Telomerase inhibitors and 'T-oligo' as cancer therapeutics: contrasting molecular mechanisms of cytotoxicity

Andrew M. Rankin[a], Douglas V. Faller[a] and Remco A. Spanjaard[b]

Telomeres, the specialized structures that comprise the ends of chromosomes, form a closed structure, or t-loop, that is important in preventing genomic instability. Forced modulation of this structure, via overexpression of a dominant-negative form of telomere repeat binding factor 2, a protein critical for maintaining t-loop structure, for example, can result in the activation of DNA-damage responses, and ultimately cellular senescence or apoptosis. This response is also seen in normal somatic cells, where telomeres steadily decrease in length as cellular proliferation occurs owing to inefficient replication of terminal telomeric DNA. When telomere length becomes critically short, t-loop structure is compromised, and the cell undergoes senescence. Telomerase, the enzyme responsible for telomere length maintenance, is overexpressed in a majority of cancers. Its lack of expression in most normal somatic cells makes it an attractive target in designing cancer therapeutics. Compounds currently under development that seek to inhibit hTERT, the reverse transcriptase component of telomerase, include nucleoside analogs and the small molecule BIBR1532. Compounds inhibiting the RNA component of telomerase, hTERC, include peptide nucleic acids, 2–5A antisense oligonucleotides, and N3′–P5′ thio-phosphoramidates. Recently, an oligonucleotide sharing sequence homology with terminal telomeric DNA, termed 'T-oligo', has shown cytotoxic effects in multiple cancers in culture and animal models. Independent of telomerase function, T-oligo is thought to mimic the DNA-damage response a cell normally experiences when the telomere t-loop structure becomes dysfunctional. In this review, the molecular mechanisms attributed to telomerase inhibitors and T-oligo, as well as their potential as cancer therapeutics, are discussed. *Anti-Cancer Drugs* **19:329–338 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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[a]Department of Medicine and Cancer Research Center, Boston University School of Medicine and [b]Departments of Otolaryngology and Biochemistry, Cancer Research Center, Boston University School of Medicine, Boston, Massachusetts, USA

Correspondence to Dr Remco A. Spanjaard, PhD, Cancer Research Center, Boston University School of Medicine, 715 Albany Street, R903, Boston, MA 02118, USA
Tel: +1 617 638 7934; fax: +1 617 638 5837;
e-mail: rspan@bu.edu

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Introduction

One of the fundamental characteristics of cancer is the ability of the tumor cells to proliferate indefinitely, free of the constraint of replicative senescence. Upregulation of telomerase, the enzyme responsible for maintaining telomere length and structure, is an important mechanism to establishing this capacity for immortality, and its aberrant activity is observed in 85–90% of cancers. The goal of this review is to discuss current research in developing drug treatment strategies that target the telomere or telomerase to inhibit tumor growth. Two classes of agents will be reviewed: agents that inhibit the telomerase enzyme, and 'T-oligo' – a DNA oligonucleotide with sequence homology to human telomeres that has generated cytotoxic responses in various cancer cell types independently of telomerase activity (Fig. 1).

Telomere biology and cancer

Telomeres, highly specialized structures comprising the ends of human chromosomes, play important roles in tumor suppression. In normal and cycling cells, telomeres lose approximately 50 base pairs of end sequence with each replication cycle as a result of the 'end replication problem', stemming from the incomplete replication of lagging-strand chromosomal DNA [1,2]. After extensive cell divisions, telomeres within cells shorten catastrophically and elicit DNA-damage responses. These cells enter mortality stage 1 and undergo senescence [3]. In this respect, telomeres function as a molecular clock, eliminating aging cells from the organism before transforming mutations acquired during multiple rounds of DNA replication can be manifested [4]. Cells that have acquired mutations inactivating the p53 or Rb tumor suppressor genes, however, are often capable of bypassing mortality stage 1 and so can continue to cycle for approximately 20–30 more divisions before telomeres become critically shortened and can no longer protect chromosome ends. These cells acquire dicentric chromosomes and genomic rearrangements, enter mortality stage 2, or 'crisis', and rapidly undergo apoptosis. Cells that escape mortality stage 2 have essentially acquired the ability to replicate indefinitely and may serve as progenitors for cancer [3,5–7].

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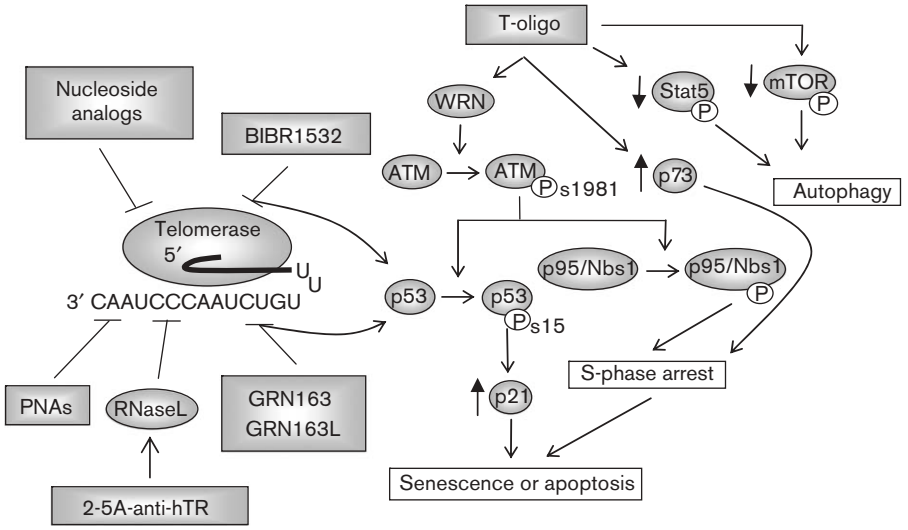
Fig. 1



