ISSN 0006-2979, Biochemistry (Moscow), 2015, Vol. 80, No. 3, pp. 251-259. © Pleiades Publishing, Ltd., 2015.
Original Russian Text © M. I. Zvereva, T. S. Zatsepin, D. M. Azhibek, O. S. Shubernetskaya, O. V. Shpanchenko, O. A. Dontsova, 2015, published in Biokhimiya, 2015, Vol. 80, No. 3, pp. 309-319.

REVIEW

Oligonucleotide Inhibitors of Telomerase: Prospects for Anticancer Therapy and Diagnostics

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Received October 23, 2014
Revision received November 14, 2014

Abstract—The activity of telomerase allows eukaryotic cells to have unlimited division potential. On its functioning, telomerase synthesizes short DNA repeats at the 3′-end of DNA within chromosomes that ensures genome stability during cell division. Telomerase is active in the majority of cancer cell types and is virtually absent in somatic cells with rare exceptions. This difference allows us to consider inhibition of telomerase activity as a possible approach to antitumor therapy. Telomerase is a nucleoprotein composed of two main components: the reverse transcriptase (hTERT), which is a catalytic subunit, and telomerase RNA (hTR), which encodes a template for synthesis of repeats. The biogenesis and features of telomerase seem very promising for its inhibition due to complementary interactions. In this review, we analyze putative pathways of oligonucleotide influence on telomerase and consider the known native and modified oligonucleotide inhibitors of telomerase, as well as possible mechanisms of their action. We also discuss the application of telomerase-targeted oligonucleotide conjugates for *in vivo* imaging of tumor cells.

DOI: 10.1134/S0006297915030013

Key words: human telomerase, telomerase inhibitors, anticancer therapy target, oligonucleotide, tumor cell imaging

Telomerase is an RNA–protein complex that elongates 3′-protruding ends of chromosomal DNA by addition of telomeric repeats, which prevents shortening of chromosomes during replication and ensures unlimited potential for cell division [1]. It is known that telomerase is active in embryonic, stem, and sex cells, in some cells with a high proliferative potential [2], and, especially, in cancer cells [3]. Telomerase activity was earlier considered only as a factor that allowed researchers to distinguish cancer cells from normal ones because in the majority of cell types in malignant tumors the telomerase activity was significantly higher than in normal tissues. However, now it is known that the role of telomerase in the cell is not limited to compensation of the replicative shortening of telomeres associated with cell aging, but the catalytic subunit of telomerase protects mitochondrial DNA from damage [4]. Thus, telomerase is a promising target for anticancer therapy.

On the other hand, a short-term activation of telomerase can be considered as a possibility to withstand cell aging [5], and therefore the search for activators and regulators of telomerase activity is a similarly promising problem as the search for its inhibitors. However, we have not found in the literature works concerning the search for oligonucleotide activators of telomerase. At present, only one patent-protected low molecular weight TA-65 telomerase activator of plant origin is described [6].

Application of telomerase inhibitors can be accompanied by side effects affecting, first of all, stem cells, where telomerase is active and necessary for maintaining regeneration potential and viability. Nevertheless, different approaches to inhibition of telomerase have been under development for more than ten years [7]. The majority of telomerase inhibitors are low molecular weight inhibitors capable of stabilizing G-quadruplexes and preventing the interaction of telomerase with DNA.

The main components of the telomerase complex are the catalytic subunit represented by a reverse transcriptase

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Abbreviations: hTERT, human telomerase reversed transcriptase; hTR, human telomerase RNA; 2′-MOE, 2′-metoxyethylribooligonucleotides; MSCs, mesenchymal stem cells; PNAs, peptide-nucleic acids.

