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Review

Targeting critical steps of cancer metastasis and recurrence using telomerase template antagonists

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ABSTRACT

Metastasis, tumor relapse, and drug resistance remain major obstacles in the treatment of cancer. Therefore, more research on the mechanisms of these processes in disease is warranted for improved treatment options. Recent evidence suggests that the capability to sustain tumor growth and metastasis resides in a subpopulation of cells, termed cancer stem cells or tumor-initiating cells. Continuous proliferation and self-renewal are characteristics of stem/progenitor cells. Telomerase and the maintenance of telomeres are key players in the ability of stem and cancer cells to bypass senescence and be immortal. Therefore, telomerase inhibitors have the therapeutic potential for reducing tumor relapse by targeting cancer stem cells and other processes involved in metastasis. Herein we review the role of telomerase in the immortal phenotype of cancer and cancer stem cells, targeting telomerase in cancer, and discuss other opportunities for telomerase inhibitors to target critical steps in cancer metastasis and recurrence.

1. Introduction

In 2000, Hanahan and Weinberg listed “hallmarks” of cancer that must be considered in order to understand the underlying determinants of carcinogenesis: a) self-sufficiency in growth signals; b) insensitivity to growth-inhibitory (anti-growth) signals; c) evasion of programmed cell death (apoptosis); d) limitless replicative potential; e) sustained angiogenesis; and f) tissue invasion and metastasis [1]. Advances in diagnostics and treatment have significantly reduced mortality from primary cancers by successfully hitting some of the cancer hallmarks. Unfortunately, most deaths from cancer are due to metastases and tumor relapse in which the tumors display resistance to standard therapies [2]. Within the last few decades, an expansion of research on cancer metastasis, angiogenesis, and replicative potential has led to increased understanding of these processes and new developments in therapeutic targeting and potential biomarkers.

Metastasis is the ability of the cancer to spread from its origin to distant locations within the body and continue to grow [reviewed in 2]. Metastasis depends on the cancer cells acquiring increased motility and invasiveness. More specifically, the main steps in metastasis include: proliferation and angiogenesis; detachment and local invasion into the stroma; detachment and circulation of tumor cells; adherence of tumor cells at secondary sites (on capillary endothelial cells or a basement membrane); extravasation; and, proliferation/survival and angiogenesis at distant sites. As described in more detail later in this review, angiogenesis is an early step in the formation of a metastasis in which extensive vascularization must occur to allow tumors to feed and grow [2]. Several growth factors as well as the endothelial cell population play key roles in sustaining angiogenesis and therefore can be subjected to targeting by therapeutics.

The hallmark of limitless replicative potential in cells is driven by the maintenance of telomeres. Telomeres are hexameric DNA sequences (TTAGGG) found at the ends of chromosomes to prevent end to end fusions and help to maintain genomic stability [3]. The telomere is also a complex structure consisting of a 3′ G-rich overhang, which wraps around to form a protective telomere loop (t-loop), and its associated capping proteins, collectively termed the telosome or shelterin [3–6]. Telomeres in the majority of normal human cells shorten with each cellular division due to the “end-replication problem.” Since DNA polymerase can only initiate

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synthesis in the 5′→3′ direction with a preformed primer, it cannot fully synthesize the 3′ terminal end of the lagging strand of linear DNA (which is based on a series of Okazaki fragments). The result is an end-replication problem in which terminal DNA is lost with each round of division. Once telomeres have shortened to a certain length, cells with intact cellular checkpoint controls (e.g., p53/Rb) undergo replicative senescence [reviewed in 3,4,7]. Cells that have lost critical checkpoint controls can bypass senescence and their telomeres continue to shorten. During this crisis period of cell proliferation and death due to short telomeres, cell death usually prevails which can limit cellular lifespan. In rare instances, the presence of critically short or dysfunctional telomeres can result in end fusions and genomic instability, which can lead to a cell acquiring mutations and the ability to progress towards cancer.

Cellular senescence is the ultimate and irreversible loss of replicative capacity occurring in primary somatic cell culture. The shortening of telomeres is thought to function as a replicometer that counts the finite number of cell divisions and triggers replicative senescence in normal diploid cells [8]. In the majority of human malignancies, maintenance of telomeres is achieved by reactivation of telomerase, an enzyme that counteracts telomere shortening, overcomes senescence and leads to cell immortalization [9]. A smaller fraction of tumors (10–15%) use an alternative recombination-based mechanism termed ALT (alternative telomere lengthening) pathway [10]. Although the mechanisms involved in the decision of a cell to activate either telomerase or ALT are unknown at present, the ALT pathway is more commonly activated in tumors of mesenchymal origin and the presence of the ALT phenotype is associated with poor prognosis [11].

The activity of telomerase to synthesize and maintain telomeres is reliant on the expression of both the RNA component (hTR) and the catalytic subunit (hTERT), the regulation of which is tightly coordinated on multiple levels by transcriptional, post-transcriptional, translational, and epigenetic mechanisms [12,13]. During early embryonic development, hTERT is transcriptionally repressed and subsequently most human somatic cells have undetectable telomerase activity. However, germline cells, stem cells, and progenitor cells exhibit telomerase activity [14]. Ectopic expression of hTERT in normal human cells leads to extension of life-span or immortalization of many cell types [15]. Additionally, constitutive expression of TERT in cancer-resistant mice, engineered by enhanced expression of the tumor suppressors p53, p16, and p19ARF, produces a systemic delay in aging accompanied by extension of the median life span [16]. In summary, telomerase plays a critical role in the immortal phenotype of cells that have indefinite proliferative potential, such as stem/progenitor cells (including the putative cancer stem cells) and cancer cells.

