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Review

Telomere targeting approaches in cancer: over the length maintenance.

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Abstract: Telomeres are crucial structures that preserve genome stability. Their progressive erosion over rounds of DNA duplication determines senescence of cells and organisms. Telomere length homeostasis is critical for cancer development then telomere maintenance mechanisms are established targets in cancer treatment. Besides telomere elongation, telomere's dysfunction impinges on intracellular signalling pathways, in particular DNA damage signalling and repair affecting cancer cell survival and proliferation. This review summarizes and discusses about the recent findings in anti-cancer drug development targeting different "telosome" components.

Keywords: telomeres, shelterins, cancer, G-quadruplex ligands

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1. Telomeres: evolution and length maintenance in aging and cancer.

Telomeres are specialized structures at chromosome ends deputed to protection and preservation of genetic information through cell duplication. Evolutionarily, telomeres are deemed to originate from introns recombination in circular DNA molecules, generating non-coding extremities[1]. Telomere repeats are species-specific G-C rich conserved sequences, (in human 5′-TTAGGG- 3′) terminating with a G-rich (or in some species both G and C-rich) overhang[2]. The extremities of linear DNA molecules fail to be completely replicated by the DNA replication machinery, then, the presence of non-coding DNA at the extremities overcame the progressive loss of terminal sequences at each cell division round [3]. Since telomeres are lost with cell duplication, several studies were conducted to find correlations between telomere length and age, showing that telomere length is reduced over age [4]. Moreover, genetic defects reducing the inherited telomere length affect offspring lifespan and the self-renewal capacity of tissues due to stem cell exhaustion.[4] Telomere shortening is accompanied by the exhibition of DNA damage response markers, that individuate dysfunctional telomeres and trigger replicative senescence.[5] Mounting evidence supports a role for telomere's dysfunction also in human ageing-related pathologies [6]. Recently, an extensive analysis of telomere length (TL) in different human tissue types and individuals clearly showed a significant correlation of TL with genetic background, gene expression and ageing. Furthermore, telomere shortening was shown to mediate aging related gene expression. In fact, telomeres can be shortened by exogenous mechanisms as oxidative stress or inflammation, and a "short-telomeres" genetic signature can drive the exhibition of aging cell phenotypes. [7] Some cells, like gametes, cancer cells and stem cells, have developed a successful strategy to overcome the replication end problem through the expression of telomerase, a ribonucleoprotein involved in counteracting the shortening of telomeric ends. Telomerase expression is strictly controlled throughout human development: if embryo stem cells have a high activity of telomerase, in the most adult somatic cells telomerase is not detectable, with the exception of lymphocytes in bone marrow and peripheral blood and a cluster of epithelial cells in the skin, hair follicle, endometrium and gastrointestinal tract [8] [9] [10]. Some human tumors (10-20% approximately) do not express telomerase and restore telomere length through alternative mechanism (ALT). Preference for ALT or telomerase activation may depend on the histological origin of the tumor, the mutational background or epigenetic mechanisms, and confers different characteristics to the cancer type in terms of prognosis and response to treatments. [11]There is also a residual number of human tumors where any detectable mechanism of telomere elongation was found (Telomere length maintenance deficient, TLM-), however, in that tumor cells with ever-shorter telomeres, initial telomere length was

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sufficient to guarantee for cell replication capacity. This demonstrates that prevention of telomere shortening is not required for oncogenesis.[12]

2. Telomere's structure

2.1 The Shelterin complex.

In mammals, telomere repeats are bound by a specific complex composed by six factors: TRF1 and TRF2 (Telomere Repeat binding Factors 1 and 2) directly bind to the telomeric DNA duplex as homodimers, POT1 (Protection of Telomeres 1) binds the G-rich single strand over-hang, and TPP1, TIN2 and Rap1 act as a bridge among the shelterin factors, holding fast the structure of the complex itself [13] (Figure 1). The shelterin complex covers the telomeric DNA and impede the activation of repair and recombination mechanisms allowing the cell to discriminate between natural extremities and DNA lesions. The members of the shelterin complex have distinct function involved in different DDR signaling and repair pathways [14] and affect telomere elongation mechanisms [8].