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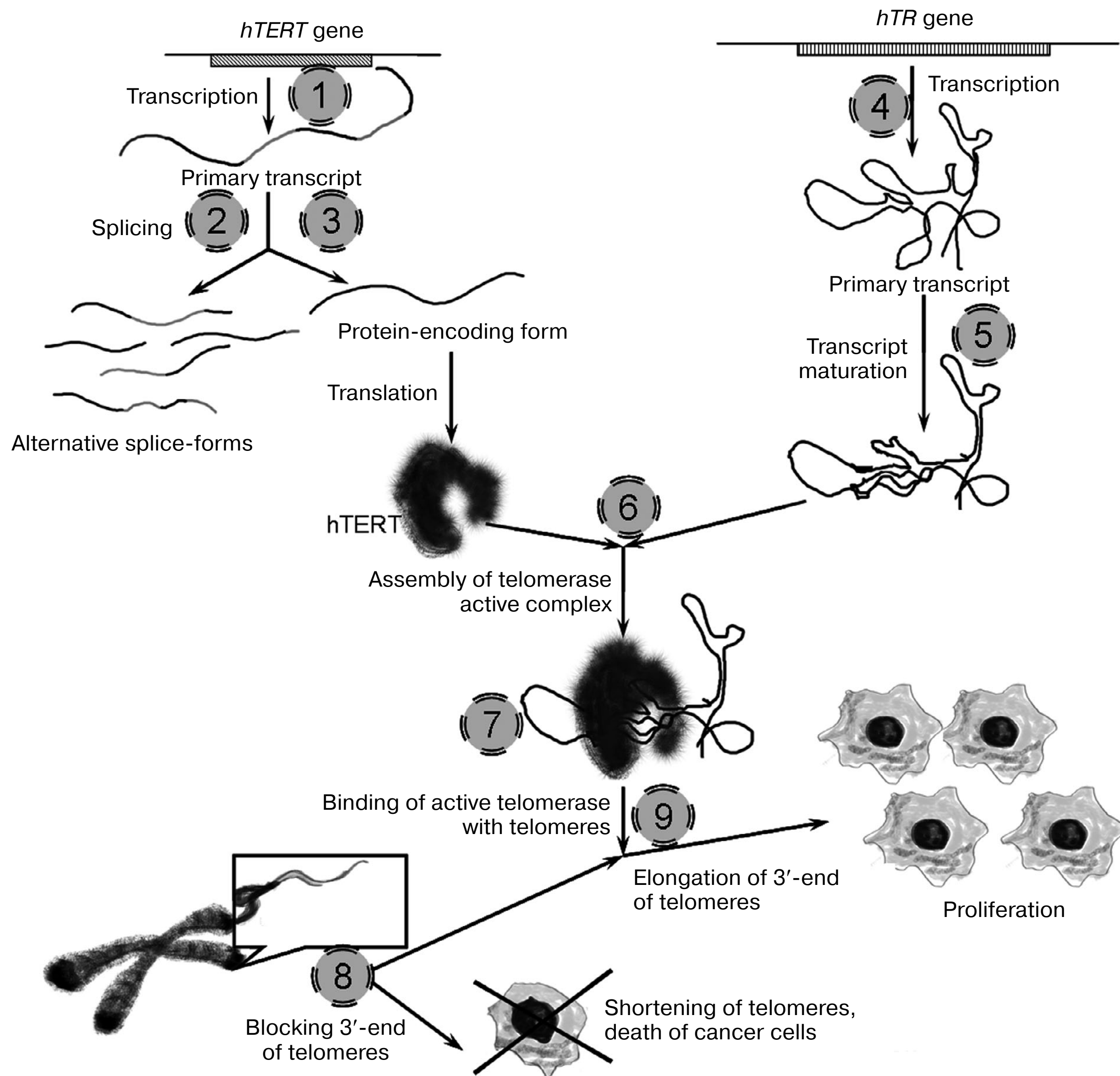
(hTERT) and telomerase RNA (hTR), which has a template region for synthesis of telomere repeats. Moreover, telomerase can function in the cell under conditions of binding additional regulatory proteins and of availability of the substrate, i.e. the 3′-terminal regions of the telomeres [1]. Thus, telomerase activity can be inhibited or its functioning can be blocked by influencing the biosynthesis, maturation, assembly, or correct interaction between the telomerase complex and the substrate. The scheme presents the main stages on which telomerase functioning can be blocked by oligonucleotide inhibitors and their targets. Main targets are processes associated with transcription of the *hTERT* (Scheme, 1-3) and *hTR* genes (4, 5), as well as a disturbance in the telomerase complex assembly (6), inhibition of enzymatic activity of the assembled telomerase complex (7), loss by telomere ends of availability for interaction with telomerase (8), and inhibition of activity of telomerase complex interacting with telomeres (9).

