The telomerase challenge – an unusual problem in drug discovery

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Telomerase is a ribonucleoprotein that is responsible for maintaining telomere length. The observation that telomerase activity is found in many types of tumors, but not in adjacent normal tissue, has led to the hypothesis that telomerase is a novel target for chemotherapy. Inhibitors of telomerase activity are essential to validate this hypothesis, and their design presents special opportunities and challenges.

Typically, when a lead compound for drug development is applied to cells in culture, a response is expected within hours. Imagine the complexity that would be added to this initial phase of drug discovery if a cellular response required weeks to manifest itself (Fig. 1). Previously straightforward assays would become cumbersome and time-consuming, while opportunities for the development of drug resistance or the appearance of experimental artefacts would increase with time. Such are the challenges likely to confront investigators aiming to discover molecules that reduce cell proliferation by inhibiting human telomerase. Solving these challenges will provide the foundation for a new class of agents for chemotherapy and for fighting parasitic or fungal infections.

Telomeres and the end-replication problem

To prevent the loss of essential genes, linear chromosomes are capped by telomeres consisting of variable numbers of nucleotide repeats (TTAGGG) in humans[1]. Replication of telomeres poses a special dilemma, termed the end-replication problem, because DNA polymerase cannot fully replicate the extreme 3′-end of the telomere during lagging strand synthesis[2–4]. As a result, in the absence of mechanisms for maintaining telomere length, telomeres will steadily shorten at an average rate of ~100 bases per cell division in culture until cellular viability is compromised[5,6], possibly resulting from the erosion of essential genes on at least one chromosome. A vigorous debate is ongoing over whether this erosion of telomere length has physiological consequences during aging of individual organisms[7–9], but what is certain is that the absence of mechanisms for maintaining telomere length would have catastrophic consequences for a species within only a handful of generations. Thus, a solution to the end-replication problem is essential, and in germ cells, stem cells, most immortal cell lines and many human tumors it is provided by telomerase[10,11].

Telomerase

Human telomerase consists of two core subunits – an RNA domain that acts as a template for replication (hTR)[12] and a protein domain that catalyzes nucleotide polymerization (hTERT)[13–15]. The RNA component of human telomerase is approximately 445 nucleotides long, although deletion experiments have shown that the minimal functional region comprises nucleotides 44–203 (Ref. 16). Within this RNA, nucleotides 46–56 (CUAACCCUAAC) serve as a binding site for telomere ends and as a template for the addition of telomeric repeats (Fig. 2). The hTERT polymerase domain is homologous to reverse transcriptase[13–15,17–19], and mutations made at conserved residues within motifs conserved between hTERT and various reverse transcriptases have been found to abolish or reduce telomerase activity[20].

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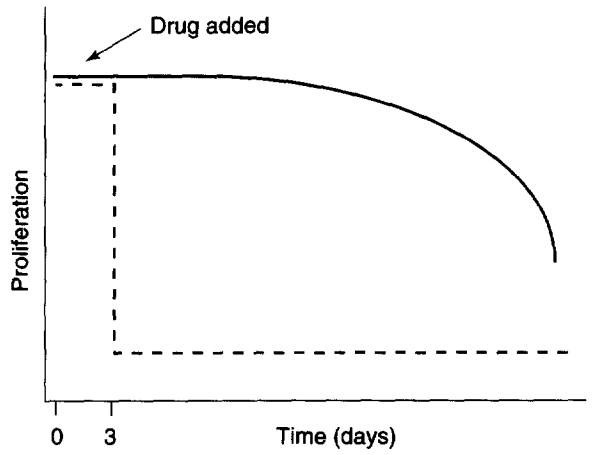

Figure 1. *Time required for assaying traditional antiproliferatives (dashed line) versus telomerase inhibitors that act through telomere shortening (solid line).*

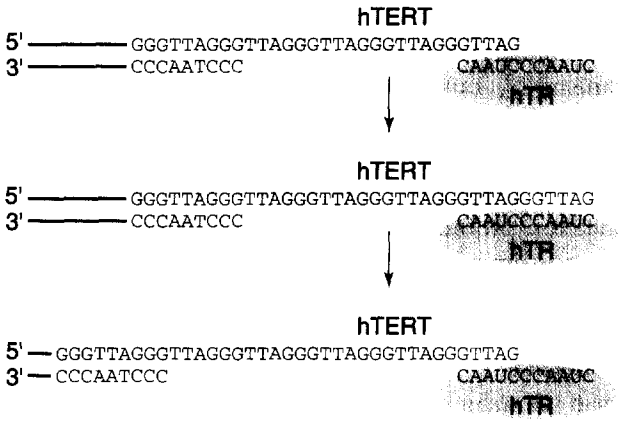

Figure 2. *Recognition of the telomere end by telomerase involves interactions between the telomere and both the hTERT and the hTR domains of telomerase. After initial recognition, the six-base telomeric repeat is added. The elongated strand then translocates, allowing addition of subsequent repeats.*