This review focuses on the targeting of telomerase and the immortal phenotype in cancer using telomerase template antagonists. One such antagonist, GRN163L, has been shown to act as a potent telomerase inhibitor with additional properties that may affect cancer cell adhesion, new vessel formation, metastasis, and drug resistance. Furthermore, telomerase has been implicated to possess extracurricular activities in replicative aging and cell survival beyond telomere maintenance [14]. Telomerase may be directly or indirectly involved in apoptosis (via mitochondria homeostasis or membrane potential), DNA damage response, stem cell and organism fitness, and regulation of chromatin architecture or gene expression (including genes involved in cell signaling, cell cycle, metabolism, cell death/differentiation, and growth factors) [14]. Therefore, while one should caution the use of telomerase as a universal and safe target for cancer therapy if normal cells may be affected, there may be increased therapeutic potential for telomerase antagonists in the fight against cancer metastasis and recurrence. Based on emerging data, we discuss the potential of telomerase antagonists for targeting cancer stem cells, hypothesized to play a major role in tumor recurrence and metastasis, as well as targeting angiogenesis, a critical step in metastasis.

2. Targeting telomerase and the immortal phenotype in cancer using telomerase template antagonists

Telomerase and the maintenance of telomeres are key players in the ability of cells to bypass senescence and be immortal. Preclinical and clinical evidence have supported the rationale of targeting telomerase as a novel form of cancer therapy [reviewed in 17]. The majority of cancer cells have increased hTERT expression, telomerase activity, and shorter telomere lengths compared to neighboring tissue and most normal cells. The telomerase complex offers several opportunities for targeting, such as genetic manipulation of expression, template antagonism or direct enzymatic inhibition [18,19]. One such target is the telomerase RNA component, hTR, which is required for the RNA-dependent reverse transcriptase function of hTERT. The steps involved in synthesizing telomeres by telomerase include: 1) the binding of the short C-rich template sequence region of hTR to the G-rich telomeric strand by Watson–Crick base pairing; 2) elongation of the telomere by hTERT, in which six nucleotides (GGTTAG) are added sequentially, driven by the template sequence of hTR; and, 3) translocation of the telomere-template heteroduplex to position it for another round of elongation (processive addition). This process can be disrupted when the extended telomere is dissociated from telomerase [17]. The hTR is not translated into peptides; therefore, oligonucleotides complementary to the hTR template region block the active site of hTERT reverse transcriptase and act as competitive enzyme inhibitors (or template antagonists). Furthermore, since hTERT may have other functions in cell survival independent of its activity [14], targeting hTR and designing direct enzymatic inhibitors will maintain hTERT expression in the cell. Since 1995, many laboratories have shown, through extensive research, the proof of principle that targeting hTR results in the inhibition of telomerase activity, progressive telomere shortening, and subsequent death of immortalized or cancer cells in vitro [reviewed in 17–19]. Preclinical animal studies have also demonstrated that telomerase template antagonists are effective in reducing the primary growth in a variety of cancers (lung, prostate, breast, ovarian, glioblastoma, leukemia, lymphoma, myeloma, for example). Furthermore, several studies suggest that telomerase template antagonists can reduce the metastasis index in animal models [20–22]. However, the mechanisms for the reduction in metastases are not completely understood. The direct telomere-shortening effects of GRN163L on the tumor cells themselves might be sufficient to explain these effects on metastasis as massive numbers of cell divisions are required for metastases to form from single invading cells. On the other hand, reduction in cell growth due to targeting the shortest telomere in a cell may result in a bystander effect on the population, or potential telomere-independent effects by the telomerase template antagonists.

Several methods for targeting the hTR to inhibit telomerase activity have been tested [18]. These include antisense oligonucleotides, peptide nucleic acids (PNAs) and chemically modified PNAs, 2′-*O*-methyl RNA (2′-*O*-MeRNA) and 2′-*O*-methoxyethyl RNA (MOE RNA), phosphorothioate (PS), N3′-P5′ phosphoramidate (NP), and N3′-P5′ thio-phosphoramidate (NPS) DNA. Further development of NPS oligonucleotides has led to the first telomerase template antagonist, GRN163L, to reach clinical trials.

2.1. GRN163L: a potent telomerase template antagonist with additional properties?

The telomerase inhibitor GRN163L is a 13-mer oligonucleotide N3′→P5′-thio-phosphoramidate lipid conjugate and represents the latest generation of modified oligonucleotides targeting hTR [23].