Recently, the stage of clinical testing started for some telomerase inhibitors, and the results indicate that telom-



Oligonucleotide inhibitors of telomerase: 1) inhibition of the *hTERT* gene transcription; 2) redirection of hTERT mRNA to alternative forms noncoding the protein or encoding inactive protein; 3) degradation of primary transcript and of the mature form of hTERT mRNA; 4) inhibition of *hTR* gene transcription; 5) degradation of the primary transcript and affecting the maturation of the hTR primary transcript; 6) disturbance of telomerase complex assembly; 7) blocking enzymatic activity of assembled telomerase complex; 8) decreased availability of telomere ends for telomerase; 9) inhibition of telomerase complex interacting with telomeres

Scheme

erase inhibitors are promising as chemotherapeutic preparations [8]. We shall consider in this review telomerase inhibition by oligonucleotides [9] due to complementary interactions with both telomerase RNA and mRNA of telomerase proteins, first of all with the telomerase catalytic subunit. The combined use of different approaches can increase telomerase inhibition efficiency.

OLIGONUCLEOTIDES FOR TELOMERASE INHIBITION

Oligonucleotides are used as effective tools in molecular diagnostics [10] and functional genomics [11], and they are considered as promising therapeutic agents [12]. Oligonucleotide-based technologies of regulation of cellular processes were shown to be effective both *in vitro* and *in vivo* [13, 14]. Different approaches are now used: antisense technology, RNA-interference, aptamers, anti-microRNAs, CRISPR, etc. The main problems associated with using oligonucleotides as therapeutic agents are caused by their low stability *in vivo* and difficulties in their selective delivery into tissues and cells [15]. Chemically modified oligonucleotides are significantly more stable and can penetrate into cells more effectively while retaining their biological activity [16]. Addition of different ligands allows researchers to obtain specific delivery of conjugates into particular cells due to receptor-mediated endocytosis [17].

Oligonucleotide inhibitors of telomerase have been created using different modifications of the sugar-phosphate framework. By now, 2′-*O*-alkylribonucleotides and oligonucleotides are described with thiophosphate, phosphamide, and thiophosphamide internucleotide bonds, as well as peptide-nucleic acids (PNAs) [18].

Both 2′-*O*-methyl- and 2′-*O*-methoxyethyl-modified oligonucleotides [19] produce more stable duplexes with complementary targets and inhibit telomerase already at nanomolar concentrations [20]. In addition to a pronounced stabilization of the duplex, 2′-MOE-modifications also promote improvement of the pharmacokinetics of the oligomer.

Thiophosphate oligonucleotides, which are the most widely distributed modified oligonucleotides [21], have also been used for inhibition of telomerase. They have high nuclease stability. However, an attempt to use them for inhibition of telomerase revealed their low specificity. Thus, the thiophosphate oligonucleotide S-ODNS inhibited telomerase due to binding with the hTERT protein and not with RNA [22]. Moreover, a 20-mer thiophosphate oligonucleotide inhibited telomerase independently of the nucleotide sequence [23]. Due to of nonspecific binding with proteins, such oligonucleotides acted as immunostimulators [24]. Thus, oligonucleotides with a small number of thiophosphate modifications, which retain the specificity on increased nuclease stability, seem promising as inhibitors of telomerase.