Both hTERT and hTR can be expressed separately *in vitro* and reconstituted to yield high levels of telomerase activity[20–22], demonstrating that they are both necessary and sufficient for function *in vitro*, although other protein or nucleic acid subunits are likely to influence telomere length regulation within cells. Moreover, ectopically expressed hTERT is sufficient to convert telomerase negative cells to telomerase positive cells[20–22].

Telomerase and cancer

Telomerase activity has been found in most types of human tumors, but not in adjacent normal cells[23–25]. This correlation has led to the hypothesis that reactivation of telomerase is necessary for the sustained cell proliferation that characterizes cancer[26–30], and that telomerase is a promising target for a class of chemotherapeutic agents that act by a novel mechanism. Supporting this hypothesis is the observation that early-stage neuroblastomas have low telomerase activity while the late-stage disease exhibits high telomerase activity[31]. A similar correlation between telomerase activity and poor clinical outcome has been reported for ordinary menigioma[32], and other studies have suggested that telomerase activity is correlated with pathologic stage[33–35] or tumor aggressiveness[35,36]. Furthermore, the level of telomerase correlates with survival rates of patients: low telomerase activity frequently leads to spontaneous remission[31] and high telomerase activity predicts a poor prognosis[32]. Conversely, transfection of the *hTERT* gene and subsequent expression of active telomerase has been shown to extend the lifetimes of normal human diploid fibroblasts[37,38]. Thus, the lack of telomerase expression appears to curb growth of rapidly proliferating cells, while an increase in telomerase permits indefinite proliferation.

Further evidence for the importance of telomerase expression for sustained cell growth comes from studies of mice that lack the mouse RNA component (mTR). These mice survived for six generations with few detectable phenotypic changes[39], but by the seventh generation highly proliferative organ systems such as the testis, bone marrow and spleen[40] appeared abnormal and the mice were no longer able to reproduce. By contrast, the highly proliferative intestinal cells appear to be normal[40]. This long lag between loss of telomerase activity and a detrimental phenotype is in agreement with the hypothesis that telomerase inhibition works through gradual telomere shortening. It is important to note that the mice used in these experiments possessed much longer telomeres than those found in most cancerous cells, and that inhibition of telomerase in human cancer would be expected to yield faster effects[41,42]. Nevertheless, the long lag is a clear warning that development of telomerase inhibitors is likely to be a lengthy and complex process that will offer stringent challenges during the drug discovery process. Mutation of the telomerase RNA of *Tetrahymena*, *Saccharomyces cerevisiae* and human cells in culture also leads to decreased cell proliferation over time[43–46]. Similarly, expression of antisense RNA complementary to hTR mRNA caused decreased proliferation of HeLa cells after 23–26 doublings[12], supporting the hypothesis that inhibition of telomerase will eventually lead to decreased cell proliferation.

Another complication to the telomerase–cancer connection is the observation that some immortal cell lines lack detectable telomerase activity[47–50]. Thus, while telomerase activity can confer extended lifespans to cells, other mechanisms exist for maintaining sustained cell proliferation. A pathway termed ALT (alternative lengthening of telomeres) has been proposed to account for this phenomenon. Evidence for the existence of this pathway is found in yeast in which a component of the telomerase holoenzyme has been deleted[45,51,52]. The cells divide normally until telomeres shorten sufficiently to affect proliferation. At this stage most cells die, but some survive and continue to proliferate through a recombination mechanism to maintain telomere length. Recently, Cech and coworkers have deleted the gene for the telomerase catalytic subunit from *Schizosaccharomyces pombe* and have shown that, while most cells die, surviving cells maintain telomeres through recombination[51].

