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Review

Targeting the telosome: Therapeutic implications

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ABSTRACT

Since telomere integrity is required to guarantee the unlimited replicative potential of cancer cells, telomerase, the enzyme responsible for telomere length maintenance in most human tumors, and lately also telomeres themselves have become extremely attractive targets for new anticancer interventions. At the current status of knowledge, it is still not possible to define the best therapeutic target between telomerase and telomeres. It is noteworthy that interfering with telomeres, through direct targeting of telomeric DNA or proteins involved in the telosome complex, could negatively affect the proliferative potential not only of tumors expressing telomerase activity but also of those that maintain their telomeres through alternative lengthening or still unknown mechanisms. This review presents the different therapeutic approaches proposed thus far and developed in preclinical tumor models and discusses the perspectives for their use in the clinical setting.

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1. Introduction

1.1. Telomeres and telomere maintenance mechanisms

Telomeres are heterochromatic structures located at the ends of chromosomes. In humans, they consist of tandem repeats of the TTAGGG sequence with a 3′ overhang that plays important structural and functional roles. Such a 3′ single-stranded telomere overhang can invade homologous double-stranded telomeric tracts, displace the G-strand and anneal with the C-strand, thus giving rise to a lasso-like structure, known as the telomeric loop (t-loop) [1]. Telomeric DNA is bound by an array of specialized proteins that form a protective structure known as the telosome [2] or shelterin [3]. Telomeres are essential to protect chromosome ends (capping function) and maintain chromosomal stability and genome integrity [1]. In somatic cells, telomeres shorten with each round of cell division due to the inability of DNA polymerase to fully replicate the end of chromosomes [4], and shortened telomeres trigger replicative senescence [5].

A hallmark of cancer cells is their unlimited proliferative potential, which can be achieved through the activation of a telomere maintenance mechanism [6]. The main mechanism for telomere elongation is the enzyme telomerase, a RNA-dependent DNA polymerase that adds multiple copies of the TTAGGG motif to the end of the G-strand of the telomere [7]. The core enzyme consists of an RNA component (hTR) that provides the template for the de novo synthesis of telomeric DNA [8] and a catalytic subunit hTERT, which has reverse transcriptase activity [9].

Recent evidence suggests that the catalytically active telomerase exists as a complex of two molecules each of hTERT, hTR and dyskerin [10]. In addition to its role in maintaining chromosome ends through the regulation of telomere length, telomerase activation has been recently implicated in providing growth-promoting properties to tumor cells [11,12]. Telomerase is present in 85–90% of human cancers [13] but is generally undetectable in somatic cells, with a few exceptions including stem cells and some highly proliferative cell types. The complex mechanisms by which telomerase activity is up-regulated in human tumors are being studied, and the altered expression of oncogenes and tumor suppressor genes seems to be involved [14,15].

An alternative mechanism for telomere elongation, the so-called alternative lengthening of telomeres (ALT, reviewed in 16), is present in a significant proportion of telomerase-negative human tumors of mesenchymal and neuroepithelial origin [16,17]. Although the molecular events leading to ALT activation in tumors are almost completely unknown, there is evidence to suggest that it relies on homologous recombination events at telomeres. Lack of telomerase gene expression in ALT tumor cells seems to be associated with chromatin remodeling at the hTR and hTERT promoters [18]. The main features of ALT include long and heterogeneous telomeres and subnuclear structures termed ALT-associated promyelocytic leukemia nuclear bodies, which contain telomeric DNA, telomere-binding proteins and proteins involved in DNA recombination and replication [16,17].

1.2. The telosome/shelterin complex

Mammalian telomeres are associated with the high-molecular-weight telosome/shelterin complex, consisting of six interdependent

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telomeric core proteins: telomeric-repeat-binding factor 1 (TRF1) and 2 (TRF2), TRF1-interacting protein 2 (TIN2), transcriptional repressor/activator protein RAP1, protection of telomeres 1 (POT1) and POT1- and TIN2-organizing protein (TPP1) [3]. TRF1, TRF2 and POT1 directly associate to telomeric DNA and are interconnected to each other by TIN2, RAP1 and TPP1. The telosome/shelterin complex allows telomeres to exist in two states: a "capped" one in which the 3′ overhang is protected from degradation and inappropriate repair mechanisms but is not accessible to telomerase, and an "uncapped" state in which telosome proteins are present but leave the 3′ overhang accessible to telomerase [19].

