

Targeting the Telomere with T-Oligo, G-Quadruplex Stabilizers, and Tankyrase Inhibitors

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Introduction

All mammalian chromosome ends are capped by telomeres, specialized DNA structures that protect chromosomes from genomic instability [1]. Telomeres are comprised of 1000-2000 tandem base pairs repeats, and their 3' ends extend beyond the 5' terminus, forming an overhang region that is comprised of a repeating TTAGGG sequence. During each cycle of cell division telomeres are incompletely replicated, and consequentially, their ends are progressively shortened. When telomeres reach a critically short length, DNA damage responses (DDR) such as senescence and/or apoptosis are triggered. Hence, telomeres are considered to be "biological clocks," as they limit the proliferative potential of most normal cells [2].

In order to safeguard the chromosome, the 3' overhang forms a specialized lariat structure, called a telomere-loop (t-loop), which serves as a protective cap [3]. The t-loop prevents the single-stranded overhang from being recognized as a double stranded break and protects telomeres from nucleolytic degradation or non-homologous end-joining [4,5]. The formation of the t-loop is facilitated by shelterin, a six-protein complex composed of TRF1, TRF2, TIN2, POT1, TPP1, and Rap1 [6], which has a critical role in telomere length maintenance [2]. All shelterin proteins except POT1 bind to double-stranded regions of the telomere. Additionally, the t-loop is partially stabilized by the guanine-rich character of the 3' overhang, which is hypothesized to form G-quadruplexes, which are four-stranded DNA structures stabilized by hydrogen bonds between guanine quartets. Increasing the amount of G-quadruplex regions has been shown to prevent telomerase, the enzyme complex that lengthens telomeres, from binding to the telomere overhang [6].

Telomerase is a DNA-reverse transcriptase that adds TTAGGG repeats to the 3' overhang, thereby elongating the telomere. It is comprised of two major components, an RNA template complementary to the 3' overhang that acts as a primer, called hTR, and a human ribonucleoprotein reverse transcriptase, hTERT, that catalytically adds nucleotides to the 3' overhang [7,8]. Telomerase has recently become an attractive target for cancer therapies because it is inappropriately overexpressed in more than 85% of cancers, while most normal cells have minimal or no detectable activity [9,10]. When telomerase is activated, telomere length is stabilized and DDRs initiated by critically-shortened telomeres do not occur, thereby allowing cancer cells to proliferate by evading apoptosis or senescence [6].

A minority of cancers, less than 15%, maintain telomere length homeostasis through the alternative lengthening of telomeres (ALT) pathway. ALT-positive cells are able to replenish telomeric DNA in a telomerase-independent manner through a homologous recombination mediated replication mechanism and are thus resistant to telomerase-based therapies [11]. However, ALT is still poorly understood, and it is possible that other mechanisms of ALT exist [12]. In addition, it is thought that drug-induced resistance to telomerase inhibitors may occur through the activation of ALT pathways in some cancers [6,13].

Cancer Therapies Related to Telomeres

Currently, most agents targeting the telomere promote telomere attrition through direct inhibition of telomerase. However, many of these agents have shown no improvements in overall survival or progression free survival in recent clinical trials [14]. Hence, other strategies to molecularly target the telomere in cancer are needed. Recently developed potential therapeutics such as G-quadruplex stabilizers, Tankyrase inhibitors, and T-oligos are telomerase-independent or inhibit telomerase indirectly (Figure 1). They can induce telomere dysfunction by interfering with telomere structure or by targeting shelterin proteins, and may circumvent activation of the ALT pathway [2].

The guanine-rich character of the 3' overhang of the telomere can form G-quadruplexes, four-stranded DNA structures (tetrads) that stack together and may be necessary for proper telomere functioning. However, it has also been demonstrated that increasing the stability of G-quadruplex structures at telomeres via small molecules can negatively disrupt telomere structure and also inhibit telomerase activity [15]. Increasing G-quadruplex content in chromosomes could also stall the replication fork during DNA synthesis, which could thereby induce DDRs [6]. Hence, G-quadruplex stabilizers such as telomestatin are being explored as anticancer therapeutics. Telomestatin is a small molecule that can facilitate the formation or stabilization of telomeric G-quadruplex structures at the 3' overhang [16]. Additionally, in some ALT positive tumors, telomestatin has been shown to be effective in causing telomere dysfunction and triggering DDRs [17]. Furthermore, studies targeting leukemia demonstrated that telomestatin not only inhibits telomerase, but suppresses proliferation and increases efficacy of other chemotherapeutic agents and small molecule inhibitors [18].

