Telomeres, the termini of chromosomes, provide essential stability to linear eukaryotic chromosomes. The enzyme telomerase is one mechanism that maintains telomeres, and is activated in 85% of human cancer cells. New studies on peptide nucleic acids (PNAs) that inhibit telomerase have demonstrated that unexpected regions of the enzyme can serve as targets for inhibitors. The novel delivery method used expands the utility of PNAs.

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Human telomerase activity was first detected in the transformed cervical carcinoma cell line HeLa in 1989 [1]. Conventional DNA polymerases and primases cannot replicate the terminal bases of a linear DNA molecule, so, in the absence of other mechanisms, chromosomes will shorten with the removal of terminal RNA primers during each round of replication [2–4]. Telomerase is an enzyme that compensates for the loss of telomeric sequences at the ends of chromosomes by adding tandem repeats of d(TTAGGG) that make up the telomere. Human telomerase is a ribonucleoprotein, consisting of a catalytic protein subunit, the telomerase reverse transcriptase component (hTERT), one or more additional proteins, and an integral RNA component (hTR) that serves as a template for the synthesis of the telomeric repeats [5]. TERT contains reverse transcriptase (RT) motifs, which are conserved among reverse transcriptase enzymes, and telomerase-specific motifs. Conserved amino acids in both of these sets of motifs are required for catalytic activity [5–7]. Human telomerase activity can be reconstituted in vitro by expressing hTERT and hTR in rabbit reticulocyte lysate [8,9].

Telomerase, immortalization and cancer
In the last ten years evidence has accumulated correlating telomerase activity with cell immortalization and cancer [3,10]. In most cultured primary somatic human cells, telomerase is inactive: telomeres shorten upon cell division and the cells eventually undergo replicative senescence. Human germ cells, which are telomerase positive, have long telomeres. But many established, immortalized cell lines also express telomerase activity, and have short telomeres that are generally maintained at a constant length as the cells are passaged [11]. Telomerase activity has been detected in over 85% of tumor samples tested [12]. The idea that telomerase may be required to maintain telomeres and stabilize chromosomes in many immortalized cells, and that its inactivation may specifically inhibit the proliferation of cancer cells, has, therefore, been gaining ground. However, there are a number of issues to consider in attempting to develop specific anti-telomerase therapies directed against cancer cells [10,13]. Three of these are listed below.

First, 15% of immortal and tumor cells have no detectable telomerase activity. Conversely, activity has been reported in some human somatic tissues, including hematopoietic and epithelial cells [3]. Antitelomerase therapies are unlikely, therefore, to be universally effective and may have side effects.

Second, telomerase inhibition via telomere shortening would not be expected to have immediate adverse effects, especially in tumor cells that do not already have extremely short telomeres. In mice, deletion of the telomerase RNA component resulted in the loss of telomeres and end-to-end fusion of chromosomes, but only in the fourth generation [14]. Six to seven generations were required before the mice developed abnormal phenotypes, which included defective spermatogenesis and decreased proliferation in testis and hematopoietic cells of the bone marrow and spleen [15] (Table 1). Because mice have longer telomeres than humans, this lag may not be so profound in humans, but most telomerase inhibitors do have a lag phase before a phenotypic effect is apparent [13]. However, two telomerase inhibitors have been described that cause immediate cell death, perhaps by triggering cell cycle arrest, so the development of telomerase inhibitors that manifest early secondary effects may, after all, be possible [13,16,17]. There have also been reports that anti-telomerase therapy could be used in combination with chemotherapy to treat certain cancers resistant to conventional chemotherapy [18].

Third, resistance to telomerase inhibitors may be possible. In yeast, deletion or mutation of the genes encoding the telomerase RNA, catalytic or associated subunits of telomerase lead to telomere shortening, eventual death of most of the population, and the selection of survivors. These survivors have rescued telomere lengths or circularized chromosomes as a result of recombination-based, alternative telomere maintenance mechanisms [3,19–21] (Table 1).
Another approach is to target G-quadruplexes, ribozymes have also been designed to cleave hTR in a variety of experimental circumstances: in vitro, in cultured cells, and in vivo using xenograft human-mouse models (Table 2). The expression of antisense telomerase RNA in immortal human cells leads to telomere shortening and cell death [24]. These inhibitors generally target the template region of the RNA component of telomerase (hTR), the telomerase active site, the catalytic component of telomerase (hTERT), or G-quartet DNA structures [25–28].

