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Potent Telomerase Antisense Oligonucleotides over the Past Two Decades

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Abstract: The ribonucleoprotein complex telomerase is responsible for maintaining telomeres length at the end of chromosomes during cell division. It overcomes the well-known end replication problem and guards against losing chromosomes ends. Telomerase activity is observed in many cancer and other immortalized cell lines. As a result of its innate ability to access to nucleic acids, telomerase is considered ideal for the antisense oligonucleotide therapies that target the RNA template of the enzyme. Over the years there has been a mass development of these agents for treating different malignancies. This review spots light to the most reliable trials that, over the past two decades, have directed antisense oligonucleotides of different chemical classes against human telomerase, analyzes their structural features and provides a possible explanation for their variant potencies and kinetics. In total, 26 anti-telomerase oligonucleotides were selected from different studies and their relative potency was discussed in view of their physicochemical properties. It was concluded that presence of 10 nucleotides, on average, targeting positions 142 – 151 of the template region is essential for telomerase inhibitory activity. Structural modifications such as 2'-OH replacement with O-alkyl, presence of sulfur atoms, introduction of steric hindrance along the backbone has improved solubility and/or stability and thus granted better activity. Attaching basic moieties to the oligonucleotide scaffold has optimized cellular delivery of the developed candidates.

Key words: Ribonucleoprotein, telomerase, antisense, oligonucleotides, cancer

INTRODUCTION

The edges of linear chromosomes, known as telomeres, are a pivotal portion of the eukaryotic genome. They represent the caps that cover the physical ends of chromosomes and function to maintain chromosomes integrity during each DNA replication round. They prevent chromosomal ends from being recognized as DNA damage and protect them against enzymatic degradation and fusion[1-3].

In humans, the DNA sequence of telomeres consists of tandem G-rich repeats, $(TTAGGG)_n$, with a single stranded 3'-end overhang. The G-rich regions in the repetitive human telomeric sequence provide high potential to adopt a four-strand G-quadruplex structure that was confirmed by several experiments using either quadruplex-specific antibodies or quadruplex-binding ligands[4].

Replication of chromosome ends is associated with DNA loss each time a cell divides. This is because the conventional DNA polymerase could not fully replicate the 3' end of the lagging strand of the linear molecule implying that chromosomal DNA is shortened at the 3' extremity of all chromosome ends within each replication round. This is commonly known as the end replication problem that eventually results in replicative senescence and cell death so as to define cellular lifespan and safeguard against extensive cellular proliferation that may lead to cancer[5]. The enzyme telomerase is a ribonucleoprotein complex that is able to do de novo DNA synthesis, adds DNA to chromosomes end and thus ensures telomeres maintenance at this region[2, 6].

Telomerase is a specialized reverse transcriptase that consists of two essential components, the functional RNA component known in humans as hTR and the catalytic protein hTERT. The RNA subunit contains a species specific sequence complementary to the G-rich sequence of the telomere and serves as a template for the telomeric DNA synthesis[7, 8]. The catalytic subunit, hTERT, represents the protein part of the enzyme that copies the RNA template. It is encoded by TRT gene that has critical domains essential for the catalysis of telomeres elongation. The introduction of a single amino acid substitution in any of these domains results in telomere shortening and cell death [9-11].

A number of proteins and functional domains are highly crucial for telomerase functioning where they mainly facilitate the assembly of telomerase subunits, access to telomeres, and activation[12]. Telomerase RNA unit contains four conserved domains devoted for this purpose. The box H/ACA and CR7 domains are dispensable for telomerase reconstitution activity *in vitro*[13]. However, the pseudoknot structure and CR4/CR5 domains are highly conserved structural domains that are of utter importance in this regards[8, 14].

Telomerase uses the 3' extremity of telomeric DNA as a starting point for extension by synthesis of repeated units of the template contained in the RNA subunit creating multiple hexanucleotide repeats per cell replication cycle[5]. With the aid of several oncogenes, telomerase up-regulation provides an unlimited division capability and results in direct tumorigenic conversion of normal human epithelial cells and fibroblasts. Up to date, telomerase activity is detected in about 85-90% of cancer tissues and 60-70% of immortalized cell lines planted in tissue cultures[15-17].