The molecule contains two functionally important domains, which are an oligonucleotide thio-phosphoramidate and a lipid group. These moieties are connected via a short amino glycerol linker (Fig. 1). The oligonucleotide portion of the conjugate has the nucleoside sequence 5′-TAGGGTTAGACAA-3′, which is complementary to the template region of hTR and designated as GRN163. The 3′-terminal adenosine contains a 3′-amino group (estimated pKa value of ~9), which should be mainly protonated under physiological conditions at pH 7.4. All the composing nucleosides contain 3′-amino groups and the non-bridging oxygen atom of the internucleoside phosphoramidate monoester groups uniformly substituted with a sulfur atom (see chemical structure in Fig. 2B). Hence, all the internucleoside linkages of this molecule are N3′→P5′-thio-phosphoramidates. This is one of the key distinguishing features of GRN163L compared to other oligonucleotides, containing either natural phosphodiester, or phosphorothioate, or N3′→P5′ phosphoramidate internucleoside groups — see Figs. 2C, D and A respectively, and the text below. GRN163L also contains one phosphorothioate diester group tethering the lipid amino glycerol and the oligonucleotide domains (see chemical structure in Fig. 1). All the internucleoside thio-phosphoramidate as well as the linking phosphorothioate groups are ionized and negatively charged under physiological conditions. GRN163L is currently synthesized and used as all-sodium salt with molecular weight of 4895 g/mol, and 4609 g/mol for the all-acid form. The compound is also well-soluble either in water or in saline solution, making this compound translatable to the clinic.

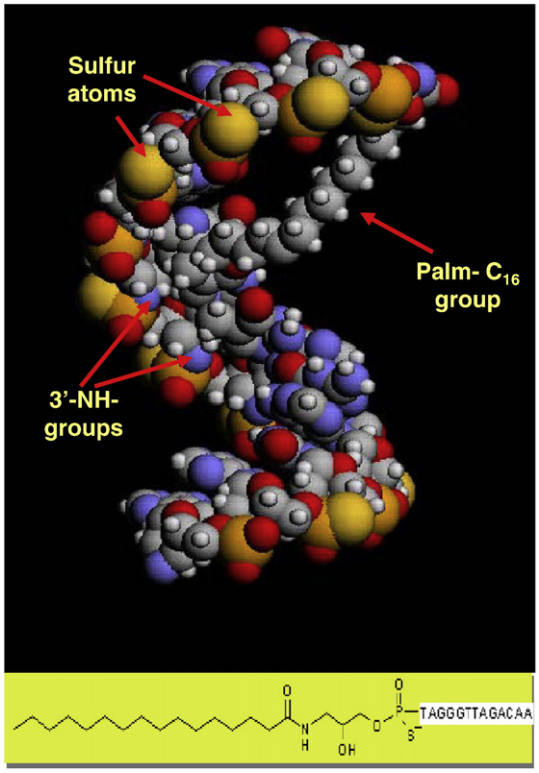

Fig. 1. Chemical structure and functionally important features of GRN163L. Space filling molecular model and chemical structure of the telomerase inhibitor oligonucleotide N3′→P5′-thio-phosphoramidate GRN163L with the nucleoside sequence 5′-TAGGGTTAGACAA-3′. All internucleoside linkages are 3′-NH-P(O)(S-)-O-5′-thio-phosphoramidate monoesters, and the oligonucleotide contains a 3′-terminal amino group (-3′-NH_2). All sulfur (=S) atoms are in yellow; the 3′-amino (-NH-) groups of the thio-phosphoramidate linkages are in blue; the palmitoyl (C_{16}) lipid group is attached to the 5′-terminus via 3-amino glycerol linker (in the molecular model the palmitoyl group is folded back towards the oligonucleotide bases).

The presence of thio-phosphoramidate internucleoside linkages in GRN163L provides for high thermodynamic stability of its complex with the targeted telomerase hTR [24–26]. Thus, the melting temperature (T_m) of a duplex formed by GRN163L and a complementary RNA strand is 69.5 °C under close to physiological conditions, in PBS. For comparison, T_m of the duplexes formed by either iso-sequential phosphodiester DNA (Fig. 2C) or phosphorothioate (Fig. 2D) 13-mer oligonucleotides is 45.2 °C or 40.5 °C, correspondingly. Moreover, thio-phosphoramidate linkages are very resistant to cellular nuclease hydrolysis, providing for a long half-life of the intact GRN163L in vitro and in vivo. At the same time, in cell-free assays, an N3′→P5′ phosphoramidate oligonucleotide is approximately 5–10 times less potent of a telomerase inhibitor than its iso-sequential thio-phosphoramidate counterpart, GRN163 (compare Fig. 2A, B). This difference in activity was attributed to possible secondary (to Watson–Crick type hydrogen bonding) interactions between sulfur atoms of the sugar-phosphate backbone and the hTERT subunit of telomerase [24–26]. Importantly, the presence of the covalently conjugated 5′-palmitic lipid group provides for better cellular uptake and higher bio-availability of GRN163L vs. GRN163 molecules. In the majority of cancer cell lined tested, IC_{50} values for telomerase inhibition were at least 5–10 times lower for GRN163L than that for GRN163. A similar trend was also observed in various in vivo animal model systems, where GRN163L demonstrated high anti-telomerase activity [17,23] for review].