In phosphamide oligonucleotides, the oxygen atom in the 3′-position of the carbohydrate residue is replaced by a nitrogen atom [25]. Complexes of such oligonucleotides with complementary targets are more stable than with natural DNAs and retain specificity. Note that duplexes of such oligonucleotides with RNA are not substrates of RNase H. Thiophosphamide oligonucleotides are more resistant to the action of nucleases than phosphamide ones, and this noticeably increases the inhibition of telomerase [26]. The best-known inhibitor of this class is a conjugate of thiophosphamide oligonucleotide with palmitic acid (GRN163L or Imetelstat) [27]. Imetelstat effectively penetrates across cell membranes due to the lipid modification, whereas the 3′-amino group increases its stability in the presence of nucleases. All nucleotides contain 3′-amino groups, and non-bridge oxygen atoms in phosphate are replaced by sulfur atoms. Thus, all internucleotide bonds are N3′→P5′-thiophosphamide ones, and this is the main specific feature of GRN163. Conjugation of palmitic acid with oligonucleotide GRN163 decreased five-fold its inhibitory activity in cellular extract, but due to the increased penetration into the cell the inhibitory effect of GRN163L in cell cultures increased 1.5-40-fold (depending on the cell line) [27]. Such oligonucleotides were used for inhibiting the active center of telomerase as antagonists of the template region of the telomerase RNA (Scheme, 7).

Peptide-nucleic acids (PNAs) are based on the N-(2-aminoethyl)-glycine framework and do not have negative charge. Peptide oligonucleotides bind with complementary nucleic acids with high affinity and specificity [28, 29]. To increase the solubility of PNAs in aqueous solutions, they are usually synthesized as conjugates with peptides. Another approach for *in vitro* delivery can be realized using a PNA duplex with a complementary oligonucleotide that allows traditional cationic lipids to be used [30]. Then the oligonucleotide within the duplex is cleaved by nucleases, and the released PNA can interact with the target. Although *in vitro* results are good, those *in vivo* are not so significant because of high toxicity of these oligomers. Nevertheless, using a PNA heteroduplex with a conjugate of oligonucleotide with asialofetuin resulted in effective delivery of PNAs into hepatocytes of mice through asialoglycoprotein-receptor-mediated endocytosis after intravenous injection [31].

Let us consider in more detail oligonucleotide inhibitors of telomerase using their classification according to action mechanism.

APPLICATION OF OLIGONUCLEOTIDES INTERACTING WITH mRNA OF TELOMERASE CATALYTIC SUBUNIT hTERT

For the most part, just the expression of the catalytic subunit hTERT correlates with telomerase activity [32],

and hTERT mRNA is used as a marker of tumorigenesis. Application of antisense oligonucleotides complementary to mRNA of hTERT inactivates telomerase, which prevents proliferation of cancer cells, and when telomeres reach the critical length, apoptosis is induced in them. An oligonucleotide (Cantide) complementary to the 3′-untranslated region of hTERT mRNA specifically decreased five-fold the active telomerase level in HepG2 cell culture as compared to untreated cells, and this promoted induction of apoptosis and death of more than half of the cells [33]. Then this oligonucleotide was shown to display antitumor activity on tumor xenografts in immunodeficient mice [34]. An increase in the Cantide concentration led to a decrease in the growth and weight of xenotransplanted hepatocellular carcinoma in mice and of a primary liver lymphoma in immunodeficient mice, and this was accompanied by an increase in survival [35]. These oligonucleotides inhibited telomerase in pathways 1 and 3 indicated in the scheme.

Another way for lowering telomerase activity by means of antisense oligonucleotides to hTERT mRNA is changing the direction of this RNA splicing (Scheme, 2). mRNA of hTERT has several splice forms, but only two of them are active in translation, and only one of the resulting proteins is functionally active [36]. In the active center of the other protein, there are no amino acids required for synthesis of the telomeric repeat (the dominant negative mutant form of hTERT). During expression only of the dominant negative form of hTERT, the cells died because of telomere shortening [36]. The ratio between the hTERT mRNA splice forms remained constant during neoplastic transformation of cervical carcinoma [37]. An artificial redirection of the alternative splicing towards increase in nonfunctional splice forms of hTERT mRNA seems to be a possible approach for decreasing telomerase activity in tumor cells [38]. However, cancer cells died only when the telomeres reached the critical length, which required much time, sometimes months, and this is a fault of this method. The tumor must be treated during this whole period, which can promote the development of resistance. However, on complete loss of hTERT, the cells enter apoptosis immediately, omitting the stage of telomere shortening. This is associated with the involvement of hTERT in many cell processes: activation of Wnt, NF-κB-pathways, inhibition of apoptosis, etc. [5].