Proposed mechanistic model for T-oligo-induced and telomerase inhibitor-induced cytotoxicity. Cytotoxic compounds displayed above the telomerase complex are direct or indirect inhibitors of hTERT. Those displayed below the same complex are inhibitors of hTERC. Cytotoxic effects observed from treatment of cells with BIBR1532, GRN163, and GRN163L are mediated in part via activation and stabilization of p53. Bold arrows adjacent to proteins with or without a phosphate modification indicate modulation of activation states or expression, respectively.

One key characteristic seen in 85–90% of cancer cells is the upregulated or persistent expression of telomerase, the key enzyme that functions to maintain telomere structure and length [8]. Consequently, these cells maintain an average telomere length of 5 kb. Recent research has identified telomere DNA content (TC) as a prognostic indicator in cancer (reviewed in [9]). In breast cancer, reduced TC in cancerous tissue relative to adjacent normal tissue is correlated with lymph node metastasis and aneuploidy [9]. It has also been associated with 5-year disease-free and overall survival. In prostate cancer, low TC is correlated with increased likelihood of disease recurrence and death [9]. The unique relationship between telomeres, telomerase, and cancer provides a relatively tumor-specific target for the design of cancer therapeutics that may spare normal cells, as most normal cells have no detectable telomerase protein, and thus provide an effective and selective cancer treatment strategy.

Telomere structure and function

Telomere structure and binding proteins

Telomeres are dynamic nucleoprotein structures comprising the ends of chromosomes, which alternate between the so-called capped and uncapped states [4]. Specifically, the capped state structure, otherwise known as the t-loop, is characterized by the looping back, and invasion of, the terminal 3′ single-stranded DNA overhang into double-stranded proximal DNA via interactions with multiple 'shelterin' complexes. This structure protects telomere integrity, and prevents end-to-end chromosomal fusions. The shelterin complex, composed of the DNA-binding proteins telomere repeat binding factor 1 (TRF1), telomere repeat binding factor 2 (TRF2), and protection of telomeres 1 (POT1), as well as three core interacting factors, Tin2, TPP1, and Rap1, functions to dynamically regulate telomere length and maintain telomere t-loop structures. The first set of three proteins interact with each other while DNA-bound, via protein–protein interactions mediated by the last three proteins [10]. TRF1 binds to double-stranded telomeric DNA with high specificity, and the number of TRF1 proteins bound to any given telomere is an indicator of overall telomere length. Overexpressing, or partially knocking-down expression of TRF1, results in a progressive shortening or lengthening of telomeres, respectively, until a new telomere length equilibrium is attained. In cells without telomerase, however, levels of TRF1 do not mediate rates of telomere shortening [11]. TRF2, a paralog to TRF1, also binds to double-stranded telomeric DNA and functions to protect the single-stranded 3′ telomere terminus from being recognized as a DNA double-strand break. Inhibiting wild-type TRF2, or overexpressing a dominant-negative form, results in a rapid induction of apoptosis, mediated predominantly through the stabilization of p53 and consequent upregulation of p53-dependent apoptotic genes as well as cyclin-dependent kinase inhibitors (CDKIs) [12]. POT1 binds to single-stranded DNA at the 3′ telomere terminus. Similar to TRF2, POT1 functions to protect telomere ends from being recognized as double-strand breaks by DNA-damage response machinery. POT1 can also serve as a negative regulator of telomere length by preventing elongation by telomerase [13].

Telomerase, the ribonucleoprotein enzyme responsible for telomere elongation, is composed of two core components: the reverse transcriptase hTERT and its associated template RNA, hTERC. Telomerase binds terminal 3′ telomeric DNA via the complementary template region of hTERC, allowing hTERT to elongate the end by one telomeric repeat. This process of binding, elongating, and releasing is continually repeated, resulting in dynamic telomere length maintenance when the telomerase complex is present [11].

Telomere function

As noted above, cells not expressing telomerase will eventually experience critically shortened telomeres and undergo senescence or apoptosis. Like this scheduled uncapping response that eliminates potentially tumorigenic cells from a population after many cell divisions, the unscheduled uncapping or resolution of t-loop structure can rapidly initiate cytotoxic responses, presumably via exposure of the 3′ single-stranded overhang to the nuclear environment. This accelerated proliferation-inhibition program is seen when TRF2 is functionally compromised and in cells from patients with the premature aging disease, Werner syndrome (WS) [14].