It has been extensively reported that G-quadruplex forming oligonucleotides (either with phosphodiester or phosphorothioate linkages) can efficiently bind to various structurally important intra- and extra-cellular proteins and thus alter their functions [reviewed in 27]. These interactions are determined, in part, by the presence of G-quadruplex structures, which may be recognized by protein ligands as structural pharmacophore groups. For example, a phosphorothioate octamer d-(TTGGGGTT), designated as ISIS 5320 (ISIS Pharmaceuticals Inc.) forms an all-strands parallel G-quadruplex structure, which efficiently binds to the HIV envelope glycoprotein gp120, and thus inhibits both cell-to-cell and virus-to-cell infections [28]. Our preliminary physico-chemical data indicate that, in addition to the formation of thermodynamically stable and sequence-specific duplexes with hTR, GRN163L is capable of forming rather stable G-quadruplex complexes under close to physiological conditions. This was determined using thermal dissociation experiments, where the change in the UV-absorption spectra was monitored at a 295 nm wavelength, which is characteristic for dissociation-association transitions of guanine quartets (Fig. 3). Moreover, the thermal dissociation experiments were conducted in practically iso-molar sodium (NaCl in PBS) and potassium (0.15 M KCl) containing buffer solutions, where strong G-quadruplex stabilizing effects of potassium ions were observed. The melting temperatures of the G-quadruplexes formed by GRN163L are 63.9 °C or 89.7 °C in the presence of sodium or potassium ions, respectively (Fig. 3). The parental iso-sequential oligonucleotide, GRN163, which lacks the 5′-lipid group, forms noticeably less stable G-quadruplexes, with T_m of 49.7 °C and 74.5 °C, respectively, under similar experimental conditions. GRN163L does not form stable G-quadruplex complexes in low ionic strength solutions, i.e., in 10 mM sodium phosphate buffer, pH 7.4, or in de-ionized water (Fig. 3).

It is estimated that the special arrangement of the G-quadruplex formed by GRN163L is structurally similar to that formed by ISIS5320, with all four strands being aligned in parallel orientation. This arrangement, we believe, is the most thermodynamically stable oligonucleotide strand orientation, due to potential lipid-lipid stabilizing hydrophobic interactions. High resolution NMR or X-ray structural data is currently not yet available, in part due to the difficulties with crystallization of GRN163L. Formation of this G-quadruplex is driven by the presence of the GGG-motif close to the 5′-terminus in the molecule. The 5′-C_{16} palmate lipid groups further

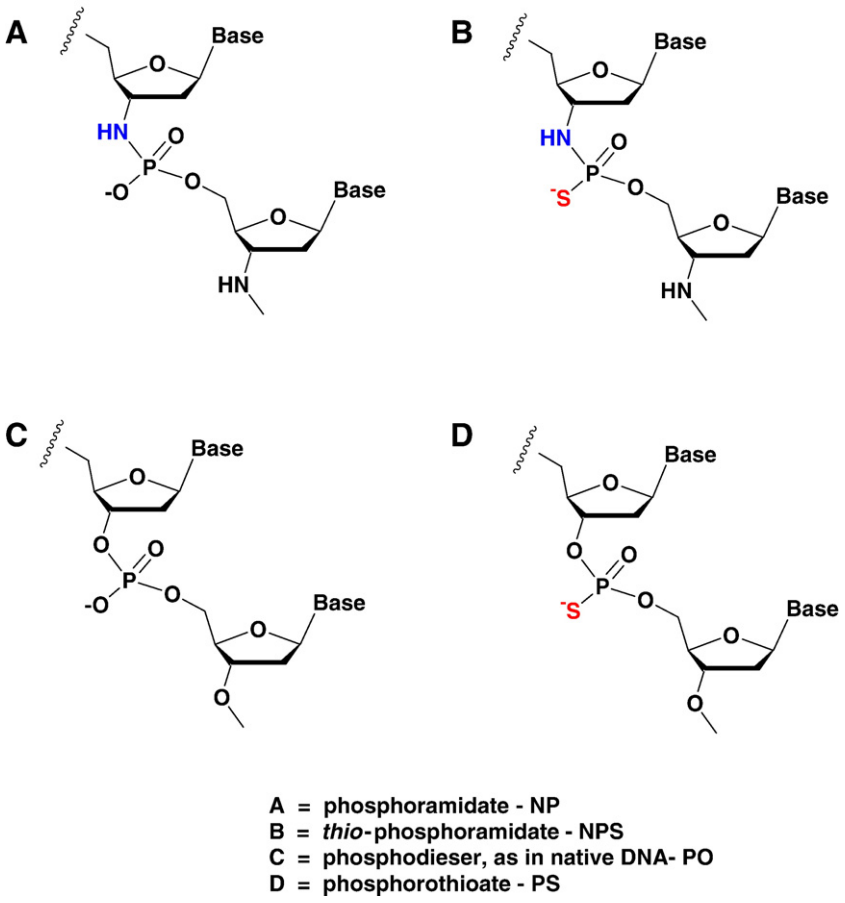

Fig. 2. General chemical structure of internucleoside linkages for representative synthetic oligonucleotides evaluated as telomerase substrates and inhibitors. (A) internucleoside N3′→P5′ phosphoramidate group, designated as NP; (B) internucleoside N3′→P5′ thio-phosphoramidate group, designated as NPS; (C) native DNA phosphodiester group, designated as PO; (D) modified DNA phosphorothioate group, designated as PS. Replacement of the bridging 3′-oxygen atom in native nucleosides by 3′-amino group results in the formation of 3′-amino-phosphoramidates (NP's); additional replacement of non-bridging oxygen atom by sulfur results in the formation of thio-phosphoramidates (NPS's). NP- and NPS-oligonucleotides are stable to cellular nucleases, and they form thermodynamically stable duplexes with complementary RNA strands, including the telomerase hTR domain.

stabilizes this four-stranded complex via hydrophobic interactions. A similar type of DNA duplex and triplex stabilizing effects via lipophylic interactions was previously reported [29]. The formed duplex with hTR is apparently more thermodynamically stable than the G-quadruplex structure of GRN163L alone.