Several biochemical protocols have been developed to assess telomerase activity *in vitro*. In 1994, Kim *et al.*[15] developed the telomerase repeat amplification protocol (TRAP) that was modified latter to the TRAP combined with enzyme-linked immunosorbent assay (TRAP-ELISA)[15-18]. A Fluorescent TRAP (F-TRAP) assay has been introduced by Aldous and Grabillto overcome the limitations of the TRAP protocol[19]. The Transcription mediated amplification (TMA), in conjunction with the hybridization protection assay (HPA) is quantitative and non-radioactive technique that is faster, as sensitive and reproducible as TRAP and is

being used also to assess telomerase activity[20]. In 2002, Xu *et al.*[21] have developed an enzymatic luminometric pyrophosphate assay (ELIPA) that has shown high sensitivity and reproducibility in measuring telomerase activity. Recently telomerase activity has been assayed using asymmetric PCR (A-PCR) on magnetic beads (MBs) followed by the application of cycling probe technology (CPT) involving RNA probe and RNase H. It's a sensitive approach that avoids the possible limitations of the previous protocols[22].

Interfering with telomerase enzyme action is considered a promising aid to overcome several types of malignancies. Beside immunotherapy and gene therapy[23], blocking telomerase activity can be achieved through different strategies. Inhibitors can either target the RNA component of telomerase, as in the case of antisense oligonucleotides, bind to the catalytic subunit (hTERT), or stabilize the G-quadruplex structure of the enzyme leading to its inactivation.

Herein, a comprehensive investigation has been conducted to view the most significant and reliable antisense oligonucleotides developed against human telomerase over the past two decades. Structural and physical features of the selected candidates have been analyzed to showcase the essential physicochemical features required for optimum enzyme inhibitory activity.

TELOMERASE ANTISENSE OLIGONUCLEOTIDES

Telomerase antisense oligonucleotides (ODNs) represent a category of inhibitors acting by blocking the RNA component of telomerase enzyme. They can be described as short stretches of DNA that are complementary to the template of the telomerase functional RNA, where they base pair with it, inhibit translation, and block the enzyme activity[24].

Table 1 shows the most successful antisense oligonucleotides developed against human telomerase enzyme through over the past two decades. The first report of successful inhibition of telomerase activity using antisense oligonucleotides was by Feng *et al.*[7] where they have used a construct expressing an antisense transcript to the first 185 nucleotides of the RNA and introduced it into HeLa cells. In this study, 33 out of 41 clones underwent crisis after 23-26 doublings. The clones that entered crisis have reduced telomerase activity and had shorter telomeres compared to those with vector alone[7, 24].

In 1998, Glukhov *et al.*[25] have reported the effect of some antisense ODNs complementary to different sites of the telomerase RNA component on the enzyme activity expressed in melanoma cell line, SK-Mel-28. The oligonucleotide **1** which is complementary to the hTR component in the region that serves as a template for the direct synthesis of telomeric repeats has shown complete inhibition of the enzyme at a concentration of 20 nM. The oligonucleotide **2** complementary to the hTR component in the proximity of its 3'-end showed a strong inhibition to the enzyme only at a concentration of 1 µM, while oligonucleotide **3**, complementary to the middle portion of the hTR component did not exert any substantial inhibiting effect on telomerase activity[25].

The 2'-*O*-Alkyl RNA is a second generation class of oligonucleotides that binds complementary sequences with high affinity when compared to other DNA and RNA oligonucleotides[26-28]. Pitts *et al.*[29] have explored the telomerase inhibitory activity of 2'-*O*-meRNAoligonucleotides **4** and **5** in human prostate tumor-derived DU145 cells. Results revealed that this second generation of antisense oligonucleotides is of higher potency and stability in terms of IC_{50} values and the melting temperatures of the complementary RNA complexes.