Another approach to indirectly inhibit telomerase is through the inhibition of a telomere-specific poly(ADP-ribose) polymerase, called Tankyrase-1 (TNKS1), which plays a role in recruiting telomerase to the telomere. TNKS1 poly(ADP-ribosyl)ates TRF1, a component of the shelterin complex, thereby releasing it from the telomere and allowing telomerase to access the 3' overhang [19,20]. In cancer cells, the upregulation of TNKS1 corresponds with increased resistance to telomerase inhibition, whereas its inhibition with small molecule

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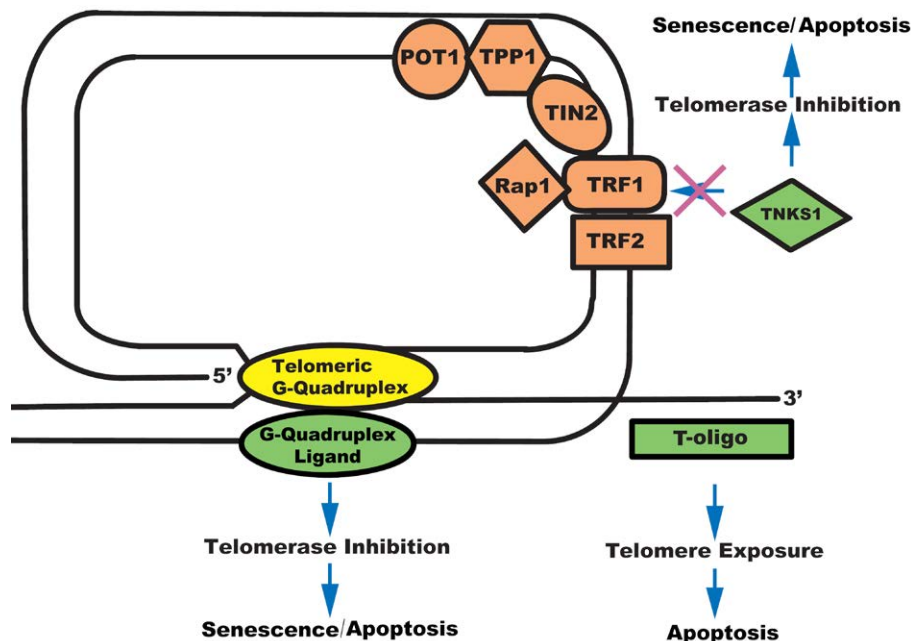


Figure 1: Novel Therapies Targeting Telomeres and Telomerase. The single stranded 3' telomere overhang folds over itself and invades the double stranded region, forming a t-loop. This process is aided by a six-protein complex called shelterin, which caps telomere ends and regulates telomerase. G-quadruplexes are formed by stabilizing hydrogen bond interactions facilitated by the guanine rich character of the 3' overhang. Increasing G-quadruplex stability can consequently inhibit telomerase. TNKS1 poly(ADP-ribosyl)ates TRF1, thereby releasing it from the telomere and allowing telomerase access to the 3' overhang. Inhibiting TNKS1 is thought to prevent poly(ADP-ribosylation) of TRF1 and hence inhibits telomerase activity. T-oligo has sequence homology with the 3' telomere overhang. T-oligo administration is thought to mimic telomere exposure and induce anticancer responses, mainly in cancer cells with minimal effects in normal cells.

inhibitors such as XAV939 reduces resistance to telomerase inhibitors, enhancing telomere shortening [21]. These studies suggest that TNKS1 inhibitors have potential as therapeutics in combination with other telomerase inhibitors. Furthermore, depletion of TNKS1 with siRNA knockdown results in telomere uncapping and increased sensitivity to ionizing-radiation, which could be potentially useful to cancer patients [22]. TNKS1 inhibitors such as IWR1, IWR2, JW55, XAV939, are currently being investigated in preclinical studies as possible novel cancer therapeutics [23-25].