The evidence described above supports the hypothesis that telomerase is associated with cancer and is critical for tumor growth. The evidence also suggests that telomerase inhibitors should exert an antiproliferative effect, even if that effect is only observed after a lag phase. In addition to these good reasons for developing telomerase inhibitors, there are concerns about drug resistance and possible side effects. The existence of non-telomerase-dependent mechanisms of telomere length maintenance is evidence that the development of drug resistance is possible through several mechanisms. These mechanisms include the ALT pathway and mutations that decrease inhibitor binding or that upregulate telomerase expression. Alternatively, cells might arise that cannot take up inhibitors or that rapidly remove them, events that are often observed during use of other chemotherapeutic agents. In addition to problems that might arise from development of drug resistance, telomerase activity has been found in proliferative stem cells[43] (such as T and B cells), leading to the suggestion that inhibitors might cause serious side effects. To counter this point, proponents of telomerase as a target for chemotherapy argue that, because cancer cells generally have shorter telomeres than stem cells and are dividing more rapidly, they might be exceptionally susceptible to telomerase inhibitors[26], sparing the stem cells.

Despite the potential problems posed by development of drug resistance and the possibility of side effects, it would be a mistake not to pursue telomerase as a target for cancer chemotherapy or prevention. Inhibitor resistance is an obstacle encountered during the development of any new antiproliferative agent. It would be naive to expect that: (1) inhibitor resistance would not also be a problem for development of telomerase inhibitors, or that (2) a priori, the development of telomerase inhibitors should not be pursued because we can already envision multiple potential mechanisms for resistance. Telomerase is unusual among drug targets because a large body of excellent basic work in telomere biology has preceded development of highly effective lead compounds, allowing us to foresee potential problems before evidence of efficacy in model systems is in hand – exactly opposite of the situation faced during most drug development.

Design of molecules that inhibit telomerase

The controversy over the potential antiproliferative effects of telomerase inhibitors is a fascinating intellectual pursuit. However, no arguments can change the fact that the potential of telomerase as a target for clinical testing of drugs for humans will remain unknown until highly potent and selective inhibitors of human telomerase are discovered and rigorously tested in relevant model systems. Inhibitors could target any one of several features of human telomerase (Fig. 3). These include: the hTERT active site; the 11-base RNA template; the 'anchor site' where hTERT interacts with telomeric DNA; the extended telomere, possibly at a G-quadruplex structure; additional, as yet unknown interacting proteins; or antisense targeting of the mRNA for either hTR or hTERT. The hTERT domain would be a preferred target in this antisense-targeting strategy because hTERT is not expressed in telomerase negative cells. In a variation on this theme, the gene for an inactive hTERT mutant could be delivered to telomerase positive cells and suppress telomerase activity though a dominant-negative approach.

A cautionary note to the design of inhibitors of human telomerase is that many agents might inhibit telomerase

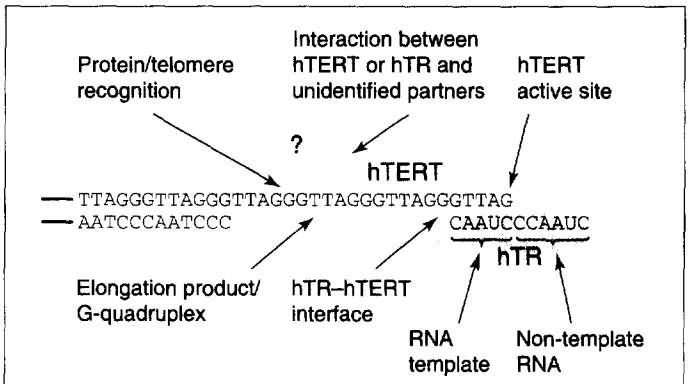

Figure 3. *Strategies for inhibition of human telomerase. Strategies for reducing telomerase activity that are not shown here include antisense oligonuceotides targeted to hTERT and hTR mRNA, expression of antisense mRNA and repression of telomerase expression.*

upon addition to cell extracts or cells in culture but lack the ability to produce telomerase-specific effects on cell proliferation. For example, addition of dimethylsulphoxide (DMSO) to cells has been observed to inhibit telomerase activity, but it is highly unlikely that DMSO interacts with telomerase specifically[53]. Rather, DMSO treatment probably induces many changes, including decreased activity or expression of telomerase, and the source of any observed phenotype might be difficult to confirm. Other examples in which telomerase activity is inhibited through mechanisms that do not directly involve interactions with telomerase are inhibitors of protein kinase C (Ref. 54) and oligonucleotides targeted against c-*myc* (Ref. 55).