Table 1: Most successful telomerase blocking antisense oligonucleotides over the past two decades

Com. No.	Sequence	IC_{50} (nM)	Cell Line	Ref.
1	5'-CCCTTCTCAGTTAGGGTTAG-3'	20	SK-Mel-28	25
2	5'-GGGGACTCGCTCCGTTCCTC-3'	1000	SK-Mel-28	25
3	5'-AACTCTTCGCGGTGGCAGTG-3'	> 0000	SK-Mel-28	25
4	5'-CAGUUAGGGUUAG-3' (2'- OMe)	8	DU145	29
5	5'-C(ps)A(ps)GUUAGGGUUA(ps)G(ps)-3' (2'- OMe)	3	DU145	29
6	5'-GGGTTAGACAA-lys (PNA)	10	HeLa	30
7	5'-TAGGGTTAGACAA-lys (PNA)	1	HeLa	30
8	5'-GCGCGGGGAGCAAAAGCAC-3' (2-5A)	NA[a]	PC3,DU145	33
9	5' GCTCTAGAATGAACGGTGGAAGGCGGCAGG 3' (NP)	0.5	Purified extract	35
10	5'GTGGAAGGCGGCAGG 3' (NP)	0.5	Purified extract	35
11	5' CGGTGGAAGGCGG 3' (NP)	1	Purified extract	35
12	5' ACGGTGGAAGGCG 3' (NP)	2	Purified extract	35
13	5' AAGGCGGCAGG 3'(NP)	> 125	Purified extract	35
14	5'-GGGTTAG-3' (ps)	8	Purified extract	36
15	5'-GGGTTAG-*fluorescein*-3' (ps)	4	Purified extract	36
16	5'-GTTAGG-3' (ps)	205	Purified extract	36
17	5'-GTTAGG-*fluorescein*-3' (ps)	40	Purified extract	36
18	5'-TTAGGG-3' (ps)	1360	Purified extract	36
19	5'-TTAGGG-*fluorescein*-3' (ps)	15	Purified extract	36
20	5'-TTAGGG-*TAMRA*-3' (ps)	44	Purified extract	36
21	5'-*acridine*-TTAGGG-3' (ps)	85	Purified extract	36
22	5'-Palm-TAGGGTTAGACAA-NH2-3' (ps)	R[b]	Different cells	37-43
23	5'-TAGGGTTAGCAA-3'(2'-*O*,4'-*C*-ethylene bridged) (ps)	NA	-------	44
24	5'-ACTCACTCAGGCCTCAGACT-3' (ps)	R	Different cells	45-47
25	5'-CAGTTAGGGTTAG-3'	> 0000	Jurkat	48
26	5'-AAACAGATTCCCTAAGAGAGTTGGG^{T} T 3'-AAACAGATTGGGATTGACTCTTCCC^{T}T	NA	HeLa	50

[a]NA: Not Available Data

[b]R: The candidate has a range of IC_{50} values based on the cell line used in the measurement.

Peptide Nucleic Acids (PNAs) represent a novel generation of oligonucleotides that has offered more advances in terms of biological stability and affinity towards the target RNA. Chemically, they are analogues of RNA and DNA in which the pentose-phosphate skeleton is replaced with a flexible, non-charged polyamide scaffold composed of N-(2-aminoethyl) glycine repeating units[24]. In the scope of

telomerase antisense PNAs, Shammas *et al.*[30] introduced a paramount study in which 11- or 13-mer PNA molecules complementary to telomerase RNA, **6** and **7**, have been developed and modified by addition of a 3'- terminal lysine residue to enhance their uptake by living cells.

Telomerase activity was assessed following the co-electroporation of the PNA molecules into HeLa cells and the developed candidates have shown promising results that opened the door for this technology as telomerase antisense inhibitors[30].

