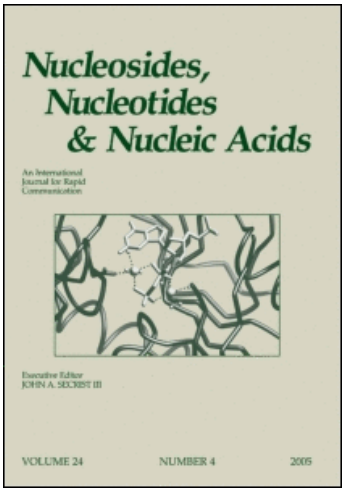

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TELOMERASE INHIBITORS – OLIGONUCLEOTIDE PHOSPHORAMIDATES AS POTENTIAL THERAPEUTIC AGENTS

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ABSTRACT

We have designed, synthesized, and evaluated using physical, chemical and biochemical assays various oligonucleotide N3′ → P5′ phosphoramidates, as potential telomerase inhibitors. Among the prepared compounds were 2′-deoxy, 2′-hydroxy, 2′-methoxy, 2′-ribo-fluoro, and 2′-arabino-fluoro oligonucleotide phosphoramidates, as well as novel N3′ → P5′ *thio*-phosphoramidates. The compounds demonstrated sequence specific and dose dependent activity with IC_{50} values in the sub-nM to pM concentration range.

INTRODUCTION

Human telomerase is a complex ribonucleoprotein reverse transcriptase, containing RNA (hTR) and protein (hTERT) subunits. The enzyme is believed responsible mainly for the synthesis of d-$(TTAGGG)_n$ telomeric repeats at chromosomal ends in constantly dividing cells. Telomerase activity is detected in the vast majority of immortal cell lines, as well as in various primary human tumors (1). At the same time telomerase activity is not detected in most somatic cells. Cells of constantly renewable tissues and germ line cells are the exceptions, where active telomerase is

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present. The observed differences in telomerase activity in normal versus tumor derived cells resulted in the hypothesis, that telomerase may represent a suitable target for the specific anti-cancer therapies. Following this appealing scientific rational, several classes of telomerase inhibitors were prepared and evaluated. Among the compounds tested are the small molecules (2), compounds capable of interacting with G-quadruplex structures (3), which telomeres might potentially form, dominant negative hTERT-derived proteins (4), and various types of oligonucleotides, including the phosphodiesters (5), 2–5A tethered phosphodiesters (6), phosphorothioates (7,8), 2′-O-methyl-phosphorothioate chimera (9), ribozymes (10), and PNA molecules addressed to the template region of hTR domain (7). In the recently reported crucial proof-of-principle studies, the treatment of tumor cell lines with the specific oligonucleotides or protein based telomerase inhibitors resulted in cellular senescence (4,11). The onset of cellular senescence was observed to be in direct correlation with the length of telomeres - cells with relatively short telomeres required much shorter treatments, than the ones with longer telomeres (4,11). Based on these initial encouraging results one can infer, that the inhibition of telomerase *in vivo* in cancer cells can lead to attrition of their telomeres, followed by the tumor cells death.

In this study we summarize our results outlining the design, preparation, characterization and biochemical evaluation of several different types of oligonucleotide N3′ → P5′ phosphoramidates as telomerase inhibitors. The obtained results indicate that these compounds are efficient and sequence specific telomerase inhibitors with a good potential for further development as therapeutic agents.

RESULTS AND DISCUSSIONS

The RNA component of telomerase (hTR), which is approximately 455 nucleotide long represents an attractive target for oligonucleotide based anti-telomerase agents. Among other functionally important regions, it contains a crucial eleven nucleotides long so-called template region, part of which serves as the template for telomere elongation by TTAGGG repeats. Another part of this region may also be involved in chromosomal end recognition by telomerase.

First, we designed and prepared several 2′-deoxyoligonucleotide N3′ → P5′ phosphoramidates addressed to the template region of hTR in order to determine optimal oligonucleotide sequence and length requirements – see Figure 1A. The oligonucleotides were synthesized using phosphoramidite transfer methodology on ABI 392 or 394 DNA/RNA automated synthesizers (12,13).