When telomeres undergo massive erosion due to replicative senescence or other stresses, the shelterin complex is less abundant to chromosomal extremities, and DDR is de-repressed leading to cell arrest and senescence[15]. DDR at eroded and/or unprotected telomeres, failing to mask linear DNA termination, activates a signaling cascade recruiting Homologous Recombination and canonical or non-canonical Non-Homologous End Joining machineries. These telomeres, considered as dysfunctional, encounter recombination events giving rise to telomeric fusions or loss of telomeric repeats.

2.2 Telomeric DNA and secondary structures

Telomere protection relies on the presence of a terminal cap-like structure called T-loop that is stabilized by the shelterin complex (principally TRF2). The presence of t-loops at telomere ends, was hypothesized almost twenty years ago given the presence of a single stranded overhang with sequence complementarity to telomere duplex, and successively observed in-vitro and in-vivo by atomic force and super resolution microscopy, [16][17].. More recently, it has been demonstrated that t-loop formation is also stimulated by telomere transcription[18]. For longtime, in fact, telomeres have been considered as silent chromatin territories. Recently, it has been found that telomeres are transcribed into long noncoding RNAs (lncRNAs) called TERRA (TElomere Repeats containing RNA). TERRA are transcribed from subtelomeric promoters, they are regulated by the methylated state of subtelomeres and are strongly upregulated in cells with alternative telomere-lengthening mechanisms (ALT). They remain associated to telomeric chromatin forming R-loops which increase the predisposition to hyper-recombination of ALT telomeres[19]. Upon DNA damage at telomeres, damage induced long noncoding RNAs (dilncRNAs) are transcribed bidirectionally starting from the DNA lesions. These RNAs are precursor of small non

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coding RNAs (named DDRNAs) and mediate the efficient transduction of signaling cascade driving cell arrest and repair[20][21]. Telomere damage induced transcription was shown to be a crucial for mediating of the expression of senescence associated cell phenotypes[22].

Telomeres are difficult to replicate regions, being constituted by heterochromatin and prone to fold into secondary structures like G-quadruplexes, t-loop, I-motifs[23]. In addition, the presence of long non-coding RNA transcribed from subtelomeric promoters, that stably interact with DNA duplex forming R-loops, [24] makes these chromosome fields enriched of topological enzymes necessary to assist replication, transcription, histone modification. Telomeres are indeed considered as "difficult to repair" chromatin, that consequently accumulate irreparable DNA damage causing senescence and aging[25]. In this regard, mutations affecting helicase, topoisomerase, histone acetylation and methylation cause telomere dysfunction and consequently aging associated phenotypes.