The use of antisense oligonucleotides against human telomerase hTR component linked to 2',5'oligoadenylate moiety have been reported by several studies to enhance telomerase blocking activity[24, 31-33]. Kondo *et al* .[31] have investigated the cytotoxic effect of a 19-mer antisense ODN, **8** that was linked to a 2-5A moiety (5'-phosphorylated 2'-5'-linked oligoadenylate) on prostate cancer *in vitro* and *in vivo*. It was shown that treatment of human prostate cancer cell lines PC3 and DU145 with this test candidate reduced tumor cells viability to 9 -18% within 6 days. The study has also shown that treatment of subcutaneous tumors in nude mice with the developed molecule has significantly suppressed the tumor growth ($P < 1000$)[33].

N3'→P5' phosphoramidate (NP) oligonucleotides stand as another class of oligonucleotides in which the 3'-oxygen in the 2'-deoxy ribose ring is substituted with 3'-amino group. These compounds have shown high specificity for RNA and DNA targets with a relatively low affinity to proteins and have demonstrated an appreciable resistance to nuclease degradation[34]. In 2002, Pruzan *et al.*[35] have reported the design of novel NP oligonucleotides complementary to the template region of telomerase hTR component with high potency in vitro against purified telomerase extract[35]. The novelty in this work is evident upon viewing the structural analysis of oligonucleotides **9** – **13** which reveals that the molecules possess different number of nucleotides complementary to variant positions on the template region of telomerase hTR. Oligonucleotide **9** is complementary to 137-166 positions of hTR ; **10** complements 137-155, **11** complements 141-153, **12** complements 142-154, and **13** complements 137-147 positions. This study has pointed out that presence of ten complementary nucleotides to positions 142-151 of hTR template region is necessary to obtain effective telomerase inhibitors[35].

Pongracz *et al.*[36] have extended the previous work and developed a novel series of relatively short (6- or 7-mer) oligonucleotides with N3'→P5' thiophosphoramidate (ps) backbone (oligonucleotides **14-21**) with pendant groups including dyes and polyaromatic functions attached to either 5' or 3' termini. The introduced oligonucleotide targetedtelomerase hTR and were varied in terms of the sequence, the pendant chemical group, and the backbone skeleton (phosphor- or thiophosphoramidate) and tested in vitro against purified telomerase extract[36]. The results of this study have shown that the shortening of the sequence length in general did not favor the stability neither the activity of the oligonucleotide candidates, however the used pendant group has shown to afford better stabilizing interactions with the protein component of telomerase and thus has enhanced the enzyme inhibitory activity. The candidates having the hydroxyl containing pendant group fluorescein were more active than those having the hydroxyl free group TAMRA. The oligonucleotides with thiophosphoramidate backbone have generally exhibited better telomerase inhibitory activity relative to their phosphoramidate counterparts.

In 2005, Herbert *et al.*[37] have developed a novel lipid modified 13-mer oligonucleotide N3'→P5' thiophosphoramidate, designated as GRN163L, **22**, and known later as Imetelstat, complementary to the template region of telomerase RNA. The candidate was able to inhibit telomerase activity in various cell

lines with seven fold lower IC_{50} values in the corresponding cell lines than that of its parent oligonucleotide GRN163.

Since the development of the promising agent GRN163L, a lot of work has been constructed to evaluate its potential as anticancer agent against different types of tumors. A dose of 1 µM of this palmitoyl domain containing candidate was able to block the telomerase activity in A549-Luc human lung cancer cells leading to progressive telomere shortening in a study by Dikmen *et al.*[38] The candidate is then proved to be efficient against MDA-MB-231 and MDA-MB-435 human breast adenocarcinoma cell lines where the treated cells have exhibited significant decrease in proliferation and invasive potential[39]. Furthermore, in an *in vivo* breast xenograft tumor and metastasis model, GRN163L was proven effective in reducing breast tumor growth and the amount of breast cancer metastases to the lung[40, 41].

Cancer stem cells (CSC) are rare drug resistant cell subsets proposed to be responsible for the recurrence of cancer and metastasis. The effect of GRN163L on putative CSC populations present in pancreatic cancer cell lines, PANC1, and breast cancer cell lines, MDA-MB231 and MCF7 was as well investigated. Not only telomerase activity was inhibited, GRN163L was also able to reduce the CSC fractions present in the used cell lines[42].