TRF2 is a critical component of the shelterin complex described earlier. When TRF2 is removed from this complex via inhibition or overexpression of a dominant-negative TRF2 mutant, telomere-specific DNA-damage foci form, followed by chromosomal end-to-end fusions and ultimately senescence or apoptosis [15]. The DNA-damage foci contain proteins typically found at sites of genomic double-strand breaks, such as 53BP1, phosphorylated forms of ataxia telangiectasia mutated (ATM), and histone H2AX, as well as the mismatch-repair (MRE11) complex [16]. In addition to these findings, loss of TRF2 results in cleavage and reduction of the terminal 3′ single strand by the ERCC1/XPF endonuclease, suggesting that under these conditions, the telomere terminus is exposed to the nuclear environment, and t-loop structure is therefore compromised [17].

The importance of maintaining proper telomere structure is evident from the study of cells from patients with WS, a condition resulting from an inactivating mutation in the gene encoding WRN, a RecQ Helicase family member. Functionally, WRN contains both 3′–5′ helicase and 3′–5′ exonuclease activities and plays important roles in nonhomologous end-joining, base-excision repair, DNA replication, and telomere structural maintenance. Cells from these patients harbor multiple abnormalities, including shortened telomeres, sharply reduced proliferative capacity in culture, premature senescence, and genomic instability. Accelerated loss of telomere length is thought to play a role in the rapid onset of senescence observed in WS cells in culture. Expression of exogenous telomerase in fibroblasts from WS patients, however, increases their replicative capacity in culture, suggesting that the premature senescence may result from the aberrant maintenance of telomere t-loop structure [18,19].

As the telomere t-loop structure seems to be critically important for maintaining cell proliferation, it may serve also as a strategic target for cancer therapeutics. Specifically, compounds that target telomerase, or other proteins critical for t-loop structure maintenance and tumor cell longevity, could provide specific avenues for selectively targeting cancer cells.

Inducing cytotoxicity by telomerase inhibition

The concept of targeting telomerase as a cancer therapeutic has been explored for over a decade, essentially since the discovery of its aberrant expression in tumors. The scientific rationale for this interest stems from several observations. First, exogenous expression of the hTERT subunit of telomerase in hTERT-deficient and normal cells is sufficient to allow them to bypass replicative senescence and continue dividing, which is one of the known hallmarks of cancer [20,21]. Additionally, exogenous expression of the hTERT subunit in cells that have been transformed and contain critically short telomeres enables them to escape crisis and continue proliferating despite genomic instability [22]. Second, knocking out hTERC in transgenic mice leads to a steady loss of telomeric DNA from one generation to the next, resulting in chromosome fusions and genomic rearrangements, which supports the protective role telomerase plays in maintaining telomere lengths [23]. The absence of telomerase in these mice inhibits the generation of skin cancer following treatment with carcinogens, demonstrating the importance of upregulated telomerase activity in tumorigenesis [24]. As most normal somatic cells do not express telomerase and because telomerase seems so important for facilitating immortalization, inhibiting its function in cancer cells may be a potent and tumor-specific clinical approach to cancer treatment. Compounds that show inhibition of either hTERC or hTERT are summarized in Tables 1 and 2, respectively.

hTERC inhibition with modified and antisense oligonucleotides

Peptide nucleic acids (PNAs) are modified oligonucleotides with a *N*-(2-aminoethyl) glycine backbone in place of the normal phosphodiester one, providing them with greater resistance to nucleases and higher specificity when annealing to complimentary RNAs or DNAs, compared with unmodified oligonucleotides, or those modified with phosphorothioate backbones. In early studies, PNAs complimentary to a portion of the

Table 1 Compounds targeting hTERC

Compound	Telomere loss?	Cell lines and treatment	Cellular and molecular response	Animal models and clinical trials
Unmodified PNAs	Yes	Gastric cancer 100 nmol/l [25]	Antiproliferative effect after 7 days	ND
		Transformed ataxia telangiectasia 1 μmol/l [26]	Antiproliferative effect after 21 days	
		Transformed cystinosis 1 μmol/l [26]	Antiproliferative effect after 49 days	
Antennapedia-conjugated PNAs	No	Melanoma 10–50 μmol/l [27]	Apoptotic morphology after 20 days	ND
2–5A antisense		Malignant glioma 5 μmol/l [28]	Antiproliferative effect after 5 days, cell death after 14 days	Treatment for 14 days suppressed tumor growth in mouse malignant glioma xenograft [28]
		Bladder cancer 0.5 μmol/l [30]	Apoptosis after 3 days	Treatment for 7 days suppressed tumor growth and reduced tumor size in mouse bladder cancer xenograft [30]
GRN163	Yes	Epidermoid carcinoma 30 μmol/l [33]	Antiproliferative effect after 5 months	10 or 20 mg/kg/day suppresses tumor growth over 8 weeks in human prostate carcinoma xenograft mouse model [33]
		Multiple myeloma 1 μmol/l [32]	Growth inhibition and death after 49 days	Seven days treatment with 150 nmol reduced tumor growth and prolonged survival in rat glioblastoma xenograft [34]
			↑p21 ↑p-p53(ser15) ↓cdc25	Three weeks treatment with 10 nmol reduced tumor growth in mouse multiple myeloma xenograft [35]
		Lymphoma 10 μmol/l [35]	Apoptotic death after 7–14 days	Three weeks treatment with 10 nmol reduced tumor growth in mouse non-Hodgkin's lymphoma xenograft [35]
				Three weeks treatment with 30 mg/kg reduced tumor volume in mouse hepatoma xenograft [39]
GRN163L	Yes	Tumorigenic breast epithelial cells 1 μmol/l [36]	Growth inhibition and apoptosis after 90 days	Three weeks treatment with5 mg/kg reduced lung tumor growth in mouse lung cancer xenograft [37]
		Lung cancer 1 μmol/l [37]	Antiproliferative effect after 3 weeks	Four weeks treatment with 30 mg/kg reduced tumor growth in mammary pads and breast cancer metasteses to lung in mouse orthotopic breast cancer xenograft [38]
				Phase I/II clinical trials for patients with refractory or relapsed chromic lymphocytic leukemia (*http://www.clinicaltrials.gov*)
				Phase I clinical trials for patients with refractory or relapsed solid tumor malignancies and advanced or metastatic non-small cell lung cancer (*http://www.clinicaltrials.gov*)
				Three weeks treatment with 30 mg/kg reduced tumor volume in mouse hepatoma xenograft [39]