Targeting telomerase activity carries the risk of inadvertently inhibiting other polymerases. Nucleoside analogs (and their triphosphate derivatives), such as azidothymidine that inhibit polyribosomes such as HIV reverse transcriptase, have also been used to inhibit telomerase, but have limited specificity and potency [25,29–33]. Several oligonucleotide derivatives have been tested as telomerase inhibitors, including DNA, phosphorothioate (PS) oligonucleotides, peptide nucleic acids (PNAs) and 2′-O-methyl (2′-O-me) RNAs (Figure 1). Hammerhead ribozymes have also been designed to cleave hTR [27,34,35]. Another approach is to target G-quadruplexes, structures that can be adopted by single-stranded G-rich regions, such as those found at the ends of chromosomes [36]. It has been postulated that such structures form at chromosome ends and that these structures are essential for proper telomerase function; several molecules that bind to G quartets have been identified that inhibit telomerase activity [26,37,38]. However, such molecules can bind both to DNA and RNA, reducing their specificity [13], and may also inhibit processes involved in immunoglobulin gene rearrangement and transcriptional regulation [38].

Directly targeting the catalytic protein component, hTERT, instead of the RNA component, hTR, may be more effective, as telomerase activity correlates best with the expression of hTERT [39]. In humans, hTR is detectable not only in immortal cancer cell lines, tumors and tissues that express telomerase activity, but also in cell lines, tumors and tissues that do not have telomerase activity [24,40]. In contrast, hTERT is expressed in all telomerase-positive cells and in only a specific subset of telomerase-negative cells thought to have long-term proliferative capacity [41]. However, to date, the only reported telomerase inhibitor that targets hTERT is the expression of a mutant catalytic subunit of human telomerase [42,43]. The effects of inhibiting hTERT by the expression of dominant negative mutants of hTERT include the inhibition of telomerase activity, telomere loss, chromosome damage, apoptosis and cell death of various human cancer cell lines [42,43].

**Table 1**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Alteration</th>
<th>Phenotype(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrahymena thermophila</td>
<td>Mutant RNA template sequences</td>
<td>Altered telomere sequence and length; nuclear and cell division defects; senescence</td>
<td>[23,48]</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>△TLC1</td>
<td>Telomere shortening; a gradual increase in generation time; cell death; survivors have rescued telomere lengths</td>
<td>[19,20]</td>
</tr>
<tr>
<td>Kluyveromyces lactis</td>
<td>△TER1</td>
<td>Loss of telomeric DNA and viability; survivors have maintained telomeres or circularized chromosomes</td>
<td>[21]</td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>△TRT1</td>
<td>Decreased telomere lengths; decreased telomerase activity; cell death</td>
<td>[24]</td>
</tr>
<tr>
<td>Human</td>
<td>Expression of antisense hTR in immortal cells</td>
<td>Telomerase expression; telomere lengthening; extension of life span</td>
<td>[49]</td>
</tr>
<tr>
<td>Mouse</td>
<td>Telomerase RNA gene knockout</td>
<td>Telomere shortening; loss of telomeres, end-to-end fusions, aneuploidy (generation four); defective spermatogenesis, decreased proliferation in tests and haematopoietic cells of bone marrow and spleen (generation six)</td>
<td>[14,15]</td>
</tr>
</tbody>
</table>

TLC1, TER1 and TRT1 are the genes encoding the telomerase RNA components of S. cerevisiae and K. lactis, and the catalytic component of S. pombe, respectively.

Alternative mechanisms of telomere lengthening also exist in humans [22]. Mechanisms of drug resistance are also likely to include mutations that decrease inhibitor binding or that upregulate telomerase expression [13].

**Telomerase inhibition**

Despite these concerns, there is evidence that telomerase inhibition could, in principle, lead to effective cancer treatments. *Tetrahymena* cells expressing mutant telomerase RNAs, like yeast telomerase RNA knockouts, show altered telomere sequences and lengths, resulting in eventual cell death (Table 1) [23]. Similarly, the identification of the RNA component of human telomerase in 1995 [24] led to a growing number of reports of inhibition of human telomerase in a variety of experimental circumstances: in vitro, in cultured cells, and in vivo using xenograft human-mouse models (Table 2). The expression of antisense telomerase RNA in immortal human cells leads to telomere shortening and cell death [24]. These inhibitors generally target the template region of the RNA component of telomerase (hTR), the telomerase active site, the catalytic component of telomerase (hTERT), or G-quartet DNA structures [25–28].

Directly targeting the catalytic protein component, hTERT, instead of the RNA component, hTR, may be more effective, as telomerase activity correlates best with the expression of hTERT [39]. In humans, hTR is detectable not only in immortal cancer cell lines, tumors and tissues that express telomerase activity, but also in cell lines, tumors and tissues that do not have telomerase activity [24,40]. In contrast, hTERT is expressed in all telomerase-positive cells and in only a specific subset of telomerase-negative cells thought to have long-term proliferative capacity [41]. However, to date, the only reported telomerase inhibitor that targets hTERT is the expression of a mutant catalytic subunit of human telomerase [42,43]. The effects of inhibiting hTERT by the expression of dominant negative mutants of hTERT include the inhibition of telomerase activity, telomere loss, chromosome damage, apoptosis and cell death of various human cancer cell lines [42,43].