In a recent study, telomere size and telomerase inhibition in response to GRN163L were measured in a panel of 10 pancreatic cancer cell lines aiming at investigating the effect of long term GRN163L exposure on the telomeres maintenance and life span of pancreatic cancer cells. The candidate inhibited telomerase in all cell lines with IC_{50} ranging from 50 nM to 200 nM. Continuous exposure of CAPAN1 and CD18 cells to the drug eventually led to crisis and to a complete loss of viability. Moreover, crisis in these cells was accompanied by activation of a DNA damage response and evidence of both senescence and apoptosis[43].

In another trial to imitate the success of GRN163L, Horie *et al.*[44] have synthesized a novel oligonucleotide **23** having the same sequence of the parent GRN163 but with 2'-*O*,4'-*C*-ethylene bridged residues[44]. The developed oligonucleotide has revealed superior properties as an antisense therapeutic application in terms of thermodynamic and chemical aspects which could be obviously attributed to the steric hindrance imposed by the 2'-*O*,4'-*C*-ethylene residues. The melting temperatures of the duplexes of the oligonucleotide **23** with the complementary RNA were higher than that of the duplex of GRN163 (85 ^{o}C versus 51^{o}C respectively). It has also shown to be more stable than GRN163 under acidic conditions (pH 5.0) and thus would show better delivery into cells by endocytosis.

Extending the work done by Wang *et al.*[45] who developed the phosphorothioate antisense ODN **24** against hTERT, known as Cantide, Du *et al.* [46] were able to investigate its antitumor mechanism of action. In this study, human hepatocellular carcinoma cells; HepG2 and SMMC-7721 were treated by Cantide then hTERT mRNA expression, telomerase activity, as well as CPP32 and ICE-like activity were investigated. At a concentration of 800 nM Cantide, telomerase activity in HepG2 and SMMC-7721 was reduced to 17% and 20% of that in control cells, hTERT mRNA levels decreased in dose dependent manner, levels of CPP32-like protease activity in HepG2 and SMCC7721 increased by 2.8 and 3 folds, and the levels of ICE-like protease activity was also increased by 2.6 and 3.2 folds respectively emphasizing the strong inhibitory effect of Cantide on tumor growth and its powerful potential as anticancer treatment[46]. Recently, Yang *et al.*[47] have reported Cantide-linked significant dose dependent reduction in tumor weight and serum lactate dehydrogenase activity in nude mouse model that is orthotopically transplanted by primary hepatic lymphoma.

In a unique trial to counteract telomerase enzyme, Diala *et al.*[48] have developed a universal method to prepare DNA-peptide conjugates by Solid Phase Fragment Condensation (SPFC), in which a DNA fragment having a free amino group prepared on solid support is reacted with a coupling agent such as carbonyl diimidazole and then with a peptide fragment bearing a single reactive amino group and the resulting oligonucleotide-peptide conjugate is cleaved from the solid support by ammonia treatment[49]. According to this study, the oligonucleotide **25** has barely shown any telomerase inhibitory activity[48]. To robustly improve its activity upon conjugation, the conjugated peptides were meant to be derived from nuclear localization signals (NLS). These peptides included NLS of SV40 T-antigen, NLS of Influenza virus nucleoprotein, and NLS of HIV-1 tat protein, **Fig. 1.**

C1: Peptide = Ac-GPKKKRKVGK-NH- (SV40 T antigen NLS)

C2: Peptide = Ac-GRKKRRQRRRPPGGK-NH- (HIV-1 Tat NLS)

C3: Peptide = -NH-ANSAAFEDLRVLS-OH (Influenza Virus NLS)

Fig. 1: Sequence of NLS derived peptides used by Diala *et al.*[48] for developing DNA-peptide conjugates.

Oligonucleotide-NLS conjugates C1, C2, C3 have shown much higher telomerase inhibitory effect in human leukemia cells, Jurkat, (43%, 38%, and 70% inhibition respectively after 48h) than the normal antisense oligonucleotide **25** that showed almost 0% telomerase inhibition[48].