Arrows adjacent to proteins with or without phosphate modifications indicate modulation of expression or activation states, respectively.
PNAs, peptide nucleic acids; 2–5A antisense, 2′,5′-linked tetraadenylate; ND, not determined.

template region in hTERC effectively inhibited telomerase activity and resulted in significant telomere length attrition and cytotoxicity in gastric cancer cells after 7 days of treatment in culture [25]. When delivered to transformed ataxia telangiectasia or cystinosis cell lines, antiproliferative effects were seen by 21 and 49 days, respectively. The marked difference in treatment time required for manifestation of antiproliferative effects to be seen may be due in part to differences in the initial telomere lengths in the cell types treated [26]. Owing to inefficient cellular uptake by conventional lipid-based delivery methods in those early studies, PNAs linked to transporter peptides were developed. Specifically, the transporter peptide antennapedia was coupled to a PNA and delivered to melanoma cells. Although delivery was greatly enhanced and telomerase activity inhibited, cytotoxic effects were still only seen after treatment intervals longer than 20 days [27].

A second method used to target hTERC is the employment of 2–5A antisense oligonucleotides. These modified oligos initiate an antiviral pathway mediated by the single-strand-specific endoribonuclease, RNase-L. Essentially, the antisense oligonucleotide is covalently bound to a 2′,5′-linked tetraadenylate (2–5A). When exposed to cells, the 2–5A motif binds and activates RNase-L. The antisense oligonucleotide motif then directs the activated RNase-L to a specific RNA substrate to be cleaved. When delivered to malignant glioma cells in culture, 79% became apoptotic within 14 days. Antiproliferative effects were seen after only 5 days. Additionally, treatment of a human glioma xenograft mouse model resulted in a 48% reduction in tumor mass over 14 days [28]. The use of cationic liposomes greatly increased potency in both cultured cells and mouse models [29]. Interestingly, cytotoxic effects were also seen in bladder cancer cells that were treated for only 3 days. When these cells were introduced into a mouse xenograft model, overall tumor size in these mice was reduced by 81% [30].

A different strategy based on alternative DNA backbone modifications to increase specificity for hTERC template

Table 2 Compounds targeting hTERT

Compound	Telomere loss?	Cell lines and treatment	Cellular and molecular response	Animal models and clinical trials
AZT	Cell line dependent	Ovarian mucinous cystadenocarcinoma 100 μmol/l [41]	Antiproliferative effect after 3 days	ND
		Uterine endometrial carcinoma 90 μmol/l [41]	Antiproliferative effect after 3 days	
		Breast cancer [42]	Antiproliferative effect cell line dependent	
		B-cell lymphoma, T-cell leukemia 100 μmol/l [43]	Proliferation normal after treatment through 238 and 100 population doublings	
AZT-TP	Yes	Ovarian mucinous cystadenocarcinoma 5 μmol/l [41]	Antiproliferative effect after 3 days ↑p53	ND
		Uterine endometrial carcinoma 4 μmol/l [41]	Antiproliferative effect after 3 days	
ddG	Yes	B-cell lymphoma, T-cell leukemia 10 μmol/l [43]	Proliferation normal after extended treatment	ND
BIBR1532	Yes	Lung cancer 10 μmol/l [45]	Cells senesced after 135 population doublings ↑p21 ↓hTERT	100 mg/kg/day resulted in 32% of treated animals with tumors greater than 1000 mm^3 compared with 70% untreated in mouse fibrosarcoma xenograft [45]
		Fibrosarcoma, breast, prostate cancer 10 μmol/l [45]	After lag phase, cells senesced	
		Primary CLL 80 μmol/l [46]	Antiproliferative effect after 14 days	
		Primary AML 80 μmol/l [46]	Antiproliferative effect after 3 days	
		Prolymphocytic leukemia 80 μmol/l [46]	Antiproliferative effect after 9 days ↑p-p53(ser15) ↓TRF2	
BRACO-19	Yes	Prostate cancer [47]	Cells senesced within 7 days ↑p21 ↑p16	Inhibition of tumor growth in early stage tumors in mouse uterine carcinoma xenograft [49]
		Ovarian cancer 2 μmol/l [48]	Antiproliferative effect after 5 weeks	
Telomestatin	Yes	Lung carcinoma 5 μmol/l [50]	Antiproliferatic effect after 4 days	ND

Arrows adjacent to proteins with or without phosphate modifications indicate modulation of expression or activation states, respectively.
AML, acute myelogenous leukemia; AZT, azydothymidine; AZT-TP, azydothymidine tri-phosphate; CLL, chronic lymphocytic leukemia; ddG, dideoxyguanosine; ND, not determined.