TRF1, TRF2 and POT1 are essential regulators of telomere structure, capping and length control. TRF1 and TRF2 specifically bind to double-stranded telomeric repeats through a Myb-like helix/turn/helix motif [20]. TRF1 is a ubiquitously expressed protein that negatively regulates telomere length by physically preventing telomerase access to telomere ends [21], whose function is regulated by TIN2 [22]. TRF1 also binds proteins with non-telomeric functions, such as poly(ADP-ribose) polymerase and tankyrase 1 [23]. Although the precise roles of tankyrase 1 are not completely understood, it has been shown that the enzyme can ADP-ribosylate TRF1, thus interfering with its negative regulation of telomere length [24]. TRF2 plays a key role in the protection of chromosome ends by stabilizing the 3′ overhang and contributing to the formation of the t-loop structure at the end of the telomere [25]. Moreover, it interacts with RAP1 [26] and brings to telomeres a variety of proteins involved in DNA damage response, including the Mre11/Rad50/Nbs1 complex, Ku heterodimer and ataxia-telangiectasia mutated (ATM) kinase [26].

As regards POT1, it has been shown that, following heterodimerization with TPP1, the protein specifically binds to and protects single-stranded telomeric DNA [27]. In addition, the POT1–TPP1 complex seems to favor the recruitment of telomerase to telomeres, thus participating in the telomerase-dependent control of telomere length [28]. However, the function of POT1 in telomere length control is still controversial, since some evidence points to POT1 as a negative regulator of telomere length, as a consequence of its ability to prevent telomerase from gaining access to 3′ telomere ends [3]. It has been demonstrated that, in association with TRF2, POT1 participates in the protection of telomeres by contributing to the t-loop formation and by regulating the nucleolytic processing responsible for the 3′ overhang formation [29]. In addition, POT1 is interconnected to TRF1 and TRF2 through TIN2 and TPP1 [30]. Evidence has been provided that the coordinated interaction among TIN2, TPP1 and POT1, not only in the nucleus but also in the cytoplasm, regulates the assembly and function of the telosome, which highlights the importance of the spatial control of telomeric proteins to guarantee the maintenance of telomere integrity [30].

In addition to the telosome/shelterin complex, mammalian telomeres interact with other factors (e.g., tankyrase 1 and 2, poly(ADP-ribose) polymerase, ATM, ATM and Rad3-related (ATR), and general DNA repair/recombination factors), which altogether cooperate to determine telomere replication, protection and stability, thus contributing to the integrity and dynamics of the ends of chromosomes [31].

2. Targeting telomerase and telomeres

2.1. Telomerase inhibitors

Since unlimited proliferative potential has been identified as one of the six hallmarks of cancer [6], telomere maintenance mechanisms have been proposed as potential targets for new anticancer interventions [32]. Due to the selective reactivation of telomerase in most human tumors, the possibility to interfere with its expression and/or functions has been actively pursued through the use of different approaches aimed to i) inhibit the enzyme's catalytic activity; ii) interfere with the expression of its components (i.e., hTR, hTERT); iii) affect the signaling pathways responsible for the post-translational modifications of the enzyme.

Among the first drugs tested for their ability to competitively inhibit telomerase activity are nucleoside analogs, which act as chain-terminators during nucleotide polymerization. Several nucleotide analogs have been proven to efficiently inhibit telomerase activity and cause telomere shortening, depending on the concentration and duration of exposure [33–37], as well as to induce antiproliferative and antitumor effects in several tumor models in vitro and in vivo [38]. A variety of non-nucleoside compounds have also been shown to inhibit telomerase [37,38], including a mixed-type non-competitive inhibitor, BIBR1532. It binds to a site in the enzyme which is distinct from those for deoxyribonucleotides and the DNA primer [39] and is one of the most potent telomerase inhibitors discovered thus far [38]. The exposure of human cancer cells of different histological origin to BIBR1532 led to progressive telomere shortening and inhibition of cell proliferation, independently of p53 gene status [37], and to a marked reduction in the tumorigenic potential following xenotransplantation into nude mice [38]. A dose-dependent cytotoxic activity of the compound was also observed in leukemia cell lines and primary cultures and was paralleled by time-dependent individual telomere erosion, loss of TRF2 and increased phosphorylation of p53 [40]. In addition, pharmacological telomerase inhibition by both nucleoside and non-nucleoside inhibitors has been shown to sensitize drug-resistant cancer cells to chemotherapeutic treatments [41].