**Selectivity of telomerase inhibitors**

One particularly interesting class of anti-hTR telomerase inhibitors is the PNAs [44], which give the highest specific in vitro inhibition of telomerase activity known [28,45,46]. Compared with standard oligonucleotides or PS oligonucleotides, PNAs bind to targeted RNA more rapidly and
with higher affinity and specificity. The lack of a phosphate backbone decreases electrostatic interactions, preventing PNAs from associating with proteins that normally bind nucleic acids. IC$_{50}$ values for the inhibition of telomerase by PNAs complementary to the template of hTR are in the picomolar to nanomolar range, whereas IC$_{50}$ values are in the micromolar range for noncomplementary PNAs. PS oligomers show lower activity and lower selectivity; the IC$_{50}$ of complementary versus noncomplementary PS oligonucleotides is 50 and 100 $\mu M$, respectively, probably a result of nonsequence selective protein binding by PS oligomers [28]. A further advantage of PNAs is their resistance to degradation by both proteases and nucleases. Very recently, David Corey and colleagues [45] elegantly demonstrated that PNAs directed to regions of hTR other than the template provide a new mode of inhibition, presumably acting prior to the assembly of the ribonucleoprotein. Initially, PNAs directed against nontemplate regions of hTR did not demonstrate significant inhibition in cell extracts [28]. But by separately expressing hTERT and hTR in a rabbit reticulocyte transcription and translation system, Corey and colleagues [45] were able to determine the effect of inhibitors on assembly of the telomerase. IC$_{50}$ values for six PNAs directed against different non-template regions of hTR were between 10 and 100 nM when the PNAs were added prior to hTERT expression. After the telomerase holoenzyme had been assembled, the same PNAs gave IC$_{50}$ values of 2 to $>100 \mu M$, indicating that the targets for these PNAs are not readily accessible once hTR has associated with hTERT. When added before telomerase assembly, PNAs targeted to the hTR template region inhibited the telomerase with IC$_{50}$ values of 0.02–0.1 $\mu M$, increasing to 0.03–0.5 $\mu M$ when the holoenzyme was used; as expected, therefore, the template region of hTR is the most accessible portion of the RNA after the holoenzyme is assembled. Using these anti-template PNAs, and an ingenious adaptation of the standard cationic lipid transfection technique to introduce partially overlapping PNA–DNA duplexes into cells, Corey and colleagues [45] show that over 85% inhibition of telomerase activity can be achieved in the DU145 prostate cancer cell line. Inhibition can also be achieved in whole cells even when the PNAs are targeted to non-template regions; a longer transfection of nontemplate-directed PNAs led to 84% inhibition of telomerase activity in one case, and 78% inhibition in another.

### Table 2

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC$_{50}$</th>
<th>Proposed target</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expression of antisense RNA</td>
<td>NA</td>
<td>hTR</td>
<td>[17,24,50,51]</td>
</tr>
<tr>
<td>Retroviral antisense</td>
<td>NA</td>
<td>hTR</td>
<td>[52]</td>
</tr>
<tr>
<td>Nucleoside derivatives: AZT-TP, 7-deaza-dATP, 7-deaza-dGTP, ddGTP, &amp; Gp4G</td>
<td>1-30 µM</td>
<td>Telomerase active site</td>
<td>[25,29-33]</td>
</tr>
<tr>
<td>Phosphorothioate DNA</td>
<td>200 pM—&lt;25 µM</td>
<td>hTR/hTERT</td>
<td>[16,28,53–56]</td>
</tr>
<tr>
<td>Phosphodiester DNA oligos</td>
<td>20–100 nM</td>
<td>hTR</td>
<td>[33,57]</td>
</tr>
<tr>
<td>Oligonucleotide with 2′-5′-oligoadenylate linkage</td>
<td>ND</td>
<td>hTR/RNas L-mediated</td>
<td>[17]</td>
</tr>
<tr>
<td>Peptide nucleic acid (PNA)</td>
<td>900 pM–100 µM</td>
<td>hTR</td>
<td>[28,45,46]</td>
</tr>
<tr>
<td>PNA–cationic peptide conjugates</td>
<td>140 nM–&gt;8 µM</td>
<td>hTR</td>
<td>[58]</td>
</tr>
<tr>
<td>2′-O-methyl RNA</td>
<td>2–300 nM</td>
<td>hTR</td>
<td>[47]</td>
</tr>
<tr>
<td>Hammerhead ribozymes</td>
<td>400 nM–10 µM</td>
<td>hTR</td>
<td>[27,34,35]</td>
</tr>
<tr>
<td>Anthraquinone derivatives</td>
<td>4–50 µM</td>
<td>G-quadruplex</td>
<td>[26,59]</td>
</tr>
<tr>
<td>Cationic porphyrin</td>
<td>≤50 µM</td>
<td>G-quadruplex</td>
<td>[37,60]</td>
</tr>
<tr>
<td>Perylenetetracarboxylic diimide</td>
<td>&gt;75 µM</td>
<td>G-quadruplex</td>
<td>[38]</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>2 µM</td>
<td>hTR transcription</td>
<td>[61]</td>
</tr>
</tbody>
</table>