The manufacturer recommended 1μmole DNA synthetic cycle was used, as well as the 3′-aminonucleoside-containing monomer building blocks and CPG-based solid supports, which were purchased from Annovis Inc. The compounds were analyzed and purified by ion exchange (IE) HPLC, and characterized by ^{31}P NMR, mass spectrometry, and by PAGE. The prepared oligonucleotides were

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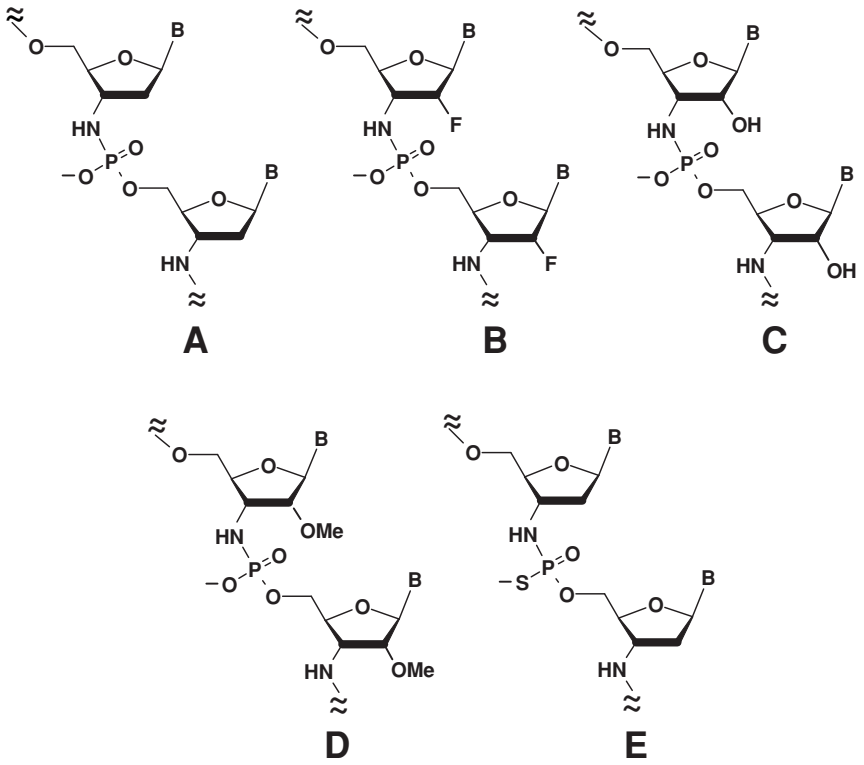

Figure 1. Structure of oligonucleotide N3′ → P5′ phosphoramidates evaluated as telomerase inhibitors.

than evaluated in biochemical assays as telomerase inhibitors, following the determination of the melting temperature (T_m) for their duplexes with the complementary RNA strand. Representative T_m data are presented in Table 3, and the obtained IC_{50} values for telomerase inhibition are summarized in Table 1.

The results indicate that IC_{50} values for the most active compounds reached the sub-nM level. Oligonucleotides with the highest anti-telomerase activity are complementary to a significant part of the hTR template region, particularly to its r-CCC segment. Moreover, anti-telomerase activity of oligonucleotides was highly sequence-dependent. Control compounds with four mismatched bases were practically inactive (more than 1000 times less active than for the fully complementary counterparts) in the assays used. These results are in a good agreement with the corresponding Tm values for the oligonucleotide duplexes: ∼72°C and ∼21°C, respectively, Table 3. The minimal oligonucleotide length for exerting high anti-telomerase activity is no less than six nucleotides, which also correlates well with the ability of these compounds to form stable duplexes with the RNA target – T_m

Table 1. 2′-Deoxyoligonucleotide N3′ → P5′ Phosphoramidates as Telomerase Inhibitors-Template Region