Antisense-mediated approaches aimed at inhibiting the expression of telomerase components have been developed through the use of chemically modified oligonucleotides (including phosphorothioates, RNA oligomers containing nucleotides with alkyl modifications at the 2′ position of the ribose, 2′,5′-oligoadenylate antisense oligomers, N3′→P5′ phosphoramidates and peptide nucleic acids). Such oligonucleotides are characterized by pharmacokinetics and therapeutic properties superior to those of conventional nucleic acid-based antisense oligomers [32]. Catalytic RNAs such as ribozymes as well as small interfering RNAs (siRNAs), which are the terminal effectors of the RNA interference pathway, have been also used as antisense-based tools [32]. The different antisense strategies explored thus far to target hTR mainly acted as template antagonists, leading to cancer cell growth arrest and reduced viability only after several population doublings as a consequence of the interference with telomere lengthening activity of the enzyme. Such evidence is in keeping with the classical mechanism by which telomerase inhibition induces a delayed cell growth arrest and death as a result of critically shortened telomeres ("slow pathway", Fig. 1).

Since such a cellular response has been shown to be largely dependent on the initial telomere length in a given tumor cell population [42], single-agent therapies based on inhibitors targeted to hTR would need long-term treatment to induce effective impairment of growth of cancer cells with relatively long telomeres. At present, the most promising oligomer targeting hTR is GRN163L, a lipid palmidate derivative of the parent thiophosphoramidate GRN163, which demonstrated in vivo activity against a panel of xenografted tumors with short telomeres [42–45] and an ability to prevent lung metastases in animal models [46]. Recent results suggest that the compound could also act in an hTR-independent fashion. Specifically, the potent antimetastatic effect of GRN163L could partially rely on its anti-adhesive properties independently of binding to hTR [47]. Interestingly, phase I/II clinical trials using GRN163L for the treatment of chronic lymphocytic leukemia patients and solid tumors have recently begun.

Several studies have shown that antisense molecules (including ribozymes, siRNAs and peptide nucleic acids) aimed at down-regulating hTERT expression are able to induce short-term antiproliferative effects and programmed cell death [32,48,49], which cannot
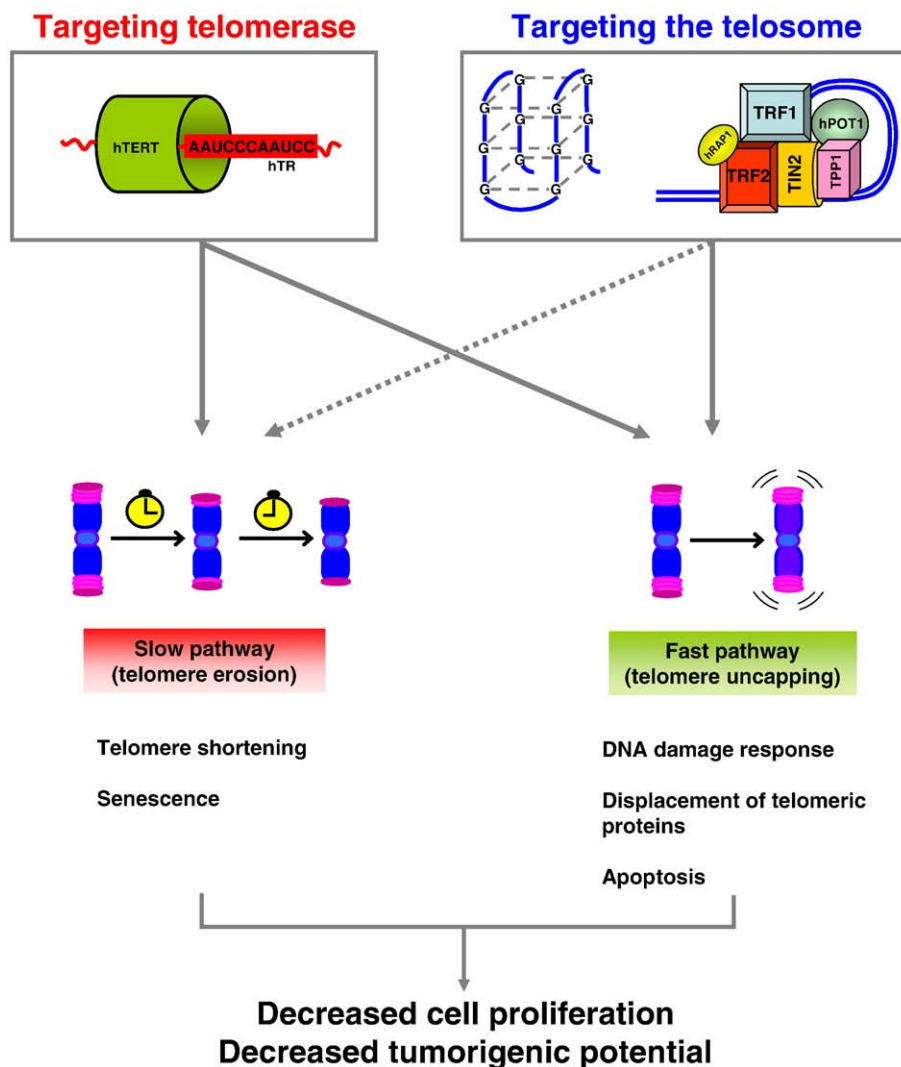
Fig. 1. Cellular consequences of telomerase and telosome targeting. Telomerase inhibitors may induce either a delayed antiproliferative effect through a "slow" pathway mainly based on telomere erosion (BIBR1532, hTR inhibitors) or a quick antiproliferative effect through a "fast" pathway mainly based on telomere uncapping (hTERT inhibitors). Inhibitors of telosome proteins and G4 ligands mainly induce telomere uncapping.