NA, not applicable; ND, not determined.

Very recently, David Corey and colleagues [45] elegantly demonstrated that PNAs directed to regions of hTR other than the template provide a new mode of inhibition, presumably acting prior to the assembly of the ribonucleoprotein. Initially, PNAs directed against nontemplate regions of hTR did not demonstrate significant inhibition in cell extracts [28]. But by separately expressing hTERT and hTR in a rabbit reticulocyte transcription and translation system, Corey and colleagues [45] were able to determine the effect of inhibitors on assembly of the telomerase. IC$_{50}$ values for six PNAs directed against different non-template regions of hTR were between 10 and 100 nM when the PNAs were added prior to hTERT expression. After the telomerase holoenzyme had been assembled, the same PNAs gave IC$_{50}$ values of 2 to $>100 \mu M$, indicating that the targets for these PNAs are not readily accessible once hTR has associated with hTERT. When added before telomerase assembly, PNAs targeted to the hTR template region inhibited the telomerase with IC$_{50}$ values of 0.02–0.1 $\mu M$, increasing to 0.03–0.5 $\mu M$ when the holoenzyme was used; as expected, therefore, the template region of hTR is the most accessible portion of the RNA after the holoenzyme is assembled. Using these anti-template PNAs, and an ingenious adaptation of the standard cationic lipid transfection technique to introduce partially overlapping PNA–DNA duplexes into cells, Corey and colleagues [45] show that over 85% inhibition of telomerase activity can be achieved in the DU145 prostate cancer cell line. Inhibition can also be achieved in whole cells even when the PNAs are targeted to non-template regions; a longer transfection of nontemplate-directed PNAs led to 84% inhibition of telomerase activity in one case, and 78% inhibition in another. These

![Chemical structures of (a) DNA, (b) PS oligonucleotides, (c) 2′-O-methyl RNAs and (d) PNA.](image-url)
nontemplate-directed PNA inhibitors represent a new class of telomerase inhibitors, and perhaps indicate new possible targets for non-PNA telomerase inhibitors.

The adapted transfection method used in this study was also previously used for the intracellular delivery of 2′-O-methyl RNA telomerase inhibitors [47], and should find wide use in studies of the cellular activities of PNAs. By using overlapping PNA–DNA hybrids instead of complementary ones, the authors expected to be able to use the DNA as a carrier, then allow the DNA to be digested by nucleasea insides the cells. The PNA would then be free to bind to its target. Microscopy and fluorescence-activated cell sorter analysis were used to confirm that rhodamine-labelled PNAs were indeed delivered into the cells with high efficiency. None of the PNA–DNA hybrids tested were immediately toxic, and the telomerase inhibition persisted for three population doublings, indicating that the amount of PNA that is delivered to the cells is sufficient to withstand the dilution caused by segregation during cellular division. After a four-week treatment telomere shortening was observed, confirming the specificity of the PNA inhibitors. Furthermore, nontemplate-hTR-directed PNAs inhibited telomerase activity to levels comparable with template-directed PNAs (upon increased time of incubation with cells), substantiating the in vitro observation that these PNAs may affect telomerase ribonucleoprotein assembly in actively dividing cells.

Conclusions and future perspectives
The discovery that PNAs directed against nontemplate regions of the human telomerase RNA can be potent telomerase inhibitors [45] offers a new set of targets for exploration and opens up the possibility that combinations of telomerase inhibitors could be used. Furthermore, the ability to effectively introduce PNAs into eukaryotic cells using a simple method and the efficient inhibition of cellular telomerase by PNAs has overcome one major difficulty for PNA research, and may have profound implications in the development of specific and potent antitelomerase cancer therapies. The question of whether PNAs can be used as effective anti-proliferative agents remains, however, to be demonstrated. This next step in the development of telomerase inhibitors will determine if targeting telomerase as an anticancer therapy will indeed be possible.

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References