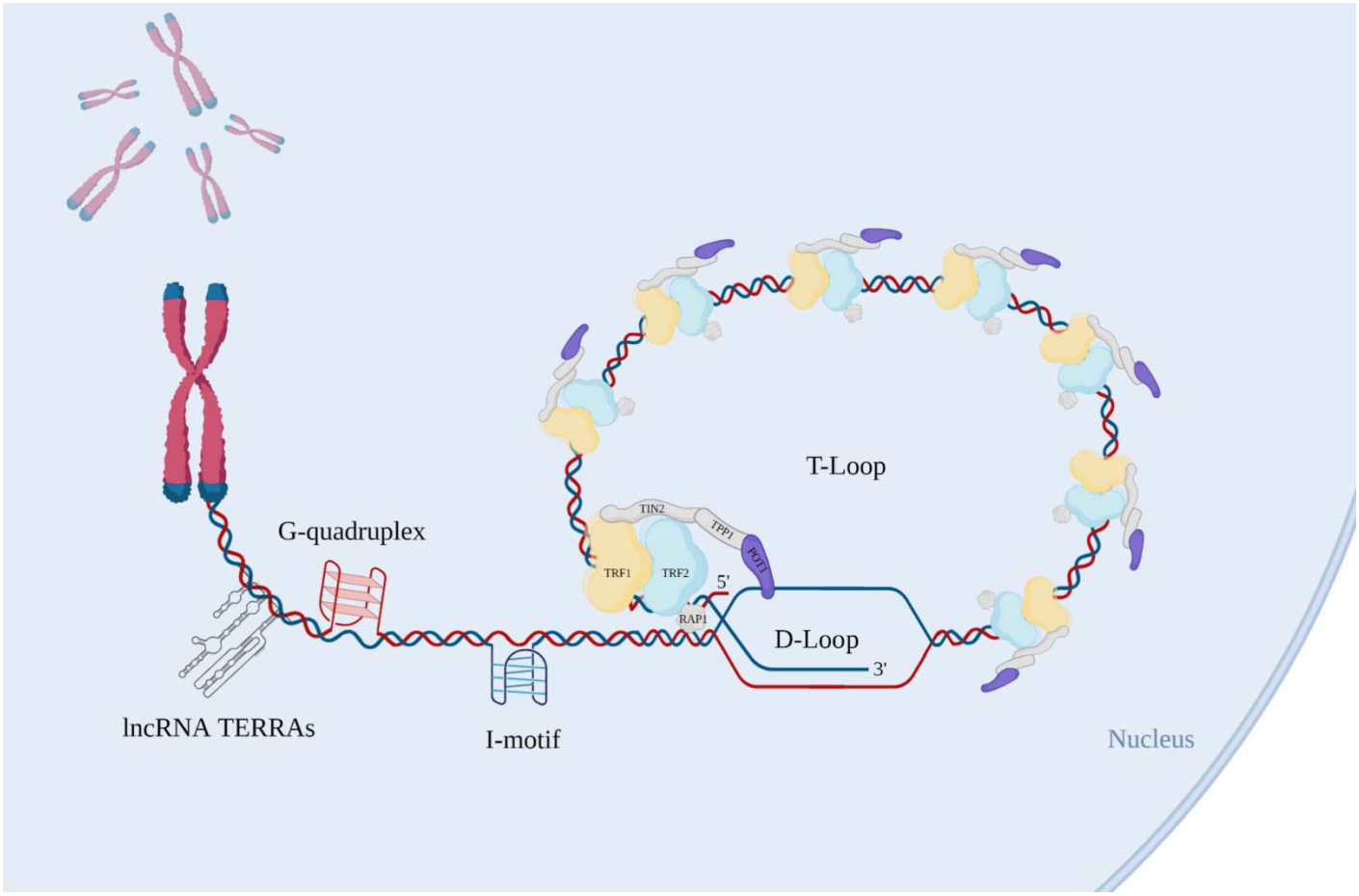

Figure 1. Molecular targets at telomeres

4. Telomere's dysfunction in cancer initiation and progression.

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In precancerous cells, bearing cell cycle checkpoints failure, shortened telomere instability generates mis-segregation and chromosome breakage during mitosis, giving rise to secondary rearrangements that fuel global genetic instability[26]. Thus, telomere protection is considered a tumor suppressive factor. Otherwise, telomere length maintenance is a prerequisite for cancer development since telomere attrition during cell divisions must be buffered in actively replicating cancer cells, to maintain an unlimited proliferative potential. [27] Telomere maintenance mechanisms are in fact considered a hallmark of cancer [28], although recently, some papers reported the existence of human tumors without any detectable telomere elongation mechanism.[12] Moreover, a pan-cancer genomics study detected hTERT (the catalytic subunit of telomerase holoenzyme) expression in ~75% of tumor samples. In these samples, telomerase reactivation occurred by point mutation (31%) or methylation (53%) in the hTERT promoter.[29] Telomerase enzymatic activity is directly correlated with cancer cell proliferation and stemness [30] and reactivation mechanisms include also gene amplification, rearrangements of gene locus. [31] Activation of telomerase coincides with other pro-oncogenes changes in adult somatic cells in the early steps of cancer development [28]. The pro-oncogenic activity of telomerase is not restricted to telomere elongation but involves interactions between hTERT subunit and signaling pathways controlling cell survival and transformation like c-myc, WNT/βcatenin, NF-kB, however, the number of identified cross-talks between hTERT and intracellular signaling is constantly growing. [32] Nevertheless, current anti-telomerase approaches, targets the telomere elongation activity only, being directed towards the catalytic site of hTERT or the RNA template.