ACTION OF OLIGONUCLEOTIDE INHIBITORS OF TELOMERASE ON hTR

Oligonucleotides complementary to telomerase RNA (hTR) inhibit telomerase through some pathways, e.g. by decreasing the lifetime of hTR in the cell [39], blocking telomerase assembly [40], or inhibiting polymerase activity of telomerase *in vitro* [41].

The lifetime of hTR in the cell can be lowered by its degradation by RNase L. This approach is based on using oligonucleotides complementary to hTR and conjugated with 5′-phosphorylated 2′-5′-oligoadenylate (2-5A), which activates in the cell RNase L responsible for cleaving single-stranded RNA [42]. Such oligonucleotides effectively and with high specificity stimulate hydrolysis of complementary RNA and thus increase 20-fold the efficiency of inhibition of telomerase activity [39].

OLIGONUCLEOTIDE INHIBITORS AFFECTING TELOMERASE ASSEMBLY

Inhibition of the telomerase assembly is based on blocking the interaction of RNA and the catalytic subunit of telomerase. Oligonucleotides complementary to hTR regions necessary for interaction with telomerase complex components affect the assembly of telomerase. In this case, telomerase activity in cells can be suppressed due to decreasing the amount of correctly assembled active telomerase [40]. Disturbances in telomerase assembly can lead not only to the loss of the telomerase activity, but also to increase in degradation of telomerase RNA because of its easier availability for nucleases. Moreover, in addition to direct steric prevention of hTR regions from interactions with other components of the complex necessary for functioning of telomerase, there is a possibility of disorder in the assembly of the RNA spatial structure element that is important for telomerase functioning, e.g. the pseudoknot [43].

Telomerase complex inhibition at the cost of assembly is advantageous because it is associated with retention of expression of telomerase complexes within the cell, which is important for executing functions of telomerase components other than supporting the length of telomeres [44]. This approach to inhibition of telomerase seems to be associated with a lower number of side effects.

Recently, a new approach was proposed for affecting human telomerase functioning at the stage of active complex assembly. This approach is based on application of chimeric bifunctional oligonucleotides containing two oligonucleotide parts complementary to functional regions of telomerase RNA and bound with differently oriented non-nucleotide linker (5′-3′, 5′-5′, or 3′-3′). Such chimeras inhibit telomerase *in vitro* at nanomolar concentrations, but in cells they are efficient at concentrations an order of magnitude lower [45].

INHIBITORS BLOCKING ENZYMATIC ACTIVITY OF TELOMERASE

Telomerase can function if hTR forms a duplex with telomeric DNA. In active telomerase, the template region of hTR is available for hybridization, and therefore

telomerase can be competitively inhibited by complementary oligonucleotides. Such oligonucleotides are effective inhibitors even in nanomolar concentrations [46]. Data on this type of inhibition obtained up to 2009 are summarized in a review by Gryaznov et al. [47]. Later it was shown on mice that in the presence of the telomerase supposed competitive inhibitor GRN163L that the sensitivity of cancer cells increased to other cytotoxic preparations, which was associated with shortening of telomeres and was accompanied by changes in the ability of cancer cells for migrating and metastasizing [48]. These effects can in particular be caused by changes in the expression of different microRNAs [49].

For application of telomerase inhibitors as antitumor agents, it is important to study the influence of such inhibitors on stem cells and possible consequences of long-term treatment. The influence of GRN163L has been studied on the growth of mesenchymal stem cells (MSCs) because they also contain active telomerase, which is necessary for proliferation. MSCs are multipotent cells that are important for maintaining the organism's homeostasis. The treatment of rat MSCs with GRN163L led to changes in the cell phenotype, the spindle-shaped cells became rounded and were separated from the surface similarly to tumor cells. GRN163L arrested MSCs in the G1 phase of the cell cycle with a dramatic decrease in the levels of mRNA and protein of cyclin D1, CDK4, and CDK6. A week after GRN163L addition, concentrations of mRNAs of these proteins and of the proteins themselves became normal, as well as the phenotype of MSCs. Thus, GRN163L did not cause irreversible changes in the self-renewal and differentiation of MSCs on cell cultivation *in vitro*, and it was supposed that the telomerase inhibition should not lead to exhaustion of the organism's stem cell pool during short-term treatment with the preparation [50].