RNA, nuclease resistance, and cellular uptake resulted in the development of N3′–P5′ thio-phosphoramidates. These compounds, though demonstrating greater ease of use and greater ability to inhibit telomerase activity compared with N3′–P5′ oligos without the added thiol group, still required long exposure times to enable telomeres in treated cells to shorten sufficiently for cellular crisis to ensue. After a lag phase of 100 days, immortalized breast epithelial cells entered senescence and at day 115 became apoptotic [31]. Shortly thereafter, the compound GRN163, a N3′–P5′ thio-phosphoramidate, was developed as a potential clinical candidate. Despite the need to deliver this compound via a lipid carrier, a wealth of data showed a high degree of effectiveness of GRN163 in terms of inducing cytotoxicity in cell culture and reducing tumor growth, size, and overall viability in xenograft mouse models. Human multiple myeloma cells treated for 28 days demonstrated critical telomere shortening, followed by cell death at day 49. Molecular analyses demonstrated that levels of p53 phosphorylated at serine 15 increased by day 14, followed by increased expression of the CDKI p21; these two molecular events are often observed in cells undergoing senescence, before apoptosis [32]. In epidermoid carcinoma cells, GRN163 produced cytotoxic effects and caused telomeres to critically shorten after approximately 5 months of treatment. When tested against a human prostate carcinoma xenograft mouse model, treatment for 8 weeks caused a significant reduction in tumor growth [33]. In a malignant glioma xenograft rat model, treatment with GRN163 for only 7 days significantly inhibited tumor growth and prolonged survival [34]. Testing of GRN163 in multiple myeloma and non-Hodgkin lymphoma xenograft mouse models resulted in tumor growth suppression after 3 weeks of treatment [35]. This high degree of variation in treatment times required for antitumor effects in cell culture and in animal models may correlate with the differences in initial telomere lengths in the tumor cells at the start of treatment. For instance, prostate cancer cells in culture show cytotoxic effects after only 3 days of treatment but also have initial telomere lengths far shorter than other cell lines tested [33].

Very recently, studies have been conducted using a lipid-conjugated form of GRN163, termed GRN163L. The compound has been used in various mouse models, and has entered phase I/II clinical trials for patients with refractory or relapsed chronic lymphocytic leukemia, a phase I clinical trial for patients with refractory or relapsed solid tumor malignancies, and a phase I clinical trial for patients with advanced or metastatic non-small cell lung cancer (*http://www.clinicaltrials.gov*). In cell culture, this modified compound effectively showed

growth inhibition and apoptosis in tumorigenic breast epithelial cells within 90 days, without the need for a lipid-based carrier [36]. In human lung cancer cells, antiproliferative effects were observed after 3 weeks of treatment. Using these same cells in a xenograft mouse model, treatment with GRN163L significantly reduced tumor formation in the lungs [37]. When delivered to mice with orthotopic breast cancer xenografts, GRN163L inhibited the growth of established tumors after 4 weeks of treatment. Moreover, the same treatment regimen inhibited breast cancer metastases to the lung [38]. The compound also significantly reduced tumor volume in mice with hepatoma xenografts after 3 weeks of treatment. Analysis of tumor sections from the mice treated with GRN163L revealed a decrease and increase in tumor cell proliferation and apoptosis, respectively, compared with those treated with phosphate-buffered saline [39]. On the basis of these xenograft tumor modeling studies, therefore, GRN163L seems to hold promise as a therapeutic agent. Current and future clinical trials will test its potential in human malignancies.

hTERT inhibition by nucleoside analogs and small molecules

One strategy aimed at inhibiting hTERT involves nucleoside analogs. A diverse spectrum of nucleoside analogs, including 6-thio-7-deaza-2′-deoxyguanosine 5′-triphosphate ddGTP, ddATP, and ddTTP, have produced significant inhibition of telomerase activity in cell extracts, but have not been thoroughly tested in cell cultures or animal models [40]. Azydothymidine (AZT), an antiviral compound currently being used as a treatment for AIDS, produces effective inhibition of telomerase activity in multiple cell lines along with progressive telomere shortening and cytotoxic effects, both of which seem to be cell line dependent. When delivered to ovarian mucinous cystadenocarcinoma and uterine endometrial carcinoma cells, antiproliferative effects were observed after only 3 days, despite a reported lack of telomerase activity inhibition [41]. In contrast, when administered to various breast cancer cell lines, antiproliferative effects were not observed until much later, but were accompanied by inhibition of telomerase activity [42]. Both B-cell lymphoma and T-cell leukemia cell lines treated with AZT demonstrated no changes in proliferation despite a significant decrease in telomere length and telomerase activity through 100 and 238 population doublings, respectively [43]. The disparity in response patterns of different cell lines to AZT is difficult to reconcile, and further studies will be needed to clarify whether off-target effects by AZT, or cell type-specific characteristics, underlie these results. Other nucleoside analogs, including azydothymidine tri-phosphate and ddI, showed similar antiproliferative effects on the ovarian and uterine cell lines described above; however, significant telomerase inhibition along with telomere length reduction was observed mainly in the uterine cells [41]. Finally, treatment of B-cell lymphoma and T-cell leukemia lines described above with ddG resulted in telomerase inhibition and telomere length reduction, but yielded no antiproliferative effects [43].