Beside telomerase and other TLM mechanisms, other telomeric proteins are found mutated or deregulated in cancer. POT1(Protection of Telomeres 1) is an essential component for telomere stability. [33] It binds both the ss and the dsDNA at telomeres directly or interacting with other shelterins (namely TPP1 and TRF1) respectively; it counteracts G-quadruplex formation [34] and attenuates ATR driven DDR.[35] Germline and sporadic mutation of *POT1* are associated with different human cancers. *POT1* is frequently mutated in aggressive forms of chronic lymphocytic leukemia. Furthermore, germline *POT1* mutations have been shown to underlie a number of hereditary familial cancer syndromes involving CLL, glioma, melanoma and colorectal cancer and angiosarcoma. [36]. Telomere binding proteins are overexpressed in cancer, this is not simply explained by telomere re-elongation, in fact, some aggressive cancers present an unbalance between telomere length and telomere binding protein expression, which can be at the basis of the presence of dysfunctional telomeres generating genome instability [37] [38]. TRF1 is over-expressed in early stages of pancreas tumorigenesis and glioblastoma progression in mouse models [39] and TRF1 SNPs were found associated with increased risk of skin cancer in human[40].

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TRF2 is upregulated in several human cancers, it is involved in immune escape and angiogenesis through different pathways [41][42][43][44]. Alterations of the shelterin complex were recently assessed in 9125 tumor samples in 33 different human cancers. TRF1 and POT1 amplification and TRF2-RAP1-TPP1 co-amplification/deletion were found associated with cancer progression defining broad molecular signatures linked to several intracellular pathways involved in oncogenesis[45]. Data collected in endometrial cancer patients suggest instead an inverse correlation between TERRA expression and cancer progression[46].

4. Targeting approaches against telomere components

4.1 Telomeric DNA secondary structures

4.1.1 G-quadruplex. Telomeric DNA is considered as a preferential target for G-quadruplex ligands and in the last two decades several molecules belonging to this class of compounds, have shown the capacity to affect both length and structure maintenance in a dose dependent manner.[47][48] G-quadruplexes were initially thought to act by binding and sequestering the G-overhang from telomerase elongation. In agreement with this, some G-quadruplex binders induced telomere shortening across population doublings. [49] In addition, G-quadruplex stabilization can displace shelterin proteins (TRF2 and POT1) and induce a rapid DNA damage response triggering cell death. [50]G-quadruplex stabilization also stabilizes DNA-loops forming in the telomeric duplex at the G-rich strand during replication, inducing replication dependent damage, or transcriptional loops (R-loops) generating transcription/translation conflicts. [51] The synergistic effect of G-quadruplex with clinically employed drugs like camptothecins and PARP inhibitors, as well as ionizing radiations, make this class of compounds still interesting and deserving investigation, although none of this compound has been approved yet for clinical use. Notably, the CX5461, the RNA polymerase I inhibitor currently employed for the treatment of XY, has been discovered to bind G-quadruplex and to target more effectively BRCA mutated cells, and this last characteristic is in common with Pyridostatin, another G-quadruplex ligand with excellent anticancer properties.[52] [53] The most studied G-quadruplex ligands with telomere-targeting properties are summarized in table 1. In addition to this list of compounds, virtual-screening and mid-high throughput screening studies have revealed other classes of compounds emerging from small molecules or natural compounds libraries, with the capability of targeting telomeres and inducing a DDR response. Thus confirming that the G-quadruplex interactive compounds are a continue source of new molecules for medicinal chemistry in anti-cancer application.[54][55][56][57][58][59]