be explained by the classical model based on telomere shortening. Interference with hTERT might therefore affect aspects of the control of cell proliferation other than telomere length ("fast pathway", Fig. 1). It has been consistently demonstrated that the early antiproliferative effects caused by hTERT knockdown are the consequence of the induction of telomere dysfunctions and a DNA damage response at the telomere level through pathways independent of the telomere-elongating activity of the enzyme [11,12].

This novel function of telomerase was first documented by Cao et al. [50], who reported that the quickly induced programmed cell death following hTERT down-regulation in human breast cancer cells could be counteracted by the ectopic expression of a mutant hTERT lacking telomerase activity. This finding has conferred to hTERT a putative pro-survival and anti-apoptotic function, which is independent of telomerase catalytic activity and likely relies on the telomere capping function of the enzyme [19]. This concept was supported by our study which showed that exposure of prostate cancer cells to a 2′-O-methyl-RNA phosphorothioate oligonucleotide targeting hTERT induced almost complete inhibition of telomerase activity as a consequence of a marked reduction of the hTERT mRNA expression level, an early decline of cell growth and apoptotic cell death without any appreciable telomere shortening [51]. Conversely, exposure of the

same cells to a 2′-O-methyl-RNA phosphorothioate oligonucleotide targeting the template region of hTR failed to interfere with cell proliferation in spite of the almost complete abrogation of telomerase activity [51].

Numerous compounds have also been shown to affect telomerase via an indirect mechanism. In fact, telomerase regulation is complex and involves several pathways that provide an impressive number of "druggable" targets. Specifically, heat shock protein 90, protein kinase C, histone deacetylase, COX-2 and tyrosine kinase inhibitors have all demonstrated an ability to inhibit telomerase activity [38]. Pharmacological agents that act on hormonal pathways such as tamoxifen [52] as well as a number of compounds from various sources, including nonsteroidal anti-inflammatory drugs and anti-oxidants [38], also negatively interfere with the enzyme's activity. However, since these molecules can affect several cellular targets, it is difficult to establish to what extent telomerase inhibition is relevant for their therapeutic activity.

Experimental data show that interference with telomerase expression and/or function leads to increased sensitivity of cancer cells to conventional anticancer drugs and radiation [32,38]. Such data corroborate the notion that telomerase represents a promising anticancer target also for the design of new combination therapies.

2.2. Interference with telosome proteins

Whereas telomerase inhibitors have been developed with the purpose to preclinically validate new therapeutic strategies aimed at inhibiting the growth of tumor cells and enhancing their susceptibility to drugs, studies carried out to interfere with the expression/function of telosome components have been mainly devoted to understand the specific role they exert in the regulation of telomere structure and dynamics [4,53,54]. As regards TRF1, long-term overexpression of the protein in the telomerase-positive HT1080 cell line resulted in a gradual and progressive telomere shortening, whereas telomere elongation was induced by expression of a dominant-negative TRF1 mutant that inhibited binding of endogenous TRF1 to telomeres. As TRF1 did not affect the expression of telomerase, it has been suggested that the binding of TRF1 controls telomere length by inhibiting the action of telomerase at the ends of individual telomeres [21].