GRN#	5′-Oligonucleotide-3′	HTR[a]	IC_{50}, nM
	hTR3′-AUGCGGGAAGAGUCAAUCCCAAUCUGUU-5′[b]		
135929	GTTAGGGTTAG	56-46	0.63
135935	GTT*GA*G *TG*TAG [c]	56-46	>1000
135993	GUUAGGGUUAG	56-46	0.8
135995	GTTAGGGTTAGAC	56-44	2.4
135998	GTTAGGGTTAGACAA	56-42	1.1
135931	TTAGGG	55-50	10000
135932	TTAGGGTTAG	55-46	2.9
135939	TTAGGGTTAGGG	55-44	2.4
135970	TAGGGTTAGAC	54-44	2.0
135930	TAGGGTTAGACAA	54-42	0.8
135973	TAGG*TG* T*GA* ACAA[c]	54-42	>1000
136001	GGGTTAG	52-46	600
136003	GGGuuAG[d]	52-46	280
136000	GGGTTAGAC	52-44	5.9
136012	GGGuuAGAC[d]	52-44	<3.2
135933	AGTTAGGGTTAG	57-46	0.38
135934	CAGTTAGGGTTAG	58-46	0.47
136004	CAGTTAGGGTT	58-48	32
136995	CAGTTAGGG	58-40	32
135971	CCCTTCTCAGTT	65-54	32
136002	CGCCCTTCTCAG	67-56	40
136009	TACGCCCTTCTC	69-58	>1000
136017	CAGuuAGGGuu[d]	58-48	9
136018	GuuAGGGuu[d]	56-48	50

[a]Position of oligonucleotides on hTR; [b]sequence of hTR target, where template region is underlined; [c]mismatched nucleosides are underlined; [d]2′-fluoro-3′-aminonucleosides are depicted as low case u.

for the 2′-deoxy N3′ → P5′ phosphoramidate hexanucleotide TTAGGG is ∼36°C under the assay buffer conditions.

Using oligonucleotide phosphoramidates we were also able to identify two other sites on hTR, in addition to the template hTR regions, that were susceptible to the oligonucleotide-induced telomerase inhibition-see Table 2.

The most active non-template addressed oligonucleotide was a 15-mer complementary to nucleotides 137–151 of hTR. It inhibited telomerase activity in the biochemical assays with an IC_{50} value of ∼0.4 nM. Other oligonucleotide compounds addressed to the adjacent hTR regions were significantly less active, except the 20-mer GRN 137155, Table 2. It is likely, that the hybridization of the 15-mer oligomer to its hTR target site which, as predicted by molecular modeling, interferes with formation of a pseudo knot structure (14), and results in a significant structural rearrangements of entire hTR molecule. This structural alteration of hTR consequently may lead to the inactivation of the whole telomerase enzyme *via* changing the spatial positioning of hTERT catalytic site

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Table 2. 2′-Deoxyoligonucleotide N3′→P5′ Phosphoramidates as Telomerase Inhibitors-Non-Template Regions

GRN#	5′-Oligonucleotide-3′	hTR	IC_{50}, nM
135940	GCTCTAGAATG	166-156	>1000
135941	GCTCTAGAATGAA	166-154	>1000
136006	GCGGCCTGAAAG	367-356	>1000
136007	TCCGTTCCTCTT	382-370	>1000
136008	GCGGGGAGCAAA	92-80	>1000
137159	GTGGAAGGCGGCAGG	151-137	0.42
137158	GCTCTAGAATGAACGGTGGAAGGCGGCAGG	166-137	1
137155	ACATTTTTTGTTTGCTCTAG	179-159	2–10

relative to the template region of hTR, or through rendering the hTR template region partially double stranded and inaccessible to telomere-like DNA primers. The 20-mer GRN 137155, Table 2, designed to hybridize to a predicted stem-loop hTR structure (14), may inhibit telomerase in a similar way. Detailed mechanism of action of non-template region addressed oligonucleotides is currently under further investigation.