In 2009, Noreen *et al.* have reported a design of partially double stranded antisense ODN **26** targeting the sequence 5'-…UUUGUCUAACCCUAACUGAGAAGGG…-3' on the hTR component of telomerase based on previously described structural properties of an ODN against the HIV polypurine tract required for the second strand DNA synthesis of HIV-1. The candidate was designed as hairpin-looped structure where the antisense strand is a 25-mer, fully complementary to the target hTR and linked by four additional thymidines (T4 linker) to the second strand that is partially complementary to the antisense strand. To impart the candidate stability against nuclease degradation, phosphorthioate modifications at each end (three bases) and in the T4 linker were implemented[50-54].

At 48 h of transfecting 50 nM of the oligonucleotide **26** to HeLa cells, the level of hTR RNA measured using qRT-PCR was strongly reduced to 32% versus 48% and 36% reduction shown when same concentration of the 25-mer antisense single stranded analogue of oligonucleotide **26** and the 21-mer siRNA were transfected respectively[50].

Using a novel tool to invade telomerase assembly, Azhibek *et al.*[55] have managed to design chimeric bifunctional oligonucleotides that contain two oligonucleotide parts complementary to the template region and functional domains on telomerase hTR which are crucial for telomerase assembly, thus each of the designed chimeras can simultaneously interact with two functional regions on telomerase hTR. The two oligonucleotide parts in the novel chimeras are connected with non-nucleotide linkers in different orientations. Three single oligonucleotides have been used in this study are shown in **Fig. 2**. Oligonucleotide M is complementary to template region on hTR with IC_{50} 100 nM against telomerase, oligonucleotide J is complementary to pseudoknot domain with IC_{50} 19 nM against telomerase, and

oligonucleotide N is complementary to CR4/CR5 domain on hTR that did not show significant telomerase inhibition *in vitro*.

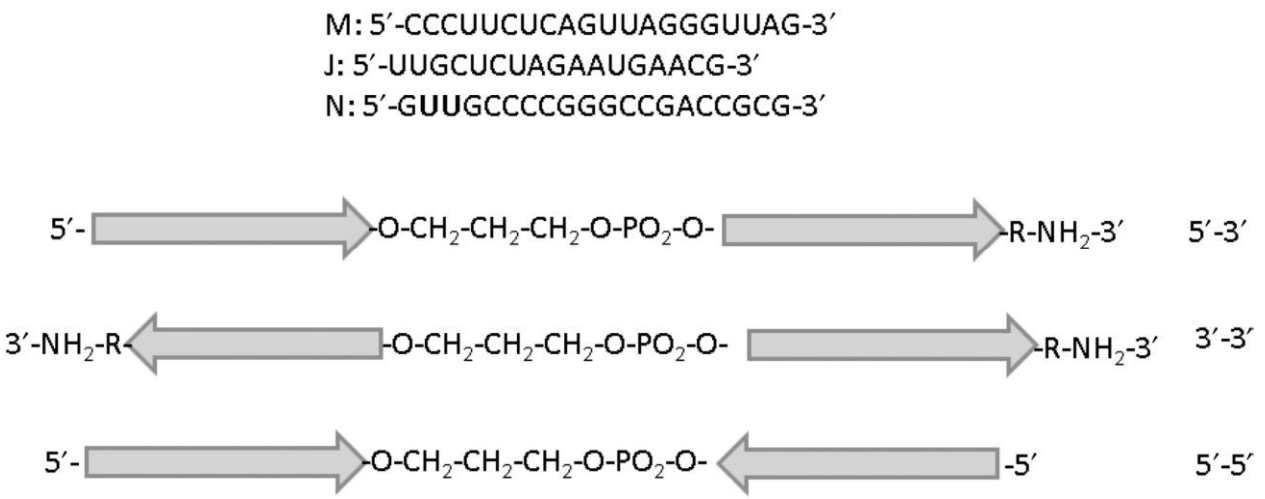

Fig. 2: Schematic representation of chimeric bifunctional oligonucleotides[55]. Arrows directed towards 3'-end of M, J, and N.