The C-rich strand of telomeres is known to form quadruplex structures in- vitro and in-vitro, namely **i-motifs**, that can coexist with G-quadruplexes or be mutually exclusive depending on the context. Some quadruplex ligands are specific for G or C-quadruplexes while other are selective (Table 1). [60][61] i-motifs binding induces telomere damage and

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cell death in cancer cells as well as G-quadruplex ligands and the rational design of new i-motifs ligand with higher specificity is a highly productive branch of drug discovery currently.

4.2 Shelterins

4.2.1 TRF1 was found upregulated in glioblastoma in human specimen and in mouse models and its downregulation impact on tumor progression validating TRF1 as a target in glioblastoma treatment. [62] Moreover, previous results showed that TRF1 inhibition impaired growth of K-Ras induced lung cancer in p53 deficient mice without significant side effects [63] [64]PI3K and AKT chemical inhibitors reduce TRF1 telomeric foci and lead to increased telomeric DNA damage and fragility sinceTRF1 is a phosphorylation target of AKT and these modifications regulate TRF1 protein stability and TRF1 binding to telomeric DNA [65][66]. In addition, TRF1 was found to be phosphorylated by multiple kinases involved in cell signaling pathway (ERK2, bRaf, mTOR) and consequently it is targeted by specific kinase inhibitors from an FDA approved library. [67]

4.2.2 TRF2 has been implicated in several cancer related pathways such as immune escape[68][69] and angiogenesis control through different mechanisms[44][70]. Since TRF2 is overexpressed in different human cancer types and in some circumstances high levels correlate with drug resistance, it has been widely proposed as target for cancer therapy. Targeting strategies currently under development consist in peptides disrupting TRF2 protein-protein interactions. APOD peptide, has been designed to mimic the TRF2 interacting domain of the exonuclease Apollo. This peptide has been shown to induce DDR and cell death, inhibiting TRF2 mediated recruitment of enzymes necessary for DNA metabolism[71]. On the basis of this first evidence, other authors reported the development of cyclic peptides with the same target[72][73]**.** TRF2 is known to undergo a series of post-translational modifications that regulate protein stability such as phosphorylation, SUMOylation, acetylation, deacetylation, ubiquitination, and Poly (ADP-ribose)ylation. [74] [75][76][77] [78] [79] This mechanistical insights into TRF2 protein stability regulation, provide the basis for indirect targeting of TRF2. SIRT6 deacetylases, for example, is known to stimulate TRF2 degradation, then its pharmacological activator could work as a TRF2 targeting agent. Extracellular signal-regulated kinases ERK1/2 regulate TRF2 phosphorylation and stability[80], consequently drugs interfering with ERK1/2 signalling could also exert a role in TRF2 targeting. A recent drug screening revealed that at least two FDA-approved compounds (AR-A014418 and alexidine·2HCl, an inhibitor of Wnt pathway and a mitochondria-targeting agent respectively) are able to induce anti-proliferative effects by downregulating TRF2 and suppressing its pro-angiogenic and immunoescaping effects. [81]While the first compound presumably exerts its effect by acting on TRF2 promoter (which is regulated by the Wnt pathway [82]), the second one acts with unexplained mechanisms. In addition, Curcusone C, another small molecule with anti-cancer property, binds to TRF2 and block its binding to DNA inducing a DDR and cell death.[83] Finally,

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chemotherapeutic drugs such as Arsencic trioxide (As2O3), clinically employed in the treatment of acute promyelocytic leukemia (APL), or the TopoI camptothecin, were shown to downregulate TRF2 levels.[84][85]