Additionally, a second generation of anti-telomerase oligonucleotide N3′ → P5′ phosphoramidates with modifications at the 2′-position of the nucleoside sugar ring was designed and synthesized (12). Evaluation of these 2′-modified phosphoramidate compounds allowed for the assessment of the influence of oligonucleotide sugar-phosphate backbone structure on their activity. Several 11- to 13-mer oligonucleotides, that were isosequential to the most active 2′-deoxy - N3′ → P5′ phosphoramidate counterparts, addressed to the hTR template, were prepared. These modified phosphoramidate oligonucleotides contained 2′-fluoro, 2′-hydroxyl or 2′-O-methyl substitutions–see Figure 1B,C,D. These compounds were assembled and isolated similarly to the 2′-deoxyoligonucleotide phosphoramidates using appropriately protected 3′-amino-5′-phosphoramidite nucleoside building blocks (12). Duplex formation properties of these 2′-substituted oligonucleotide phosphoramidates with RNA strands were evaluated using thermal de-naturation experiments and the corresponding duplex T_m values are summarized in Table 3. Substitution of the oligonucleotide phosphoramidate 2′-hydrogen atom with either a 2′-hydroxyl, (resulting in generation of the natural RNA mimetic), or a 2′-O-methyl, or a 2′-fluoro group results in a noticeable stabilization of the duplexes, formed by these 2′-modified compounds with their RNA complements.

The most thermally stabile duplexes were formed by oligonucleotides containing 2′-fluoro groups. The observed stabilization of the duplexes is likely dictated by the increase in proportion of C3′-endo or N-type of nucleoside sugar puckering for the 2′-substituted oligonucleotide phosphoramidates.

The 2′-modified oligonucleotide phosphoramidates were evaluated as telomerase inhibitors in biochemical assays, and the results are summarized in Table 4. The data obtained indicate, that the activity of studied oligonucleotide

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Table 3. Oligonucleotides and Melting Temperatures (T_m) of Their Duplexes with RNA Strands

Expt	5′-Oligonucleotide-3′	Type[a]	T_m,°C	ΔT_m,°C[b]
1	GTTAGGGTTAG	*d/po*	44.2	–
2	TAGGGTTAGACAA	*d/po*	45.2	–
3	GTTAGGGTTAG	*d/np*	72.1	27.9
4	TAGGGTTAGACAA	*d/np*	72.4	27.2
5	TAGG*TG*TA*AG*CAA	*d/np*	∼21	−51.3[c]
6	GUUAGGGUUAG	*r/np*	80.0	35.8
7	TAGGGUUAGACAA	*r/np*	82.0	37.8
8	GU^{f}U^{f}AGGGU^{f}U^{f}AG	*2′-F/np*	84.3	40.1
9	GTTAGGGTTAG	*d/nps*	71.5	27.3
10	TAGGGTTAGACAA	*d/nps*	70.0	24.8
11	GTTAGGGTTAG	*2′-OMe/np*	78.2	34.0
12	TAGGGTTAGACAA	*2′-OMe/np*	82.3	37.1

[a]*po, np, 2′-F, nps and 2′-OMe* correspond to phosphodiester, N3′ → P5′ phosphoramidate, 2′-ribofluoro- N3′ → P5′ phosphoramidate, N3′ → P5′thiophosphoramidate, and 2′-OMe- N3′ → P5′ phosphoramidate internucleoside groups, respectively; [b]ΔT_m relative to isosequential natural phosphodiester counterparts; [c]ΔT_m relative to fully complementary compound, exp. 4, and the mismatched nucleosides are underlined.

phosphoramidates is dictated primarily by their ability to hybridize and form stable complexes with their target sites on hTR, and not by the nature of their sugar phosphate backbone. The more DNA-like 2′-deoxy phosphoramidates have similar activity to their more RNA-like 2′-substituted counterparts. Importantly, these