Most data on the role of telomere-related proteins have highlighted that the telosome acts as a physical cap to protect the ends of chromosomes from being recognized as natural double-strand breaks, thereby making it possible to distinguish normal ends from double-strand breaks and avoiding the activation of DNA damage checkpoints. The notion that deprotected telomeres activate a DNA damage response gained support from the evidence that DNA damage response factors, such as 53BP1, γH2AX, Mre11, and the phosphorylated form of ATM, can localize at uncapped telomeres, forming cytological structures referred to as telomere dysfunction-induced foci (TIF) [55]. The protective (i.e., capping) function of telosome proteins has been primarily assigned to TRF2. Inhibition of TRF2 through the overexpression of a dominant-negative mutant of the protein (TRF2$^{\Delta B\Delta M}$) – able to heterodimerize with the endogenous TRF2 and to block its binding to telomeric DNA [56] – caused loss of the 3′ overhang and resulted in a high frequency of chromosome end-to-end fusions. Such a response is a consequence of the activation of DNA ligase IV-dependent nonhomologous end joining and activation of the ATM kinase/p53 DNA damage response pathway, ultimately leading to cell death in several in vitro models [3]. Such evidence indicates TRF2 as an early component of the DNA damage response system, as also pointed out by the finding that overexpression of TRF2 decreased the accumulation of γH2AX at photo-induced breaks in human cells [57].

It has been also demonstrated that mice overexpressing TRF2 in the skin show critically short telomeres and are susceptible to UV-induced carcinogenesis as a result of deregulated XPF/ERCC1 activity [58]. Furthermore, the expression of TRF2 in hTR-null mice led to chromosome instability, induction of DNA damage and a high frequency of telomere recombination [58]. The latter evidence has suggested that increased expression of TRF2, along with telomerase deficiency, can represent an event that could sustain tumorigenesis by helping to bypass the cell proliferation barrier imposed by short telomeres through the derepression of the ALT pathway, hence promoting the growth of tumors with high chromosomal instability [59]. In addition, mouse models knocked out for TRF2 showed embryonic lethality, and cells derived from TRF2- and p53-null embryos were characterized by loss of telomere protection and a massive increase in end-to-end fusions [59].

It has also been reported that in checkpoint-compromised telomerase-positive human fibroblasts, TRF2 inhibition promoted heritable changes that increase the ability to grow in soft agar but not tumor growth in nude mice. This transforming activity was associated to telomere instability but was independent of an altered control of telomere length. Such a finding supports the idea that telomere dysfunction might contribute to cancer progression even at late stages of the oncogenesis process, after the telomerase reactivation step [60].

In contrast, combined siRNA-mediated knockdown of TRF2 and hTR (by using a lentiviral vector bearing a mutant hTR template and a shRNA targeting hTR) resulted in an additive inhibitory effect on the growth of melanoma cells, which indicates a function of TRF2 in sustaining cancer cell proliferation [61]. In addition, RNAi-mediated inhibition of TRF2 expression partially reversed the resistance phenotype of the multidrug-resistant variant SGC7901 gastric cancer cells, thereby suggesting a possible role of TRF2 in drug resistance of gastric cancer [62].

Dysfunctional telomeres can also be induced by interference with expression of components of the telosome other than TRF2. It has been reported that activated ATM directly phosphorylates Pin2/TRF1 on the conserved Ser(219)-Gln site in vitro and in vivo and that transfection of tumor cells with a Pin2 mutant refractory to ATM phosphorylation on Ser(219) potently induces mitotic entry and apoptosis and increases radiation hypersensitivity [63]. In contrast, Pin2 mutants mimicking ATM phosphorylation on Ser(219) completely failed to induce apoptosis and also to reduce radiation hypersensitivity of A-T cells. The phenotype of the phosphorylation-mimicking mutants is the same as that resulting from inhibition of endogenous Pin2/TRF1 in A-T cells by its dominant-negative mutants [63]. These results demonstrated that ATM interacts with and phosphorylates Pin2/TRF1 and suggest that Pin2/TRF1 may be involved in cellular response to DNA double-strand breaks [63].