Additionally, there is an apparent correlation between G-quadruplex forming ability of oligonucleotide phosphoramidates and their effects on cellular morphology and cell adhesion properties. GRN163L has been shown to be capable of inducing a rounding-effect on cellular morphology and prevent cell attachment to a substrate for various cell lines in a telomerase activity-independent manner [22,30]. However, its mismatch control compound (5′-Palm-TAGG***TG***TA***AG***CAA, mismatch sequences in italics), having the same nucleoside composition, but lacking the GGG-motif (and therefore unable to form stable G-quadruplex, with T_m of 29.2 °C in PBS), does not affect the cell adhesion and morphology properties under similar experimental conditions. GRN163L was observed to not have an effect on cell growth, morphology, or adhesion in normal fibroblasts, nonmalignant cell lines, or normal tissues in animal models [17] and relevant references therein, [22,30].

2.2. Status of telomerase template antagonists in clinical trials

GRN163L is the most advanced direct enzymatic telomerase inhibitor that has reached cancer therapeutic clinical trials [17]. As described earlier, the chemistry of GRN163L allows its potential to be transitioned to the clinic. GRN163L exhibits a higher melting temperature, formation of stable duplexes with single-stranded RNA, resistance to degradation and drug efflux mechanisms (drug resistance), and higher affinity and specificity for its target. GRN163L entered clinical trials for cancer patients in 2005 and currently there are six trials for multiple tumor types (chronic lymphocytic leukemia, multiple myeloma, lung, breast, and advanced solid tumors). Correlative studies include investigating the number of circulating tumor cells, which may constitute the "seeds" for metastasis, after treatment with GRN163L. Recently, GRN163L entered a Phase I/II clinical trial for patients with locally recurrent or metastatic breast cancer (Trial ID: NCT00732056, www.clinicaltrials.gov). This trial is in combination with paclitaxel, a mitotic inhibitor, and bevacizumab, an anti-angiogenic agent and monoclonal antibody against vascular endothelial growth factor (VEGF). Important to note is that these early stage trials do not address in depth the combination effects of GRN163L with other forms of therapy; therefore, the future design of combination trials is eagerly anticipated. The use of anti-telomerase agents in clinical trials is still in its earliest stages for complete analyses of outcomes, efficacy, tolerance, and toxicity on normal tissues [31].

3. New therapeutic potential for telomerase antagonists: targeting critical steps in cancer metastasis and recurrence

3.1. Targeting telomerase in cancer stem cells: a potential target for tumor recurrence and metastasis

Recent evidence has suggested that the capability to sustain tumor growth and metastasis may reside in a small number of cells, termed