Table 4. Effects of Oligonucleotide Structure on Telomerase Activity

Expt#	5′-Oligonucleotide-3′	Type [a]	IC_{50},nM
1	TAGGGTTAGACAA	*d-np*	0.8
2	TAGGGTTAGACAA	*d-nps*	0.4
3	TAGGGTTAGACAA	*r-np*	0.5
4	TAGGGTTAGACAA	*r-npm*	0.5
5	TAGGGTTAGACAA-Fluor[b]	*d-np*	0.75
6	GTTAGGGTTAG-Fluor[b]	*d-np*	0.3
7	TDGGGTTDGDCDD	*d-np*	0.3
8	TAGG*TG*TA*AG*CAA[c]	*d-np*	>1000
9	TAGG*TG*TA*AG*CAA[c]	*d-nps*	110
10	CAGTTAGGGTTAG	*d-np*	0.6
11	CAGTTAGGGTTAG	*d-nps*	0.05

[a]*np*, *nps*, *npm* correspond to N3′→P5′ phosphoramidate, thiophosphoramidate and 2′-OMe-phosphoramidate oligonucleotides respectively; [b]Fluor correspond to fluorescein attached to 3′-aminogroup; [c]mismatched nucleosides are underlined.

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results strongly suggest, it is unlikely that the anti-telomerase activity of various types of DNA- and RNA-like oligonucleotide phosphoramidates results from any specific, but sequence independent, high affinity interactions with only the protein component of telomerase - hTERT.

Additionally, a new class of oligonucleotide analogues – oligonucleotide N3′ → P5′ *thio*-phosphoramidates (Fig. 1E) was prepared and evaluated as telomerase inhibitors. These compounds retain high affinity to complementary RNA strands, similar to the parent phosphoramidates, yet are much more acid resistant (15). It was demonstrated before, that the oligonucleotide phosphorothioates are efficient, but highly not sequence specific inhibitors of telomerase (7,16), and that the main site of their interaction with the enzyme was likely the protein hTERT, rather than the RNA component, hTR (8). We decided to combine the attractive properties of two classes of oligonucleotides – high RNA binding affinity of the phosphoramidates and protein interactive capabilities of oligonucleotide phosphorothioates in a single molecule. The structure of oligonucleotide N3′ → P5′ phosphoramidates may allow these compounds to bind in a sequence specific manner to the targeted template region of hTR, and then create, *via* PS-groups-protein contacts, secondary stabilizing interactions with the regions of hTERT in proximity to the hTR hybridized oligonucleotide.

Oligonucleotide N3′ → P5′ *thio*-phosphoramidates were first tested in biochemical assays, and the results are summarized in Table 4. The data demonstrate high and sequence specific telomerase inhibiting activity for the template region addressed *thio*-phosphoramidate oligonucleotides. They were as active, or more active than the isosequential phosphoramidate oligonucleotides. The IC_{50} value for the most potent 13-mer reached ∼50 pM, exp. 11, Table 4. Notably, mismatched control compounds were significantly less active (IC_{50} values were 100–250 folds higher), than the fully complementary oligonucleotides. At the same time, the *thio*-phosphoramidate mismatched control compounds were more active telomerase inhibitors, that the isosequential phosphoramidates, but significantly less active then the mismatched or random sequence oligonucleotide phosphorothioates. This would suggest that *thio*-phosphoramidate compounds might have some sequence – independent affinity for hTERT protein, which is in turn significantly lower, than that for the oligonucleotide phosphorothioates.

We next studied the effects of oligonucleotide N3′ → P5′ phosphoramidates on telomerase activity *in vitro* in cells. Various immortal cell lines with different levels of telomerase activity were tested, and telomerase activity was determined as a function of the oligonucleotide concentration, both in presence or absence of lipid-based uptake enhancers. The results are presented in Table 5. A typical gel profile with *in vitro* analysis of inhibition of telomerase activity by oligonucleotides is presented in Figure 2.