To confirm action through a telomerase-dependent mechanism, inhibitors of human telomerase should meet the following criteria: (1) addition of inhibitors should reduce telomerase activity in cell extract; (2) addition of inhibitors should eventually lead to observable shortening of telomeres; (3) addition of inhibitors should cause cell proliferation to decrease, and the time necessary to observe decreased proliferation should vary with initial telomere length; (4) chemically related molecules that do not inhibit telomerase in cell extract should not cause decreased cell proliferation or telomere shortening. The progress of investigations of described inhibitors is discussed below and outlined in Table 1.

Nucleotide inhibitors

Telomerase shares substantial identity with reverse transcriptases, so it is not surprising that nucleotide analogs known to inhibit polymerases, such as AZT (azidothymidine)[56,57], 7-deaza-ATP (Ref. 58), ddG (Refs 54,59) and carbovir[60], also inhibit telomerase. Unfortunately, these molecules also inhibit other polymerases, making it difficult to relate changes in cell proliferation to inhibition of telomerase. Identification of nucleotide inhibitors that are selective for telomerase may be able to take advantage of reverse transcriptase inhibitors synthesized during programs to investigate inhibition of other polymerases, some of which may have been poor inhibitors of their original targets. The recent cloning of *hTERT* and possession of reconstituted enzyme will facilitate development of protocols for mass screening of these derivatives, possibly using recently developed chemoluminescence assays for polymerase activity[61,62]. Identification of *hTERT* should lead to high-resolution structural information, and this should also facilitate development of nucleotide analogs.

G-quadruplex interactive agents

An approach to telomerase inhibition that has been the focus of much attention is the design of small molecules that bind G-quartet structures. This strategy is based on the assumption that the single-stranded G-rich regions at telomere ends will form quadruplex structures, and that these structures are essential for proper functioning of human telomerase. Conclusive evidence for the formation of G-quartets within cells is lacking, and inosine-substituted oligonucleotides that lack the capacity to form G-quartets continue to act as primers for both *Tetrahymena*[63] and human telomerase (X. Jia and D.R. Corey, unpublished). In spite of these open questions regarding the importance of G-quartet formation within cells, the uniqueness of the G-quartet structure makes it an attractive target for inhibitors designed to disrupt regulation of telomere length through inhibition of telomerase.

Two classes of molecules – anthraquinone derivatives[64] and cationic porphyrins[65] – have been demonstrated by NMR spectroscopy to interact with G-quadruplex structures. Both molecules also inhibit telomerase *in vitro* when present at concentrations of 1 μM or above by reducing the processivity of the enzyme. Chemically related molecules that bind to G-quadruplex DNA with lower affinity are less inhibitory, supporting the suggestion that these inhibitors act by binding to G-quartets and not by merely intercalating into DNA or RNA or by some nonselective interaction with protein. Although this represents a highly innovative

Table 1. Reported inhibitors of human telomerase activity[a]

Inhibitor	Proposed target	Refs
Expression of antisense RNA	hTR	12,79
Peptide nucleic acid	hTR template	66,71
Phosphorothioate DNA	hTR template/hTERT	71,73,80
2′-*O*-methyl RNA	hTR template	72
Retroviral antisense	hTR template	81
Phosphodiester DNA oligos	hTR template	80,82
Anthraquinone derivatives	G-quadruplex/telomere	64
Cationic porphyrin	G-quadruplex/telomere	65
Perylenetetracarboxylic diimide	G-quadruplex/telomere	83
Nucleoside derivatives	hTERT active site	56–60,84
Tea catechins	Unknown	90
Oligonucleotide with 2′–5′ A linkage	hTR/RNase L -mediated	74
Small molecules	Different protein	53–55,85
Ribozyme	hTR	86,87
Phosphorothioate DNA	Anchor site	88
Expression of antisense RNA	hTERT	89

[a]hTR and hTERT are essential domains in telomerase.

approach, the fact that these planar aromatic molecules are likely also to bind to DNA and RNA when added to cells might lead to a propensity for nonselective associations that will limit their efficacy, particularly if they need to be administered for weeks before an effect is observed. That caveat being noted, it is important to remember that the G-quadruplex interactive compounds described to date are only experimental leads, and that standard approaches in medicinal chemistry could serve to optimize efficacy and specificity.

Oligonucleotides as inhibitors

The 11-base template within hTR is intrinsically accessible to binding by telomere ends and this recognition is essential for maintaining telomere length, suggesting that telomerase should be an ideal target for inhibition by oligonucleotides that target this sequence. We have found that peptide nucleic acids (PNAs) complementary to the template inhibit telomerase with IC_{50} values of 1–10 nM (Ref. 66). The potential for PNAs to be lead compounds for drug development has been increased by the recent development of methods for delivery of PNAs within cells, including the use of import peptides[67–69] and lipofection of DNA–PNA hybrids (S.E. Hamilton and D.R. Corey, unpublished). In addition, we have also examined inhibition by phosphorothioate DNA and 2′-*O*-methyl-RNA, which exert sequence-specific effects within cells as well as animals. These compounds are already in clinical trials, enhancing the potential for development of practical telomerase inhibitors in the near term[70].