There were similar morphological changes in breast cancer cell line MCF7 treated with GRN163L [51]. Due to loss of cell adhesion, GRN163L caused rounding of the cells independently of the initial expression of hTR or of the length of telomeres in these cells, but the detailed mechanism of this effect is not clear. Thus, GRN163L induced the loss of adhesion in lung cancer cells A549 due to decrease in expression of E-cadherin, which resulted in disorders in the cytoskeleton substantiated by changes in actin, tubulin, and organization of intermediate filaments. Thus, the cells lost the majority of contacts, stopped dividing, and remained in the G1 phase of the cell cycle, which was accompanied by a decrease in the expression of the template metalloproteinase-2. These effects of GRN163L did not depend on decrease in the catalytic activity of telomerase, and it was supposed that this could increase therapeutic efficiency of GRN163L due to lowering the adhesion and proliferation of cancer cells under normal conditions [52]. However, perhaps a decrease in cancer cell adhesion in the organism is associated with increased risk of metastasizing, especially after withdrawal of the preparation (when the proliferative potential of the cells recovers), if disorders in the contacts and the decreased adhesion will result in accidental migration of the cancer cells and their arbitrary retention, for instance, in lymph nodes.

Unexpectedly, GRN163L-caused inhibition of telomerase was accompanied by an increase in the sensitivity of cancer cells to ionizing radiation [53]. A GRN163L-based preparation named Imetelstat also influenced the cell sensitivity to other chemotherapeutic agents. Thus, in the case of chronic lymphocytic leukemia (CLL), it increased the sensitivity of primary lymphocytes to fludarabine *in vitro* [54]. This effect was observed in lymphocytes isolated from clinically resistant patients and with cytogenetic abnormalities. The Imetelstat-mediated sensitization of lymphocytes to the cytostatic preparation fludarabine was not associated with the activity of telomerase, but it depended on the basal expression of Ku80. This occurred because Imetelstat prevented the interaction of the Ku70/Ku80 heterodimer with hTR. The effect of the oligonucleotide complementary to the binding site of Ku70/80 with hTR on the CLL lymphocytes under the influence of fludarabine was similar to the effect of Imetelstat, which suggested a functional interaction of the Ku family proteins and hTR in human cancer cells [54]. The effect of Imetelstat was studied on stem cancer cells, which were cell subpopulations resistant to many pharmaceuticals and possibly responsible for cancer recurrences and metastasizing [55]. Imetelstat decreased the fraction of cancer cells *in vitro*, and differences in the telomerase activity or the length of telomeres did not correlate with the increased sensitivity of the stem cancer cells to the preparation. Therefore, it was supposed that an alternative action mechanism of Imetelstat on the stem cancer cell subpopulation should exist, which should be independent of telomere shortening [55], but the mechanism of such action is still unknown.

Imetelstat displayed good results in clinical testing on patients with thrombocythemia. Among side effects was a toxic action on the liver in 90% of treated patients. The high price of Imetelstat is also an obstacle for its wide application in therapy [56].

The combined application of antisense oligonucleotides concurrently acting on hTR and hTERT leads to an increase in the efficiency of inhibition of telomerase activity, to suppression of cell growth and their death mainly through apoptosis, and cell cycle inhibition, as shown on human colon cancer cell line SW480 [57].

INHIBITORS INFLUENCING TELOMERE AVAILABILITY FOR TELOMERASE

Telomerase acts on protruding 3′-terminal regions of telomeres. Sequences of telomeres are capable of produc-

ing a specific G-quadruplex structure in which the terminal fragment is not an effective substrate of telomerase. Aromatic ligands binding with G-quadruplexes stabilize this structure and decrease the efficiency of the telomerase action, which has been shown for various low molecular weight compounds [58]. Oligonucleotides containing intercalators can also stabilize DNA quadruplexes and can inhibit telomerase and thus prevent substrate recognition [59]. This pathway of telomerase inhibition is described only in one work. This approach does not seem promising because of a great number of telomeric repeats (throughout the whole genome), which can also interact with such oligonucleotides and change their own structure, which possibly will change expression of the adjacent genes.