A second strategy to inhibit hTERT has relied on the development of small molecule inhibitors that bind directly to, and inhibit, telomerase. Perhaps the most promising candidate thus far is designated BIBR1532. In early studies, this small molecule demonstrated very specific telomerase activity inhibition, coincident with steady erosion of telomeric DNA length [44]. Treatment of a lung cancer cell line for over 120 days resulted in a nearly complete inhibition of cell proliferation, which corresponded approximately to 135 population doublings. These cells underwent morphological changes consistent with senescence, including enlargement, development of multiple nuclei and a vacuolated cytoplasm, and increased levels of senescence-associated β-galactosidase activity. Similar outcomes were observed in breast cancer, fibrosarcoma, and prostate cancer cell lines. Interestingly, the functional status of p53 did not seem to play a factor in determining responsiveness, as both p53-deficient and p53-proficient cell lines responded similarly to the compound. Treatment of a fibrosarcoma xenograft mouse model resulted in 32% of treated mice with tumors greater than 1000 mm^3 compared with 70% of untreated control mice [45]. Interestingly, high concentrations of BIBR1532 induced an acute cytotoxic effect on primary leukemia cells taken from acute myeloid leukemia and chronic lymphocytic leukemia patients after 3 and 14 days, respectively, of continuous exposure. Treatment levels that were 3–8-fold higher than necessary for inhibition of telomerase activity resulted in end–end fusions, phosphorylated p53 at serine 15, and decreased expression of TRF2. These results lead to the conclusion that whereas induction of dysfunctional telomeres likely accounted for most of the observed cytotoxicity, other 'off-target' mechanisms could not be ruled out [46].

As observed with the majority of telomerase inhibitors described above, cytostatic and cytotoxic effects in the form of senescence or apoptosis, respectively, occur only after a prolonged treatment lag phase, during which time telomere lengths in the cells decrease steadily. Although the specificity afforded by some of these potential drugs makes them markedly superior to many current cytotoxic compounds used in cancer treatment, the extended treatment time required may limit their use as cancer therapeutics. As suggested above, the design of drugs intended to inhibit the ability of a cell to maintain proper telomere t-loop structures may offer new advantages over telomerase inhibition in targeting cancer cells for destruction, as cytotoxic effects would occur much more rapidly.

One recently developed class of drugs, termed G-quadruplex interacting ligands, is thought to cause a disruption in the normal telomere t-loop structure by stabilizing the so-called G-quadruplex structures within the 3′ single-stranded overhang. These modified nucleotide interactions result in a four-stranded DNA structure that effectively inhibits telomerase binding, POT1 binding, and telomere elongation [47]. Both short-term and long-term cell type-specific cytotoxic effects are observed, depending upon the concentration of compounds used. For example, BRACO-19, a tri-substituted acridine compound, when delivered to prostate cancer cells, induced senescence within 1 week of treatment. These effects were accompanied by chromosomal end-to-end fusions and increased expression levels of the CDKIs p21 and p16 [47]. When delivered to ovarian cancer cells for a longer length of time and at subcytotoxic concentrations, however, antiproliferative effects were observed only after 5 weeks of exposure. Telomerase inhibition and gradual telomere shortening were also observed [48]. Treatment of a uterine carcinoma xenograft mouse model resulted in inhibition of tumor growth in early stage tumors [49]. Similarly, the compound telomestatin has been used effectively against lung carcinoma cells. At high concentrations, the cells underwent cell cycle arrest within 4 days. By decreasing the concentration but extending the length of treatment, a delayed cell cycle arrest was observed, which eventually pushed the cells into a senescent-like state [50]. Degradation of the 3′ single-stranded telomere overhang occurred during treatment and was followed by an early DNA-damage response consisting of increased levels of γ-H2AX colocalized with telomere termini [51].

Inducing cytotoxicity by mimicking telomere exposure with T-oligo

Recent research into molecular mechanisms of tanning responses has resulted in the synthesis of DNA oligonucleotides with a sequence largely homologous to the 3′ single-stranded overhang of a human telomere. Early studies leading up to the development of these oligos began when melanocytes in culture were treated with thymidine dinucleotides (pTT) and other 5-mer and 9-mer oligonucleotides that had varying homologies to human single-stranded telomeric DNA. Within days of exposure, cells showed an increase in melanogenesis, a heightened level of DNA repair activity, and cell cycle arrest. Expression of p53 and its target genes were increased as well [52].

Notably, similar cellular and molecular events are observed upon experimental disruption of the telomere using dominant-negative TRF2 [53–55], as well as in cells that have experienced critically shortened telomeres [16,56]. Cytotoxic effects elicited by these events are thought to be due to exposure of the single-stranded 3′ telomeric overhang to the nuclear environment. Owing to this correlation, and because the oligos used were similar in sequence to human single-stranded telomeric DNA, it was hypothesized that the cytotoxic effects were due to the initiation of a signal that mimicked an unscheduled uncapping of the telomere [52].