4.2.3 POT1 is the most recurrently mutated gene of shelterin complex in cancer. Indeed, mutations affecting the interaction domains of POT1 to the ssDNA or TPP1 are associated with multiple types of human malignancies such as glioma, familial melanoma, mantle cell lymphoma, chronic lymphocytic leukemia and cardiac angiosarcoma. [36]In addition, it is frequently upregulated in therapeutic and radiation-resistant cell lines. This makes it an attractive target as therapeutic intervention against POT1-related cancers. The first attempt to identify specific POT1 inhibitors comes from a high-throughput time-resolved fluorescence resonance energy transfer (TR-FRET) screen for agents hampering POT1/ssDNA interaction. The yielded compound, the bis-azo dye Congo red (CR), was able to competitively inhibit POT1 binding to telomeric DNA in vitro. [86]Recently, a virtual high-throughput screening (vHTS), designed against a ZINC library, has led to the identification of two selected natural compounds as promising inhibitors of POT1, which deserve to be further exploited as lead to develop potent and selective molecules against POT1. [87]

5.3 TERRA

TERRAs have an established role in telomere protection and genome integrity (recently revised in [88]). Growing evidence not surprisingly attributes an anti-cancer role to TERRA [45][46][89], however its targeting could be detrimental especially for ALT cancer cells[90]. TERRA is prone to fold into G-quadruplex structures that can be bound and stabilized influencing intracellular TERRA levels and localization. TERRA transcription was shown to be affected directly by acridine derivatives [91] and indirectly by Quindoline derivatives acting on TRF2 levels which in turn regulates TERRA expression [92]. TERRA stabilization could also occur as a consequence of sequestering from degradation upon direct binding by G-quadruplex ligands [90].

Agent	Telomeric targets	Mechanism	Synergism/synthetic lethality	Anti-cancer effect	Ref.
RHPS4 and derivatives	G-quadruplex i-motifs	TRF2 POT1 delocalization Replication perturbation DDR activation	Camptotecins PARPi Ionizing radiations	ALT cells Glioblastoma Colorectal cancer	[93][94] [95][96] [97][98] [99]
BRACO19	G-quadruplex i-motifs	T-loop disassembly POT1 downregulation DDR activation	Cis platinum	Lung cancer Breast cancer Glioblastoma	[100][101] [102]

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Telomestatin	G-quadruplex	POT1 and TRF2 displacement G-overhang loss DDR activation	Imatinib Vincristin Ionizing radiations	Glioma Neuroblastoma Sarcoma ALT cells Leukemia	[103][104] [105][105] [106][107] [108]
Naphtalene diimmides	G-quadruplex	DDR activation	Ionizing radiations	Glioma Pancreatic cancer	[109][110] [111][112] [113]
Pyridostatin	G-quadruplex i-motifs	DDR activation	BRCA1/2 mut	Colon cancer Renal cancer	[114][53]
Perilene coronene derivatives	G-quadruplex	DDR activation	N.A.	Colorectal cancer	[115][116]
AKT inhibitors	TRF1	TRF1 downregulation	N.A.	Glioblastoma	[65][67]
APO D41 peptides	TRF2	DDR activation	N.A.	N.A.	[71]
Curcusone C	TRF2	DDR induction	N.A.	Ovarian cancer Endometrial cancer	[83]
Quindoline derivatives	G-quadruplex	TRF2 delocalization TERRA downregulation	N.A.	N.A.	[117]
BPBA	G-quadruplex	TERRA stabilization	N.A.	ALT cells	[90]

Table 1. Summary of compounds with *in-cellulo* assessed telomere-targeting activity and mechanism of action.

6. Conclusions

Telomeres are nucleoprotein complexes involved in genome stability, cell proliferation, cancer predisposition and aging. Their homeostasis is subjected to a complex interaction network including multiple regulation levels. Beside telomerase inhibition and consequent telomere attrition, telomere targeting gather different approaches directed toward DNA, RNA, and proteins. Telomere's dysfunction, intended as DNA damage response activated at telomeres, can trigger genetic instability and cell death in tumor cells, giving the rational for pursue investigating in telomere targeting approaches in cancer as single agents and in rational based combinations with standard therapeutics.

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