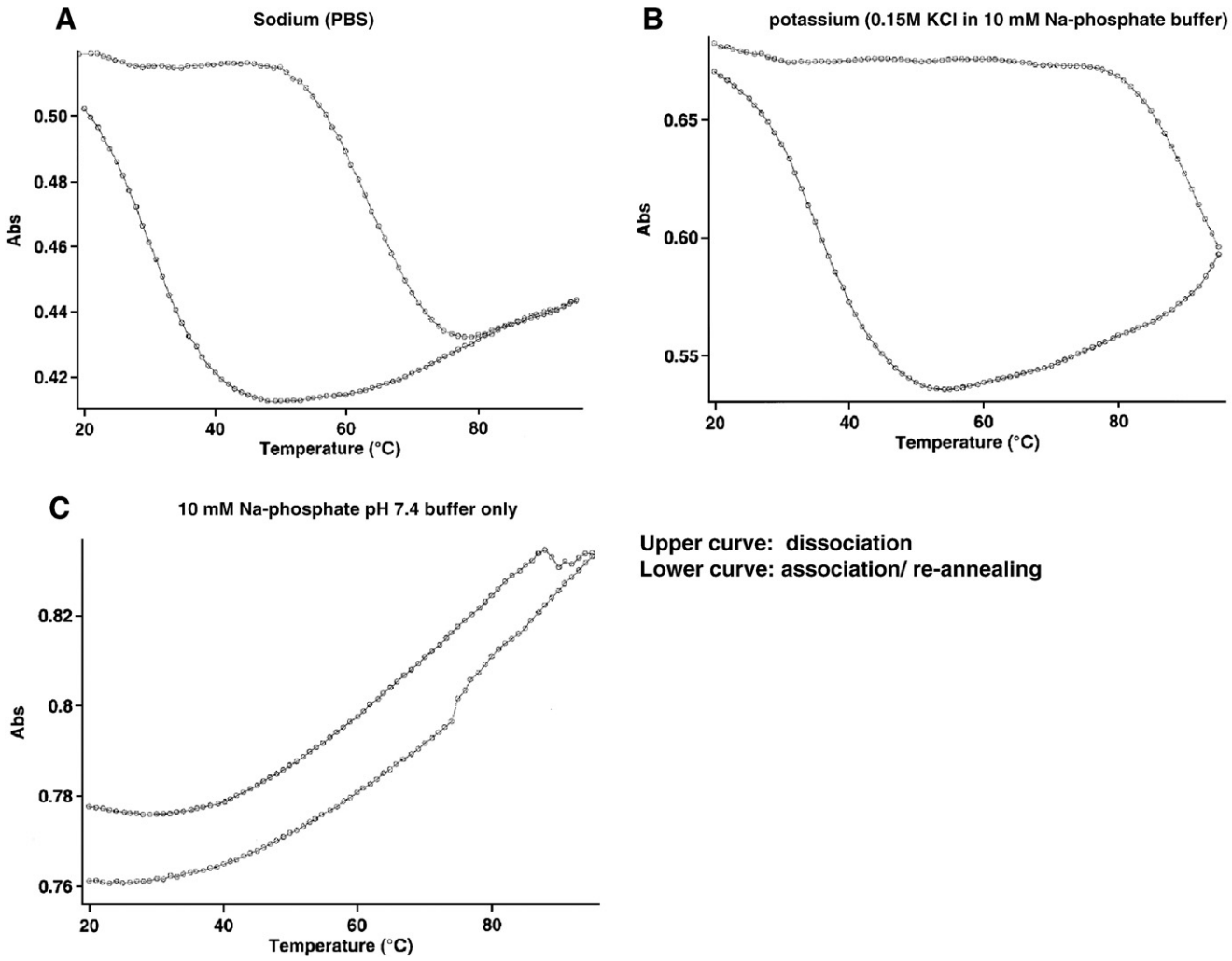

Fig. 3. Thermal dissociation and association curves for G-quadruplex formed by GRN163L. Thermal dissociation experiments were conducted using a Cary 1E spectrophotometer. Change in UV absorbance was recorded at 295 nm. A temperature gradient of 1 °C/min was used for to acquire both dissociation (upper curve) and association, or re-annealing (lower curve), where a significant lagging effect for the re-annealing process was observed (which is characteristic for a relatively slow *tetra-molecular* reaction of G-quadruplex formation). Panels A - C correspond to the dissociation-association experiments conducted in sodium (PBS), potassium (0.15 M KCl in 10 mM Na-phosphate buffer) and 10 mM Na-phosphate pH 7.4 buffer only, respectively.

cancer stem cells or tumor-initiating cells [32–34]. The cancer stem cell population is proposed to be highly tumorigenic, to contain self-renewal properties, to differentiate to give rise to a diverse progeny and a heterogeneous tumor, to possess efficient efflux mechanisms for drug resistance, and to undergo continuous proliferation. Cancer stem cells have been recently identified in several cancers based on the expression of stem cell-like cell surface markers as has been historically utilized for hematological cell/cancer cell types [35–37]. Continuous proliferation and self-renewal upon mitogen stimulation, in addition to telomerase activity, are also characteristics of normal stem/progenitor cells. As a putative stem/progenitor cell population with similar characteristics for indefinite replicative potential, cancer stem cells should be subjected to similar telomere maintenance and telomerase regulation as other stem/progenitor cells. Interestingly, while it has been accepted that stem/progenitor cells express telomerase activity, stem/progenitor cells actually exhibit gradual telomere shortening during their life-span in culture. So while the expression of telomerase in these cells may not be sufficient to prevent telomere shortening, the expression of low levels of telomerase activity in these cells may permit their continuous cell turnover and overall survival or function [14]. On the other hand, recent evidence has shown that the adult stem cell compartment is enriched with the longest telomeres of a tissue [38]. Consistent with in vitro data, the telomeres were observed to shorten during age in the normal adult stem cell compartment and this coincides with a decrease in stem cell function during aging. Recent findings with breast cancer cells suggest that isolated side populations of $CD44^{+}/CD24^{-/low}$ cells, which show similar characteristics and cell surface markers of stem/progenitor cells, also exhibit telomerase activity and short telomere lengths similar to the parental cancer cell line [37]. These results suggest that the stem cell-like tumor initiating cells may be prime targets for telomerase inhibitor treatment as part of the therapeutic regimen for cancer.

Li et al. have shown that the intrinsic resistance to chemotherapy in breast cancer patients, which can result in tumor relapse, resides in a small subpopulation of cancer stem cells [39]. Since a characteristic of stem cells is efflux (such as in the efflux of the Hoechst dye) and drug resistance [39,40], it is imperative to develop novel therapy that can effectively enter these cancer stem cells and reduce tumor re-growth. Therefore, it will be important to determine whether oligonucleotide telomerase template antagonists, such as GRN163L, can be effectively taken up by the putative cancer stem cells. Emerging evidence suggests

that GRN163L can target drug-resistant cancer cells and restore sensitivity to therapy that may be due to both telomere-dependent and independent mechanisms [21,41]. Goldblatt et al. have shown that GRN163L can effectively inhibit telomerase in trastuzumab-resistant breast cancer cells, reduce cell growth in vitro, and restore sensitivity to these breast cancer cells to trastuzumab [41]. Therefore, telomerase inhibitors have the potential to target telomerase, telomere maintenance, drug resistance, and the replicative potential of cancer stem cells for effective cancer therapy.