The collected data indicate, that oligonucleotide phosphoramidates inhibit telomerase activity in the majority of tested cell lines in a dose and sequence dependent manner. The observed IC_{50} values for telomerase inhibition by lipid

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Table 5. IC_{50} Values for *In Vitro* Inhibition of Telomerase by 2′-deoxyoligonucleotide N3′ → P5′ Phosphoramidate–TAGGGTTAGACAA

	IC_{50}, μM[a] Lipids	
Cell Type	(−)	(+)
U87	5	nd
293	5	0.1
ACHN	>100	0.3
Caki-1	>100	0.2
PC-3	~30	0.2
A431	>100	0.3
A549	>100	>10
HME50-5E	20	0.5
BJ-hTERT	10	0.5

[a]IC_{50} Value for MM control TAGG*TG*TA*AG*CAA with or without lipids: >100 μM.

carrier-formulated oligonucleotide phosphoramidates are significantly lower, then those for the non-formulated compounds. This may suggest relatively inefficient cellular uptake or unfavorable intracellular distribution of oligonucleotide phosphoramidates in the cell lines used. Importantly, inhibition of telomerase activity in cells by oligonucleotide phosphoramidates resulted in the concomitant reduction in the length of their telomeres, as was expected from the model for the role of telomerase in telomere maintenance.

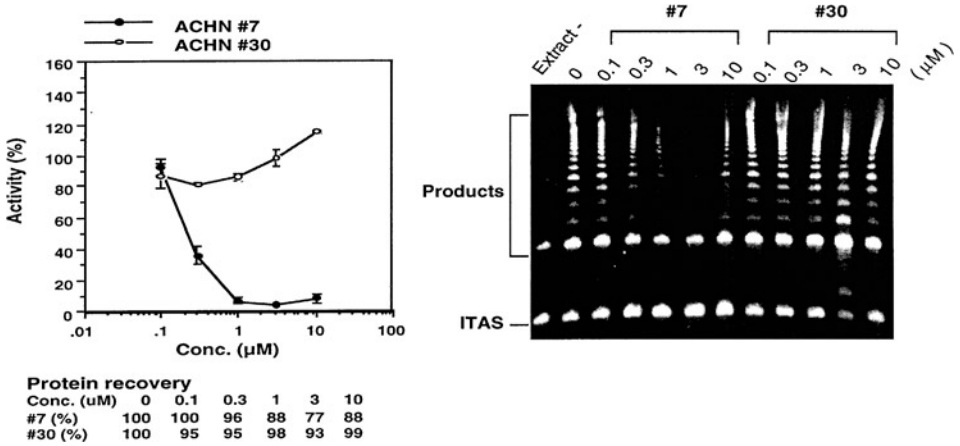

Figure 2. Analysis of telomerase inhibition in ACHN cells treated for 24 hours with Lipofectamine 2000 formulated oligonucleotide phosphoramidates by TRAP assay; #7 and #30 correspond to compounds GRN 135930 and 135973 in **Table 1**, respectively; ITAS is an internal control. Total protein recovery was measured to demonstrate the lack of acute cytotoxicity for the phosphoramidate oligonucleotides.

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The obtained results demonstrate that the properly formulated oligonucleotide phosphoramidates can be used as highly efficient and specific telomerase inhibitors in cells. Further studies may identify cell lines where the used phosphoramidates will be more efficacious without the help of uptake enhancers. Moreover, *in vivo* animal models, where cellular uptake and biodistribution might be very different from the oligonucleotide behavior in cells, may serve as better indicators of the therapeutic potential for the telomerase inhibiting phosphoramidates.

Oligonucleotide N3′ → P5′ *thio*-phosphoramidates are currently under *in vitro* and *in vivo* evaluations and the results will be reported in a due course.

In summary, several classes of oligonucleotide N3′ → P5′ phosphoramidates were prepared and studied as telomerase inhibitors. The telomerase inhibiting activity of these compounds is dependent on their ability to form stable duplexes with critical segments of the telomerase RNA component hTR, and not by the nature of their sugar phosphate backbone. The most active oligonucleotides exerted IC_{50} values for sequence specific telomerase inhibition in biochemical assays in the sub-nM to pM range of concentrations, and in cells IC_{50} values were in nM to μM concentration range. The results obtained warrant further development of these compounds as efficient telomerase activity regulating agents with good therapeutic potential.

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