A novel anticancer agent that targets the telomere is T-oligo, an 11-base oligonucleotide (GTTAGGGTTAG) homologous to the 3' telomere overhang. T-oligo is thought to mimic the physiological signal of telomere exposure, since it elicits DDRs similar to those induced by telomere dysfunction after ectopic expression of a non-functional TRF2, an integral component of the shelterin complex [6]. T-oligo accumulates in the nucleus and rapidly induces DDRs mediated by ATM, p53, E2F1 and p95/NBS1 and their downstream targets, resulting in cell cycle arrest, senescence, apoptosis, and differentiation [6,26-28]. Interestingly, T-oligo has little or no effect on most normal cells [26,29] and does not affect telomerase activity [30,31]. The disparity in response between malignant cells and normal cells suggests the possibility that these altered responses may be due to pre-existing gross DNA abnormalities and loss of normal cell-cycle checkpoints, leading to apoptosis or a markedly differentiated or senescent phenotype [26,28].

In vitro studies have shown that T-oligo is highly effective in reducing viability and growth of melanoma, lung, prostate, ovarian, breast, and colorectal cancer cells [26,27,29,32-34]. Treatment with T-oligo demonstrated upregulation of several tumor associated antigens in colorectal cancer, which have roles in inhibiting proliferation and

are lost in poorly differentiated cancers [29]. T-oligo treatment also induces the upregulation of several melanoma differentiation proteins which are currently the targets of melanoma vaccine therapies [28]. T-oligo was also used in combination with other molecularly targeted therapies *in vitro*. Combination treatment of T-oligo with a tyrosine kinase inhibitor or histone deacetylase inhibitors currently in clinical trials demonstrated additive inhibition of cellular growth [29,33]. Furthermore, when combined with ionizing radiation treatment, T-oligo increased radiosensitivity and synergistically inhibited cellular growth both *in vitro* and *in vivo* [34]. Other derivatives of T-oligo, such as the guanine-enriched T-oligo (GGTT)₄, have exhibited increased cytotoxic effects in certain cell lines compared to T-oligos (telomere overhang sequence), which may be due to increased G-quadruplex formation by (GGTT)₄ [32,33].

T-oligo also stimulates various anti-cancer responses *in vivo*, such as reduction of tumor burden and metastatic potential in mice, with no detectable toxicity [6,26,35]. Our lab has recently shown that tumor volume in mice with SW1573 and H358 lung cancer xenografts was reduced by 80 and 88%, respectively, after treatment with T-oligo for seven weeks. Another *in vivo* study in melanoma reported that, when compared with controls, T-oligo reduced metastases by 92-95% and tumor volume by 84-88% [28]. T-oligo also demonstrated its ability to elicit its anti-tumor effects by inducing senescence [28] and inhibiting angiogenesis in melanoma and lung cancer [26,35,36].

Despite its effectiveness, T-oligo has limited stability *in vitro* and *in vivo* due to degradation by nucleases. To enhance its stability and delivery, our lab has recently complexed T-oligo with a positively charged helical polypeptide, PVBLG-8 (PVBLG). As a result, T-oligo's cellular uptake improved on a log scale and its ability to inhibit cellular growth and reduce tumor burden in mice was significantly enhanced.

Melanoma tumors grown on the flanks of SCID mice and subsequently treated with T-oligo in the presence or absence of PVBLG showed an 89.1% and 68.1% reduction in tumor size, respectively, compared to control [35].

Conclusion

Telomeres and telomerase have emerged as attractive cancer therapeutic targets due to their important roles in cancer cell immortality. One limitation of therapies that target telomerase is that several generations of cell divisions are required before telomeres are critically shortened and apoptosis and senescence is induced. Within that time, malignant cells may become resistant to treatment and lengthen their telomeres via activation of the ALT pathway. Moreover, the effects of telomerase inhibition on stem cells with telomerase activity are presently unclear and require further study [2]. Hence, other therapeutic modalities that target telomerase are needed. Preliminary studies have demonstrated suppression of telomerase activity by using TNKS1 inhibitors, such as XAV939, which can eliminate resistance to telomerase inhibition and may be used in combinatorial clinical therapies in the future [21]. In addition, therapies that induce telomere-based DDRs in a telomerase-independent manner have recently emerged. G-quadruplex stabilizers, like telomestatin, facilitate the formation and stabilize G-quadruplex structures and can disrupt telomere structure while also preventing telomere elongation by inhibiting telomerase. Furthermore, telomestatin can increase the effectiveness of other chemotherapies in combinatorial treatments [18]. T-oligo has demonstrated significant anti-cancer effects both *in vitro* and *in vivo* in a variety of cancers and is thought to induce telomere dysfunction by mimicking the overhang and forming G-quadruplex structures (Figure 1). However, more studies are needed to elucidate its mechanism of action and overcome its delivery limitations [2].

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