It has also been reported that TRF2 cooperates with POT1 to maintain telomere integrity [64]. The over-expression of exogenous POT1 blocks the erosive effect on the 3′ overhang and the occurrence of chromosome abnormalities detectable in different cells expressing the dominant-negative form of TRF2 [64]. It has been recently reported that TRF2 and POT1 act independently to repress the ATM- and ATR-dependent DNA damage responses [65]. In fact, telomere damage generated by TRF2 loss primarily activated an ATM-dependent response. Deletion of TRF2 from Atm$^{+/+}$ cells led to induction of TIF and phosphorylation of Chk2, whereas such a response was largely abrogated in Atm$^{-/-}$ mouse cells [65]. In contrast, the role of POT1 in repressing the activation of ATR kinase at natural chromosome ends was demonstrated by evidence that TIF formation in POT1a/b double knockout mouse cells was significantly reduced when ATR was inhibited by using a short hairpin RNA [65].

The role of POT1 in protecting telomeres has been also evaluated in breast cancer cells exposed to anti-POT1 siRNAs [64]. Specifically, the POT1 knockdown resulted in the induction of apoptosis, consequent to telomere dysfunction, increased expression of p53 and Bax, and concomitant decrease of Bcl-2 levels, in breast cancer cells [64] and in a marked increase of TIF in the G$_1$ phase of the cell cycle in HeLa cells and in primary and hTERT-expressing BJ fibroblasts [29]. In addition, fibroblasts responded to POT1 depletion with strongly reduced proliferation and induction of senescence, independently of the presence of telomerase [29]. A role for POT1 in carcinogenesis has been further suggested by findings showing that deletion of the POT1 gene in mice resulted in chromosomal instability and increased telomere recombination [59].

It has been reported that transient knockdown of TPP1 in mouse embryonic cells initiates a robust ATR-dependent DNA-damage response and that its long-term knockdown elicits primarily an ATM-dependent DNA-damage response. Although the molecular mechanism contributing to this difference in damage signaling is not yet clear, biochemical analysis suggests that TPP1 not only binds POT1, but also interacts with TIN2 to stabilize TRF1–TIN2–TRF2 interaction [66]. In addition, disrupting the nuclear export signal of TPP1 resulted in a telomeric DNA-damage response and telomere length deregulation [30].

Depending on the genetic makeup of the cells, dysfunctional telomeres can lead to the induction of either apoptosis or senescence. In this context, analysis of the extent to which the cellular level of telomere dysfunction and p53 gene status affect these cellular responses in mouse liver has been conducted through TRF2 inhibition by TRF2$^{\Delta B\Delta M}$ [67]. It has been shown that the level of telomere

dysfunction correlated with the level of TRF2$^{\Delta B \Delta M}$ protein expression, resulting in chromosomal fusions, aberrant mitotic figures and aneuploidy of mouse liver cells. Such alterations provoked p53-independent apoptosis but a strictly p53-dependent senescence response in distinct cell populations. Specifically, apoptosis was associated with a higher expression of TRF2$^{\Delta B \Delta M}$, whereas cellular senescence was associated with low levels of dominant-negative TRF2 expression, providing evidence that induction of senescence or apoptosis in vivo depends on the cellular level of telomere dysfunction and on p53 gene function [67]. Accordingly, it has been demonstrated that overexpression of dominant-negative mutant TRF2 caused apoptosis in tumor cells whereas normal fibroblasts went into senescence [68].

2.3. G-quadruplex stabilization

G-quadruplexes (G4) are a family of nucleic acid secondary structures stabilized by G quartets (each composed of four guanines, held together by a cyclic arrangement of 8 hydrogen bonds), which form in the presence of cations and are thought to play a role in key biological processes. The in vivo existence of G4 in oncogene promoters and telomeres has been demonstrated through the use of specific ligands [69,70]. The human telomeric G-overhang can fold into several different intramolecular quadruplex structures that differ for the position of the adjacent loop regions. The crystal and solution structures of G4 formed by human telomeres have recently been elucidated, and this is boosting the rational development of novel effective compounds [38]. The number of known G4 ligands has grown rapidly over the past few years (reviewed in 38). All of them target G4 structures by a reversible process, whereas no selective irreversible G4 alkylator has been thus far described. Features shared by many of these ligands include a large flat aromatic surface, the presence of cationic charges, and the ability to adopt a terminal stacking mode.