In studies using an 11-mer telomere homolog oligonucleotide ('T-oligo'), p53-pathway-compromised Jurkat cells that were treated for 3 days demonstrated an acute S-phase arrest, followed by detection of high levels of cleaved caspase 3, indicating apoptosis. Interestingly, levels of the p53 homolog, p73, were greatly increased upon exposure, suggesting that p73 possibly facilitated the apoptosis. In human melanoma cells that have wild-type p53, a similar induction of p73 was seen. Expression levels of the transcription factor E2F1 were also increased, suggesting functional Rb may serve as a mediator in the cytotoxic responses [52]. Additionally, p53-proficient breast cancer cells treated with T-oligo undergo cell cycle arrest and apoptosis after 4 days of treatment whereas cells that failed to apoptose became senescent after 7 days [57]. At present, the importance of p53 in facilitating T-oligo-dependent cytotoxic responses remains unclear. In our own studies, several cancer cell lines that harbor mutant p53 proteins show variable levels of cytotoxic responses when treated with T-oligo, suggesting the presence of other important signaling mediators (unpublished observations).

Interestingly, as mentioned above, some cell types undergo senescence instead of apoptosis when treated with T-oligo. Which of these programs is initiated seems to be cell type dependent. In an effort to establish the importance of the cell cycle regulators Rb and p53 in T-oligo-induced senescence, dermal fibroblasts engineered to lack a functional p53 pathway via ectopic expression of a dominant negative p53, or Rb pathway via ectopic expression of mutant CDK4, or both were used. A functional T-oligo response was gauged by induction of the p53-regulated CDKI p21. Although p21 levels in cells with a compromised Rb pathway were comparable to those seen in normal cells, there was abrogation of p21 expression in cells with a compromised p53 pathway and in cells with both the p53 and Rb pathways compromised. Additionally, the senescence-associated increases in β-galactosidase activity in normal cells when treated with T-oligo did not occur in cells with both p53 and Rb pathways compromised [58]. These studies speak to a role for p53 and Rb in cell cycle arrest, and potentially senescent responses induced by T-oligo, but do not address requirements for the apoptotic response.

Efforts to identify proteins critically important for the induction of the S-phase arrest upon treatment with T-oligo led to studies focusing on the p95/Nijmegen

breakage syndrome-1 (p95/Nbs-1) protein, a critical component of the Mre11 DNA-damage response complex. p95/Nbs-1 was identified previously as a downstream target of ATM phosphorylation and a key mediator in establishing ionizing radiation-induced S-phase arrest in fibroblasts [59]. To determine the relevance of p95/Nbs-1 in cellular responses to T-oligo exposure, cells from a patient with Nijmegen breakage syndrome, which lack functional p95/Nbs-1, were treated with T-oligo for 24 h. These cells did not undergo the expected S-phase arrest seen in normal cells in response to T-oligo exposure. Additional data supporting a role for p95/Nbs-1 in transducing the signal initiated by T-oligo exposure is the finding of new phosphorylation of p95/Nbs-1, a modification indicative of its activation, which is observed when cells are exposed to T-oligo for 2–4 days [60].

In searching for the earliest molecular responses to T-oligo treatment, efforts were focused on identifying potential mediators of the hypothesized DNA-damage response in addition to p95/Nbs-1. The phosphorylation of ATM, a DNA-damage sensor protein, plays an important role in mediating downstream signaling following DNA damage by ionizing radiation. Among the phosphorylation targets of ATM are serine 15 of p53, threonine 68 of Chk2, serine 139 of histone H2AX, and serine 343 of p95/Nbs-1 [59,61–63]. Cells from ATM patients, which lack functional ATM proteins, were exposed to T-oligo and displayed markedly reduced levels of phosphorylated p95/Nbs-1 and p53 at serine 15 [60]. Other studies using breast cancer cells demonstrated an increase in levels of the phosphorylated and activated form of ATM, and its downstream targets, histone H2AX and p53, following exposure to T-oligo [57].

Additional data have suggested a potential role for WRN, an important mediator of nonhomologous end-joining, base-excision repair, and DNA replication, in the mechanism of action of T-oligo [64]. Cultured cells harboring a dysfunctional WRN protein exhibit rapid induction of senescence owing to critically shortened telomeres, suggesting that telomere structural maintenance is also mediated in part by WRN [18,19]. In initial studies, it was observed that cells treated with T-oligo modified with phosphorothioate linkages and those modified with a 3′ phosphoropropyl amine group, both of which make them less susceptible to exonucleolytic degradation, did not exhibit activated p53 or phosphorylated histone H2AX following exposure to T-oligo. These findings led to the hypothesis that the WRN protein may be important in mediating T-oligo cytotoxicity. Importantly, when T-oligo was delivered to cells derived from a patient with WS, there was a sharp reduction in levels of phosphorylated serine 15 on p53 compared with controls. Furthermore, knocking down expression of the WRN protein with small interfering RNA (siRNA) before T-oligo treatment resulted in relatively decreased levels of phosphorylated p53, and histone H2AX, as well as levels of phosphorylated and activated ATM. Finally, cells in which WRN protein levels were knocked down by siRNA failed to upregulate senescence-associated β-galactosidase activity in response to T-oligo, in contrast to control cells with intact WRN protein. Thus, WRN is potentially a major mediator of T-oligo-mediated cytotoxicity [64].