To develop the chimeras, the single oligonucleotides were then connected together either via 5'3', 5'-5', 3'-3' ends with 1,3-propane diol linker as shown in Fig. 2[55]. For the 5'-3'chimera Mc3N, the inhibitory effect was slightly better than for M alone with IC_{50} of 35 nM. In case of the 5'5'chimera M*c3N (inverted M) and 3'-3' chimera Mc3N* (inverted N), the inhibitory activity increased almost two times when compared to Mc3N with IC_{50} of 15.7 and 13.5 nM respectively. For Nc3M chimera, inhibitory activity decreased with IC_{50} of 76 nM. In the case of J containing chimeras the inhibitory activity significantly reduced for Jc3N in comparison with J alone and was not detectable in inverted forms. Mc3M showed very little inhibitory effect, although M*c3M (first M inverted) showed an IC_{50} value of 11.9 nM[55].

DISCUSSION

The ultimate role of a therapeutic antisense oligonucleotide is to hinder the transfer of a genetic information into the corresponding protein. To achieve this target, an oligonucleotide can either act as a steric blocker to inhibit the translation machinery or lead to mRNA degradation via the activation of RNase H enzyme that hydrolyzes RNA strand of RNA/DNA duplex[56].

A chemically non modified oligonucleotide is a polynucleotide made of single stranded DNA or RNA molecule in which nucleotide units are bonded together via phosphodiester bonds. Oligonucleotides are designed to bind complementary to a specific sequence in one of the targets contained in cells. Intracellular endonucleases and exonucleases rapidly degrade oligonucleotides to the mononucleotide monomers which then dephosphorylated to the corresponding nucleosides that were shown to explore cytotoxic and antiproliferative effects[57]. This provides a strong rationale for the development of new generations of chemically modified oligonucleotides that function mainly to resist enzymatic degradation and hence provide better kinetics and safety profiles. Methylphosphonate oligonucleotides are among the first

chemically modified oligonucleotides developed in which the non-bridging oxygen atom of the phosphate group is replaced by methyl group along the whole nucleotide chain[58]. Despite the optimum biological stability of these neutral oligonucleotides, they have shown poor solubility and cellular uptake which might be attributed absence of charges. This could explain why such a category of oligonucleotides is not developed as telomerase antisense inhibitors.

Another important modification on oligonucleotides structure involves replacing the 2'-hydroxyl function on the ribose sugar by O-alkyl moiety, most commonly methyl. This kind of modification is to save the charge of the oligonucleotide so as to confer appropriate solubility and cellular uptake. Moreover, this kind of modification is found to grant the oligonucleotide more stability through formation of high melting heteroduplexes with the target RNA and better affinity as well.

This is obvious when comparing the IC_{50} value of oligonucleotide **4**[29] to that for oligonucleotides **1**, **2**, and **3**[25]. Phosphorothioates is a group of oligonucleotides in which a non-bridging oxygen atom is substituted by sulfur. This group of oligonucleotides has been widely studied because of their unique chemical and biological features that have led them to be introduced into clinical therapeutic trials[59]. They have been found to be stable against cellular nucleases although their chemical backbone does by no means support this fact. They are able to activate RNase H activity, shown to be highly soluble and have superb antisense activity. These features would explain the better activity of the oligonucleotide **5** relative to **4**. However, some cons have been reported for the phosphorothioate based oligonucleotides, where this backbone has shown length dependent but sequence independent high affinity to different cellular proteins including mainly heparin binding growth factors such as vascular endothelial growth factors, platelet derived growth factors, acidic and basic fibroblast growth factors[60, 61]. Further attempts, therefore, have been developed to outdo the previous noticed problems.