OLIGONUCLEOTIDE INHIBITORS OF TELOMERASE FOR DIAGNOSTICS OF CANCER

An alternative application of antisense oligonucleotides capable of inhibiting telomerase for imaging cancer cells has been described [60]. Antisense oligonucleotides were first used for noninvasive studies in 1994 on mammary tumor-bearing mouse model. The antisense oligonucleotide was represented by a 15-mer phosphorothioate oligodeoxyribonucleotide containing In-111 for documentation of the constitutive expression of the c-Myc gene [61]. Afterwards, antisense oligonucleotides have been actively developed for imaging tumor cells [62]. In 2010, molecular imaging with antisense oligonucleotides was named a new interdisciplinary field capable of providing the potential for early detection of oncologic diseases and elucidating mechanisms of their appearance on the molecular level and for assessment of therapy [63]. Tumor-specific mRNAs, including mRNA of hTERT, were proposed for imaging. Thus, a conjugate of the antisense thiophosphate oligonucleotide with a gadolinium chelate complex complementary to the region of hTERT mRNA was selectively accumulated in the hTERT-transformed cells [64]. Therefore, this oligonucleotide was used as a molecular probe for an active imaging of tumors by magnetic resonance tomography. Later such conjugate was used for reliable imaging of tumor xenotransplants in immunodeficient mice [65]. The authors of this work concluded that the technetium-99-modified antisense oligonucleotide to hTERT mRNA could be promising as a molecularly specific diagnostic marker of a tumor-caused lesion as a molecular probe for the imaging of tumors by magnetic resonance tomography [65]. There is no doubt that conjugates of antisense oligonucleotides to hTERT mRNA will be used for *in vivo* imaging of human telomerase as a new approach for diagnostics of oncologic diseases.

Oligonucleotide inhibitors of telomerase are now modified only by a limited number of known methods [16]. Nevertheless, increase in stability of inhibitors or improvement of their functioning *in vivo* are observed. Using the latest achievements in chemistry of nucleic acids is expected to improve the known oligonucleotide inhibitors of telomerase and promote the creation of new ones.

However, shortages of oligonucleotide inhibitors of telomerase as antitumor preparations should be also noted. First, although in the majority of the cell malignization cases the proliferative potential and the constant length of telomeres are provided by telomerase activity, an alternative recombination-based mechanism of telomere elongation (the ALT-mechanism) also exists. Thus, inhibition of telomerase can lead to triggering the ALT-mechanism [66], which is equivalent to appearance of resistance to telomerase inhibitors. Second, at the initially very long telomeres in the transformed cell, it is unreasonable to use inhibitors of telomerase because too many cell divisions will be required for them to display their effect, and this is another shortcoming of such preparations [67]. However, it is known that on using some approaches for influencing telomerase (see Scheme) a rapid cellular response can be obtained that will overcome this shortcoming. The third problem is that the application of oligonucleotide inhibitors of telomerase as antitumor preparations is associated with high price and difficult synthesis of modified oligonucleotides. However, an increase in the efficiency of telomerase activity by antisense oligonucleotides concurrently influencing hTR and hTERT and an increased influence on tumor stem cells suggest that oligonucleotide inhibitors of telomerase can be promising in combined antitumor therapy, but not as a universal preparation.

The search for and development of oligonucleotides activating the enzyme due to changes in the efficiency of splicing or in influence on telomerase processivity as putative anti-aging agents is another potential line of studies.

Moreover, the successful application of oligonucleotide inhibitors of telomerase for diagnostics of oncologic diseases provides a basis for further searches for oligonucleotides influencing the activity of telomerase.

This work was supported by the Russian Scientific Foundation (project 14-24-00061).

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