of cancer patients.

Another potential way to circumvent the lag phase and to induce direct telomere damage in tumor cells is by small-molecule G-quadruplex-interactive agents which lead to rapid onset of senescence or apoptosis. It will be crucial to identify compounds with low toxicity, high efficacy and specificity at nanomolar concentrations in order to translate these concepts into the clinic. A further perspective would be to inhibit not only the activity of telomerase but to suppress or knockdown hTR or hTERT, as was demonstrated by siRNAs. In addition to effects on telomeres, other response pathways related to angiogenesis and metastasis seem to be specifically involved. However, the translation of this approach seems to be more sophisticated than using drugs.

Apart from the effects on tumor cells, it is often argued that telomerase inhibition might also affect proliferation of highly proliferative organs such as germ cells, stem cells and lymphocytes which express low levels of telomerase activity. However, since they have in general longer telomeres than tumor cells, telomere loss is likely to be moderate without reaching critical telomere dysfunction [178] (see also Fig. 1). Given the detrimental effects of conventional cytotoxics on the hematopoietic stem cell pool, this issue should not raise too much concern. However, clinical studies need to address this question carefully.

In summary, translation of the biology of telomeres and telomerase into the clinic has started (inhibitors, immunotherapy, oncolytic virus therapy), and has to be seen as the result of extensive basic research during the last 15 years. Whether or not we are entering a new era of targeted therapy for cancer can only be answered by the outcome of well-designed clinical trials.

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