T-oligo also seem to confer cytotoxicity by inducing autophagy in certain cell types. Treatment of two different malignant glioma cell lines with T-oligo-induced nonapoptotic cell death after 3 and 5 days of treatment. The induction of autophagy was evidenced by an increase in acidic vesicular organelles and an increase in their colocalization with the autophagy associated LC3 protein. Expression of the LC3 protein was enhanced as well. Molecularly, mTOR and STAT3 signaling pathways were implicated in the regulation of the observed autophagy [65].

In contrast to the cytotoxicity induced by T-oligo exposure in cancer cells, several studies have demonstrated the relative lack of cytotoxic effects of T-oligo in normal cells. When treated with comparable amounts of T-oligo, normal mammary epithelial cells underwent significantly less apoptosis and demonstrated increased cell viability compared with breast carcinoma cells [57]. Similarly, treatment of normal human astrocytes with T-oligo conferred little to no cytotoxic effects compared with those seen in malignant glioma cells [65]. The same phenomena were observed in normal B and T lymphocytes, which do not display cytotoxic responses when treated with T-oligo compared with B-cell and T-cell lymphomas (unpublished observations).

In attempts to establish the utility of T-oligo as a potential cancer therapeutic, several mouse models have been studied. In a breast xenograft mouse model, MCF-7 breast cancer cells were injected into the tail vein of mice. The mice received two rounds of two injections per day for 5 days of either 60 nmol of T-oligo or diluent control. Within 6 weeks, all diluent-treated mice died or were killed owing to extensive weight loss. In contrast, 75% of the T-oligo-treated mice lived through 30 weeks without developing weight loss or pathological symptoms [57]. Using a melanoma xenograft mouse model, the ability of melanoma cells to metastasize following treatment with T-oligo was determined. MM-AN melanoma cells were treated with 40 μmol/l T-oligo, a complementary control oligo, or diluent for 48 h, and were then injected into the tail vein of severe combined immunodeficiency mice. After 40 days, all of the mice were killed and examined for macroscopic malignancies on internal organs. Mice injected with diluent-treated or control oligo-treated melanoma cells lost significant body weight, and displayed signs of severe illness. In contrast,

mice receiving T-oligo-treated melanoma cells seemed healthy and increased in weight. In addition, these mice displayed an 85% reduction in volume of tumor metastases compared with control mice. In another melanoma xenograft model, the ability of T-oligo to reduce tumor growth in preexisting tumors was gauged. Severe combined immunodeficiency mice received injections of MM-AN melanoma cells and 72 h later were treated twice daily with 60 nmol of T-oligo, control oligo, or diluent. After 22 days, when the experiment was ended, T-oligo-treated mice exhibited an 84% reduction in tumor growth compared with diluent or control oligo, respectively [66]. In a glioma mouse xenograft model, glioma cells pretreated for 24 h with T-oligo, control (complimentary) oligo, or diluent were inoculated intracranially into nude mice. Within 42 days, mice inoculated with diluent-treated or control oligo-treated cells died, whereas four of six mice inoculated with glioma cells pretreated with T-oligo survived to day 72. The remaining two mice were killed at day 85 and displayed no clinically detectable tumors. In addition, the mice showed no signs of weight loss or neurological deficits, unlike the control-treated animals [65]. The ability of T-oligo to dramatically reduce the tumor burden in cancer-bearing mice, while also sparing them the deleterious side effects observed with conventional chemotherapy, warrants further research into future cancer therapy.

Although the full mechanism underlying the antitumor effects of T-oligo has yet to be completely worked out, the striking potency of this class of agents against cancer cell lines and in mouse models, within a relatively short time frame of exposure indicates its potential as a cancer therapeutic. Additionally, T-oligo treatment of normal cells does not elicit permanent, cytotoxic effects. As current cancer therapy often depends on using cytotoxic agents simultaneously to limit side effects (off-target activities), while achieving additive or synergistic cytotoxic effects on cancer cells, it may be plausible to combine T-oligo with conventional cytotoxic compounds, in the hope of increasing antitumor activity without increasing toxicity. Indeed, recent work demonstrates the ability of T-oligo to induce cell death in cultured B-cell lymphoma lines in a synergistic manner when combined with the cytotoxic compound vincristine. Additionally, combining T-oligo with subtherapeutic levels of CHOP, an often used combination chemotherapy regimen for non-Hodgkin lymphoma, in a mouse B-cell lymphoma model reduced tumor burden in a synergistic manner compared with treatment of T-oligo alone [67].

Conclusion and future perspectives

With the initiation of early clinical trials using GRN163L, a new approach to selectively target cancerous cells is being studied in the clinic. Analyses of these studies will determine whether the long lag phase required for emergence of cytotoxic effects of this compound will limit the application of this novel class of potential therapeutics. Ideally, a cancer drug should not only selectively target transformed or cancerous cells, but should act rapidly, before resistance to this therapeutic modality could arise. In-vitro data suggest that T-oligo and G-quadruplex interacting ligands are both tumor-selective and have rapid onset of action. Telomere-targeted therapeutics thus represent a novel and promising approach as tumor-specific modalities.

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