3.2. Potential for telomerase antagonists to target angiogenesis

Angiogenesis is required for invasive tumor growth and metastasis and constitutes an important point in the control of cancer progression. During tumor progression and metastasis, environmental and genetic changes induce an "angiogenic switch" through upregulation of angiogenic factors or downregulation of angiogenesis inhibitors. Frequently, overexpression of angiogenic factors, such as vascular endothelial growth factor (VEGF), allows tumors to make the angiogenic switch. Anti-angiogenic therapy targets proliferating endothelial cells instead of cancer cells and may reverse this "angiogenic switch" preventing growth of tumor vasculature [42,43]. The correlation between increased angiogenesis and poor outcome has been established in many types of tumors such as breast cancer, renal cancer, prostate cancer, colon cancer and melanoma [43,44].

Telomerase, which is highly expressed in advanced stages of most cancers, may also contribute to tumor progression through still undefined cell growth-promoting functions [14]. Vascular endothelial cells were observed to express hTERT mRNA by in situ hybridization on human astrocytic tumor sections [45]. Additionally, a significant correlation was observed between the level of hTERT mRNA expression and the proliferation rate of the endothelial cells within the tumor vasculature as well as the histological grade of the tumor. Since hTERT expression by vascular endothelial cells is absent in non-neoplastic granulation tissues and in normal brain, it is possible that diffusible angiogenic factor(s) released by the tumor might act on the endothelial cells [45,46]. When telomerase activity was inhibited in glioblastoma cell lines via retroviral transduction of an small-interfering RNA (siRNA) targeting hTERT mRNA, decreased cell proliferation, increased apoptosis, and reduced angiogenesis was observed in the hTERT-siRNA expressing xenograft tumors compared with controls [47]. Furthermore, telomerase expression has been shown to correlate with VEGF expression in breast carcinomas [48]. The increase in hTERT, hTR and VEGF expression during malignancy may also be attributed to hypoxia stimulation of transcription [49]. Zaccagnini et al. showed that telomerase activation is essential for the VEGF dependent vascularization of ischemic tissue [50]. Adenovirus-mediated transfer of the wild-type hTERT gene in ischemic rats induced development of new capillaries whereas delivery of a dominant negative mutant of TERT failed to promote capillarogenesis. Additionally, the inhibition of telomerase by 3′-azido-3′-deoxythymidine (AZT) in VEGF-treated endothelial cells strongly reduced capillary formation and failed to prevent apoptosis [50]. Similarly, when Falchetti et al. inhibited telomerase activity of human endothelial cells, either by RNA interference (RNAi) or by a dominant negative allele of hTERT, tubule formation was abolished and survival of the endothelial cells in tumor xenografts were significantly reduced [51].

All these findings suggest a contribution of telomerase activity to angiogenesis. The expression of telomerase by tumor vascular cells and endothelial progenitor cell proliferation appears to be an early event during the tumor progression. In this view, anti-telomerase therapy would be effective against new vessel formation by decreasing proliferation of the endothelial progenitor cells in addition to inhibiting neoplastic cell proliferation. It has been demonstrated that telomerase-deficient (Terc−/−) mice with critically short telomeres show a striking impairment in angiogenesis. Therefore, cancer therapies based on eliciting telomere shortening (i.e., by telomerase inhibition) would contribute to tumor size reduction not only by a cytolytic effect but also by slowing or preventing angiogenesis [52]. Alternatively, the emerging evidence of GRN163L's additional properties as an anti-cancer agent, in addition to its effects as an anti-telomerase agent, may also play a role in this context. In support of this concept, the chorioallantoic membrane (CAM) assay can be used to determine the in vivo effects of GRN163L on blood vessel growth. The CAM assay is well established and widely used as an in vivo model to examine angiogenesis and anti-angiogenesis by topical administration of new study agents [53]. It is an alternative approach to small animal models and provides a natural environment of growing blood vessels [54,55]. Preliminary findings of the CAM assay data suggest that GRN163L at 5–10 mg/kg doses inhibits in vivo angiogenesis without a cytotoxic effect. As shown in Fig. 4, physiological angiogenesis was observed in the form of some allantoic vessels in