Due to the inability of telomerase to extend a G4-folded telomeric substrate, G4-interacting agents were first evaluated as telomerase inhibitors. In agreement with the initial paradigm for telomerase inhibition, long-term exposure of human cancer cells with subtoxic doses of some G4 ligands (such as disubstituted triazines and telomestatin) induced progressive telomere shortening and replicative senescence [71–74]. However, in several other studies, it was shown that G4 ligands, including BRACO 19 and RHPS4, were able to cause a short-term antiproliferative response (mainly in terms of induction of apoptosis) that cannot be simply explained by telomerase inhibition. Specifically, the observation that BRACO 19 and other G4 ligands caused chromosome end-to-end fusions, telophase bridges, together with the appearance of p16-associated senescence, led to the proposal that the target of G4 ligands is the telomere rather than telomerase [75,76].

G4-stabilizing agents were also shown to inhibit the growth of ALT cell lines. Specifically, the quinoline-based G4-ligands 115405 and RHPS4 were both able to cause growth inhibitory effects in the SV40-immortalized human fibroblast cell line GM847 that expresses the ALT phenotype [71, 77]. In addition, 2,6-pyridine-dicarboxamide derivatives, displaying strong selectivity for the G4 structures, were found to induce antiproliferative effects in the telomerase-negative human osteogenic sarcoma cell line Saos-2, which maintains its telomeres through ALT mechanisms [78]. These findings further corroborated the hypothesis that the antiproliferative effect of G4 ligands was largely independent of the presence of active telomerase [75,77]. In accord with this hypothesis, it has also been reported that G4 ligand-induced dysfunction at the level of telomeres (which under normal conditions prevent the recognition of natural chromosome ends as double-strand breaks) provokes a DNA-damage response in human tumor cells, as evidenced by the formation of γH2AX foci that partially co-localize at the telomere.

Telomestatin also induces degradation of the G-overhang and a reduction of telomeric repeat fragment, with a concomitant displacement of POT1 and TRF2 from their telomere sites in cancer but not in normal cells [79,80]. Based on this evidence, a consistent mechanism of action schema is now emerging for G4 ligands in tumor cells, which initially involves alteration of the G-overhang structure followed by degradation through a DNA-damage repair pathway and release of POT1 from telomeres. In addition, G-overhang degradation induces a further t-loop instability followed by anaphase bridges and telomere loss associated with TRF2 release from telomeres [38].

Evidence that G4 ligands selectively limit the growth of cancer cells without affecting the viability of normal cells [80], together with their ability to exert an antitumor activity against a variety of human tumor xenografts (including some inherently resistant to chemotherapy) and to inhibit the development of metastases in selected in vivo models [81], provide compelling rationale for the clinical use of these compounds to target the limitless replicative potential of malignant cells for broad-spectrum cancer therapy. In addition, very recent data showing the good pharmacodynamic profile of RHPS4 and its ability to potentiate the antitumor activity of cytotoxic agents widely used in cancer treatment, such as taxanes [82] and camptothecins [81], strongly suggest this drug as a promising anticancer agent.

However, notwithstanding the promising results obtained in preclinical models, G4 ligands have not yet reached the clinical setting. One main reason is likely related to the fact that the exact structure(s) of human telomeric G4 is still controversial. It has been shown that telomeric G-overhang can fold into several intramolecular quadruplex structures that differ by the position of the adjacent loop regions [83]. The knowledge of the intact human G4 structures formed in vivo under physiological conditions is a prerequisite for structure-based rational design of compounds able to exert a clinical activity. In this context, it has been experimentally demonstrated that different conformations of G4 play a pivotal role in molecular recognition and govern G4 binding to small molecules. For example, it has been reported that the G4 ligand TMPyP$_4$ shows preferential binding to the parallel G4 over its antiparallel counterpart [84].

3. Conclusions and perspectives

Data obtained from preclinical studies on the effects of telomerase inhibition, accomplished through genetic and pharmacological approaches, provide a compelling argument to indicate that the enzyme is a well-validated target for cancer therapy. It emerged that telomerase inhibition mainly leads to tumor growth impairment through a slow pathway mediated by progressive telomere erosion, even though it has been shown that direct targeting of hTERT expression is able to induce a fast antiproliferative response that relies on telomere uncapping (Fig. 1). The differences in telomerase expression, telomere length and predicted stem cell kinetics in normal versus tumor tissues suggest that no major safety concerns should be expected with the clinical use of anti-telomerase therapies.