Structural Analysis of a PNA-based oligonucleotide reveals a totally neutral molecule that is supposed to inhibit the target RNA through rather a steric hindrance, RNase H independent, mechanism because these molecules are not substrates for any kind of RNases. They are able to form strongly stable duplexes or even triplexes with DNA or RNA[62]. Despite the inability to recruit RNase H activity, this class of oligonucleotide has shown to be very potent telomerase inhibitors with IC_{50} values in the nano-molar rang, see oligonucleotides **6** and **7**. Absence of electrostatic repulsion as a result of lacking the negative charges along the whole backbone is a possible contributor to the relatively high affinity of this oligonucleotide class. On the other hand, this might be a cause for poor solubility that does not favor the cellular uptake which is the case also in N3'→P5' phosphoramidate and thiophosphoramidate. This might be offset by virtue of the polypeptide bond extended all over the PNA skeleton or the pseudo- peptide bond (PONH, PSNH) on the phosphoramidate and thiophosphoramidate skeleton. The introduction of Lysine moiety to the candidates developed by Shammas *et al.*[30] also adds a plus in this regards. It worth to mention that N3'→P5' thiophosphoramidate (oligonucleotides **14-21**) have shown better telomerase activity than their phosphoramidate analogues[36]. In an explanation of this, gas phase laser spectroscopy studies have proven that despite the smaller electronegativity value of the sulfur relative to oxygen atom, sulfur is able to show a strong potential as oxygen to act as hydrogen bond acceptor. In some situations sulfur would be even stronger acceptor of a hydrogen bond where the system affording the hydrogen bond has a geometry that ensures the best hydrogen bond angle required for maximizing the strength of the bond[63, 64].

The enzyme 2'-5' oligoadenylate synthetase-dependent ribonuclease known as RNase L is an interferon induced ribonuclease that is activated by binding of 2',5'-linked oligoadenylates and is proven to demolish

targeted RNA sequence at the site of activation[65, 66]. That is why the novel oligonucleotide **8**[33] contained 2-5A moiety as a basic structural feature to develop 2-5A-anti-hTR so as to recruit and activate RNase L.

It has been believed that endocytosis and pinocytosis are among the major mechanisms adopted by antisense oligonucleotides to penetrate inside the cells[67]. Oligonucleotides are characterized by being highly hydrophilic, unstable, and easily degraded by nucleases [68] making it an uneasy for cell internalization and nuclear localization which in turn may lead to inadequate pharmacological response.

It is noticeable when viewing the above described telomerase antisense oligonucleotides that several trials have been implemented to enhance cellular uptake and efficiency. Encapsulating the developed nucleic acid derivative in a cationic liposomes, oligonucleotides **8**[33] and **24**[69] lead to dramatic improvement of the telomerase inhibitory activity. Using the developed liposome carrier has lowered the IC_{50} of **24** from 25.24 µM when using the free oligonucleotide to 1.88 µM when it was complexed to the liposome carrier[69]. These cationic carriers show strong affinity to the negatively charged cell membranes and thus improve the cellular penetration of the contained agent. For the same purpose, the positively charged basic Lysine units under physiological conditions has been loaded to oligonucleotides **6** and **7**.

Coupling an oligonucleotide covalently to basic peptides characterized by having the potential of membrane translocation is another attempt to enhance the oligonucleotide penetration through cell membrane to ensure direct delivery to the cytoplasm then into the nucleus[70, 71]. Diala *et al.*[48] have adopted this concept to improve the cellular delivery and hence the telomerase inhibitory activity of oligonucleotide **25**[48].

Coping with the era of nanotechnology, Beisner *et al.* have developed chitosan coated polylactidecoglycolide (PLGA) nanoparticles[72] to mediate efficient delivery of the telomerase inhibitor **5**[29] in a concentration of 4 µM into human non-small lung cancer cells A549 in approximately 80% of which telomerase activity have been detected[73- 75]. During the long-term treatment (15 weeks), it was shown that telomerase activity has been reduced by approximately 80%. Moreover, significant telomerase shortening from 5.9 kb to 4 kb in the human A549 cells was also reported[72].

Lipofectamine mediated gene transfection tactic has been used recently by Qi *et al.*[76] who have constructed recombinant retrovirus vector with an antisense DNA complementary to the telomerase hTERT and introduced it into the ovarian tumor cells ES-2. This delivery approach seemed effective where telomerase activity and the proliferation of the transformed cells were significantly retarded.

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