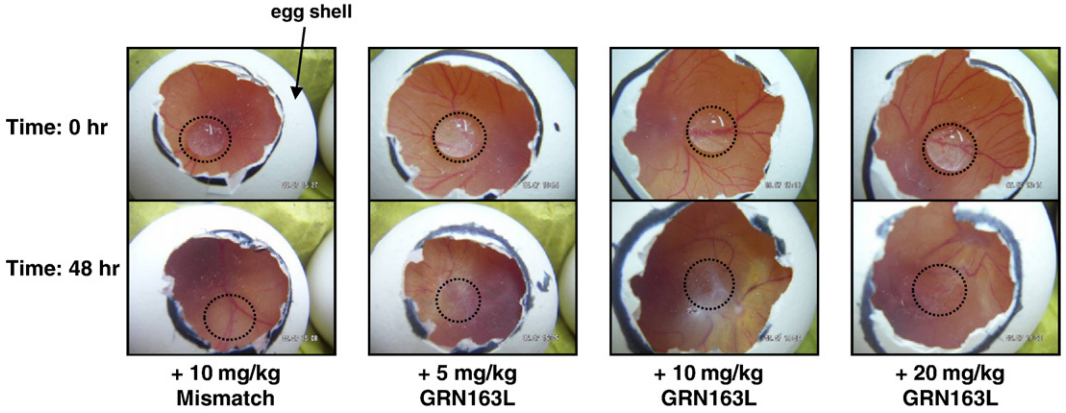

Fig. 4. The oligonucleotide telomerase template antagonist GRN163L has potential to reduce vascular development as demonstrated by the chorioallantoic membrane (CAM) assay. The anti-angiogenic effect of GRN163L on vascular development was evaluated using the CAM assay [53]. For the assay, Atak-S fertilized chicken eggs were obtained from Tavukculuk Institution (Ankara, Turkey) and incubated at 37 °C and 85–90% relative humidity throughout the experiment. On day six, the eggs were divided in 4 groups, each group consisting of six eggs, and a window was opened in each egg to allow subsequent access to the CAM. GRN163L and Mismatch control oligonucleotide solutions were prepared in PBS at 3 different concentrations (5 mg/kg, 10 mg/kg, and 20 mg/kg). GRN163L solution (100 μl) was placed on the surface of each CAM (dotted circle, time 0 h), whereas control CAMs received only mismatch solution, and then all eggs were sealed with transparent tape. Following 48 h, CAMs were screened and photographed with a digital camera (Konica Minolta, Tokyo, Japan). The dotted circle at 48 h indicates the placement of the solution at time 0 h.

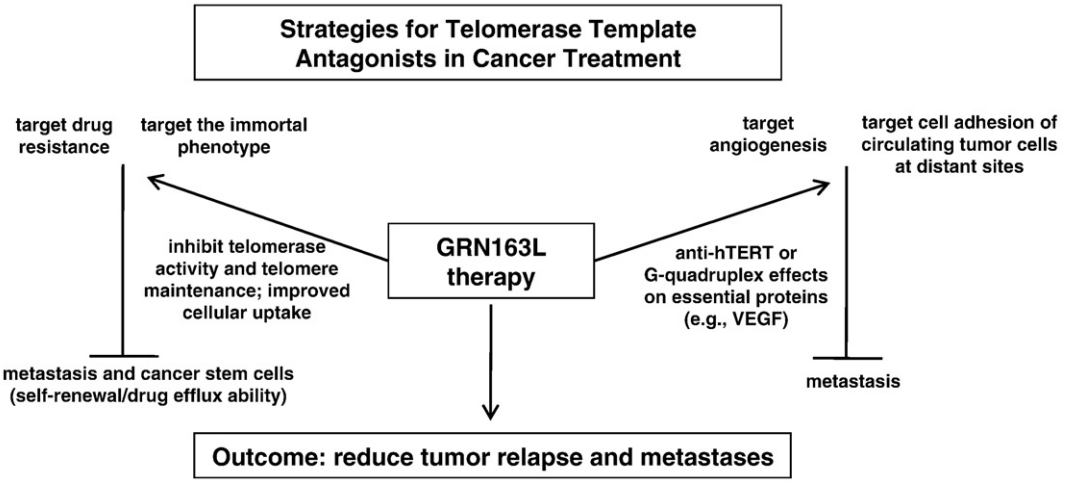

Fig. 5. Strategies for targeting metastasis and the immortal phenotype of cancer/cancer stem cells using telomerase template antagonists.

the mismatch control oligonucleotide-treated eggs, whereas a significant reduction in CAM area was appreciable macroscopically in the GRN163L treatment group after 48 h incubation. Although 5–10 mg/kg GRN163L was well tolerated in these eggs, higher doses (20 mg/kg) caused toxicity in 40% of the eggs. Considering these data and its role as a potent telomerase inhibitor, GRN163L may have therapeutic potential as an anti-angiogenic agent in the treatment of cancer; however, further animal studies are needed to show that GRN163L administration is safe and effective.

4. Conclusions and therapeutic perspectives

Although the main role of telomerase in aging and cancer has been well documented, accumulating evidence suggests that telomerase has important roles in angiogenesis, metastasis and cancer stem cells in addition to its classical function in telomere length maintenance. Continued research will identify the novel biochemical properties of telomerase which will provide additional insights in aging and cancer. GRN163L, as a potential G-quadruplex forming oligonucleotide, may have additional anti-cancer effects beyond its function as a telomerase inhibitor (i.e., anti-metastatic). Fig. 5 summarizes strategies for targeting metastasis and the immortal phenotype of cancer/cancer stem cells using telomerase template antagonists. As these agents, as well as other telomerase-based therapies, are currently in clinical trials, careful analysis of their effects on different tissues should shed light on their potential as therapy as well as insight into telomere/telomerase biology in normal, stem, and cancer cells.

Potential conflict of interest disclosures

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