At present, the only telomerase inhibitor in the clinic is GRN163L, which is currently used in phase I/II trials as a single agent for the treatment of chronic lymphocytic leukemia and solid tumors. In addition, based on preclinical evidence suggesting a chemosensitizing effect of telomerase inhibition, the drug is also used in combination with paclitaxel and carboplatin for the treatment of non-small cell lung cancer patients [85]. Another important clinical application that could be envisaged for GRN163L (as well as for other compounds able to inhibit telomerase and/or induce telomere dysfunction) is in combination with radiation therapy. In this context, it has been recently demonstrated that in the MDA-MB-231 human breast cancer cells, exposure to GRN163L resulted in progressive telomere shortening and significantly enhanced radiation sensitivity in vitro and in vivo, likely as a consequence of an impairment in radiation damage repair [86]. An issue that needs to be addressed for

the clinical use of telomerase inhibitors is related to the choice of the best patient population. In this context, the recent finding that a significant fraction of solid tumors of mesenchymal and neuroepithelial origin expresses the ALT phenotype instead of telomerase [87,88], and as a consequence is not likely to be affected by telomerase inhibitors, suggests that telomere maintenance mechanism status should be determined in individual patients before starting an anti-telomerase treatment. Finally, as for all mechanism-based therapies, the validation of pharmacodynamic markers of clinical activity is warranted.

At the preclinical level, it has been demonstrated that the use of compounds that selectively target components of the telosome complex or specialized telomere structures, such as G4, can quickly induce programmed cell death and activate a DNA-damage response in a variety of tumor models (Fig. 1). In addition, biochemical determinants of DNA-damage response (i.e., phosphorylation of H2AX) have been proposed as surrogate markers of tumor response to be used in clinical trials. Interestingly, some of these compounds were able to induce antiproliferative effects also in ALT cells, and one of them, telomestatin, was shown to selectively induce early cell death in cancer cells, with normal cells remaining viable much longer. Also widely debatable, it cannot be excluded that protein composition at the telomere may differ, quantitatively and/or qualitatively, in normal versus tumor cells and that normal cells may have a higher degree of telomere stability.

Although a comprehensive survey of telomere-related proteins in normal and cancer cells has not yet been done, initial evidence suggests that an altered expression of telomeric protein could be involved in the development of a malignant phenotype. In this context, a gradual increase in the expression of TRF1, TRF2, and TIN2 was detected according to the progression of hepatocarcinogenesis, with a marked increase, paralleled by inversely correlated telomere lengths, in hepatocellular carcinoma compared to high-grade dysplastic nodules [89].

Again, genes encoding for telomere-associated proteins have been reported to display different patterns of expression in human breast cancer specimens and in normal breast tissues, suggesting different and sometimes opposing roles in mammary carcinogenesis [90]. Specifically, POT1 transcription levels demonstrated a compelling trend to be lower in malignant tissues and much lower in those patients who develop recurrent disease, indicating that POT1 may act as a tumor-suppressor gene [90].

It has been reported that subsets of tumors of different histological origin, including liposarcoma, osteogenic sarcoma, peritoneal mesothelioma and glioblastoma multiforme [87,88,91,92], possess no apparent telomere maintenance mechanism (telomerase or ALT), revealing that these tumors probably use a mechanism that has not yet been identified. However, even though it cannot be excluded that the presence of a constitutively active telomere maintenance mechanism is not a stringent requirement for this small fraction of tumors, it is possible to hypothesize that a therapeutic interference with telomere homeostasis through the use of telosome/telomere-interacting agents could negatively affect cell proliferation also in these malignancies.

Although several small molecules have been demonstrated to exert at a preclinical level a strong antiproliferative and antitumor effect, as a result of the direct interference with telomeres (mainly through G4 stabilization), none of these compounds is under clinical development. This observation poses a significant question concerning the reliability of the preclinical models that are currently used to evaluate the therapeutic potential of these compounds. It suggests that more sophisticated human models (including three-dimensional and organotypic cultures as well as animal models that address the problems of tumor heterogeneity and slow replication of tumor stem cells) should be used to obtain a more realistic proof of the potential of the proposed therapeutic approach.

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