Phosphorothioate DNA oligomers were relatively potent inhibitors, but this inhibition is not sequence selective[71]. Phosphorothioate DNA has enhanced potential for interaction with proteins, and nonsequence-selective inhibition is probably the result of binding of the phosphorothioate backbone to hTERT. Inhibition by fully phosphorothioate-substituted 2′-*O*-methyl-RNA was similarly potent but exhibited only tenfold selectivity for match versus mismatch sequences[72]. The lesser sequence selectivity for inhibition by fully phosphorothioated DNA or 2′-*O*-methyl-RNA does not necessarily imply that these oligomers cannot be used to inhibit telomerase *in vivo*, but it does complicate their development as candidate antiproliferative agents because it will be more difficult to prove that effects are being exerted through a telomerase-dependent mechanism. Phosphodiester-linked 2′-*O*-methyl-RNA, however, proved to be a highly selective inhibitor and displayed a potency comparable with the best PNA inhibitors[72]. Introduction of phosphorothioate linkages at the 3′- and 5′-termini of the 2′-*O*-methyl-RNA increased stability towards nuclease digestion without sacrificing potency or specificity. This modified 2′-*O*-methyl-RNA inhibited telomerase within cells upon delivery with cationic lipid. The modified 2′-*O*-methyl-RNA and related oligomers should prove to be valuable reagents for investigating the effect of telomerase inhibition within cells and animals.

There have been two reports of anti-telomerase oligonucleotides causing cell death soon after addition, results that are contrary to the dogma that telomerase inhibitors require a lag phase before their effects become apparent. One involves a hexamer oligonucleotide directed to the template region of hTR (Ref. 73), while a second contains a 2′–5′ adenosine linkage that is meant to direct RNase L-mediated cleavage to a non-template region predicted to be accessible[74]. These intriguing reports could indicate that telomerase inhibitors can function by a mechanism other than gradual telomere erosion. For example, it is interesting to speculate that oligonucleotide-directed RNase L cleavage might be damaging telomerase so that it can bind but not extend the telomere, thus inducing cell-cycle arrest and rapid antiproliferative effects. More extensive studies will be necessary to support these surprising observations and it will also be interesting to test the effects on telomere length maintenance of telomere-directed oligonucleotides.

Cancer and beyond: applications for inhibition of telomerase

The likelihood that a long lag phase will be required before decreased cell proliferation is observed suggests that telomerase inhibitors will not be well suited as a primary treatment for cancer. It seems more reasonable to expect that telomerase inhibitors will be administered as chemopreventive agents or after the removal of bulk tumor mass by an initial round of chemotherapy or surgery. There is also a danger that the inhibition of telomerase within normal cells that proliferate extensively, such as germ cells and some somatic stem cells, might result in undesirable side effects. The magnitude of such side effects will have to be evaluated, but cancer cells might be especially susceptible to telomerase inhibitors because of their high proliferative capacity and already critically shortened telomeres.

Discussion of telomerase inhibitors has been almost entirely confined to their use in chemoprevention and chemotherapy. In theory, telomerase inhibitors have the potential for broad usage. Telomeric repeats have been characterized in several parasitic organisms[75–77], and telomerase activity has been observed in *Plasmodium falciparum*[78]. These observations suggest that it might be possible to take advantage of the differences between the host and the parasite or fungal enzymes to design selectively toxic drugs. Unlike the application of telomerase inhibitors

to cancer, inhibitors of telomerase from other organisms would not affect telomerase in host stem cells, thus reducing the risk of undesirable side effects.

Conclusion

Our knowledge of the basic biology of telomere length maintenance suggests that discovery of drugs that target telomerase would represent a new class of therapeutic agents that act through a novel mechanism. Balanced against this attractive possibility is the sobering realization that the likelihood of a long lag-phase for pharmacological efficacy combined with the potential for inhibitor resistant phenotypes will hinder 'business as usual' approaches to inhibitor development that ignore the complexity of telomere biology. Telomerase is a complex enzyme that offers many targets for the design of inhibitors, and their development and testing will provide the only sure way to address the telomerase challenge to drug discovery.

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