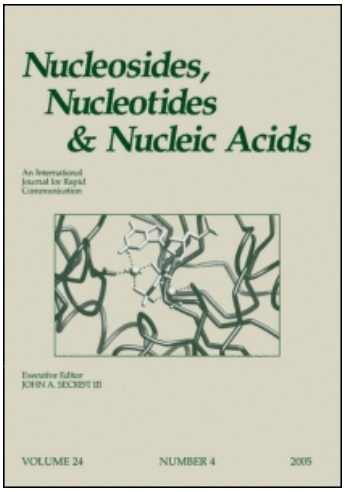

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 09 August 2003

To cite this Article Gryaznov, Sergei , Asai, Akira , Oshima, Yuko , Yamamoto, Yoshihiro , Pongracz, Krisztina , Pruzan, Ronald , Wunder, Ellen , Piatyszek, Mieczyslaw , Li, Shihong , Chin, Allison , Harley, Calvin , Akinaga, Shiro and Yamashita, Yoshinori(2003) 'Oligonucleotide N3' → P5' Thio-phosphoramidate Telomerase Template Antagonists as Potential Anticancer Agents', Nucleosides, Nucleotides and Nucleic Acids, 22: 5, 577 — 581
To link to this Article: DOI: 10.1081/NCN-120021958
URL: http://dx.doi.org/10.1081/NCN-120021958

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 22, Nos. 5–8, pp. 577–581, 2003

Oligonucleotide N3′ → P5′ Thio-phosphoramidate Telomerase Template Antagonists as Potential Anticancer Agents

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ABSTRACT

Human telomerase is a reverse transcriptase that is expressed in essentially all cancer cells, but not in the vast majority of normal somatic cells. Therefore, the specific inhibition of telomerase activity in tumors might have significant beneficial therapeutic effects. We have designed and evaluated oligonucleotide N3′ → P5′ thio-phosphoramidates as telomerase template antagonists. In biochemical cell-free assays 11-13-mer thio-phosphoramidate oligonucleotides demonstrated sequence specific and dose dependent inhibition of telomerase with pico-molar IC_{50} values. Optimization of the oligonucleotide sequence and length resulted in the identification of a 13-mer-oligonucleotide *thio*-phosphoramidate GRN163 as a drug development candidate. In cell cultures GRN163 was able to inhibit telomerase activity in the absence of cationic lipid with ~1 μM IC_{50} values. Telomerase inhibition by GRN163 produced gradual telomere shortening, followed by cellular senescence and/or apoptosis of cancer derived cell lines.

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DOI: 10.1081/NCN-120021958
1525-7770 (Print); 1532-2335 (Online)

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Key Words: Telomerase; Inhibitors; GRN163.

INTRODUCTION

Human telomerase is a complex ribonucleoprotein reverse transcriptase containing two essential subunits – RNA (hTR) and protein (hTERT).[1,2] The enzyme synthesizes hexanucleotide $d(TTAGGG)_n$ telomeric repeats at chromosomal ends of dividing cells, employing DNA telomeres as primers. Telomerase activity is detected in the majority of immortal cell lines, as well as in various primary human tumors and reproductive tissues, but not in most somatic cells.[3] Somatic stem cells of constantly renewable tissues have low or transient levels of telomerase activity. The observed differences in telomerase activity in normal versus tumor derived cells led to the hypothesis, that telomerase may represent a suitable target for highly specific anti-cancer therapies. In this work we present our results related to the design, characterization and biochemical evaluation of oligonucleotide N3′ → P5′ thio-phosphoramidates as telomerase inhibitors.

RESULTS AND DISCUSSION

The RNA component of telomerase (hTR), which is 451 nucleotides long, represents an attractive target for oligonucleotide based anti-telomerase agents. Among other putative functionally important regions, it contains a crucial eleven nucleotide long template region, part of which serves as the template for telomere elongation by d-(GGTTAG) repeats.[3] Various types of modified oligonucleotides, including 2′-deoxyoligonucleotide N3′ → P5′ phosphoramidates (NP's) and several 2′-substituted NP's were used for telomerase inhibition studies.[4] Here we describe the development of a new class of oligonucleotide analogues – N3′ → P5′ thio-phosphoramidates (NPS) as telomerase inhibitors. In the design of these compounds we combined attractive features from two types of oligonucleotides: N3′ → P5′ phosphoramidates and phosphorothioates. We attempted to unify in a single type of molecules the high RNA binding affinity and sequence specificity of the phosphoramidates with protein interactive capabilities of phosphorothioate (PS) oligonucleotides. The structure of NPS oligonucleotides, we believed, might allow these compounds first to bind, in a sequence specific manner, to the targeted template region of hTR, and then create, via PS-group-protein contacts secondary stabilizing interactions with the regions of hTERT protein located in proximity to the hybridized oligonucleotide. Several NPS oligonucleotides were prepared and first tested in biochemical (cell-free) assays, (Table 1). The data demonstrate high and sequence specific telomerase inhibiting activity for NPS molecules addressed to the hTR template region. These compounds were noticeably more active than the parental isosequential phosphoramidate oligonucleotides. The IC_{50} values for the most potent 13-mers NPS molecules reached 26–44 pM, (expts 1 and 4, GRN163 and GRN940, Table 1). This increase in activity, relative to cognate NP counterparts (IC_{50} values of 400–600 pM, expt 12, Table 1), might be attributable to the formation (in addition to the base-pairing) of other stabilizing interactions between the oligonucleotide thio-phosphate groups and

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Table 1. Oligonucleotide N3′ → P5′-*thio*–phosphoramidates as telomerase inhibitors – IC_{50} values in biochemical "flash-plate" assay.

Expt.	GRN#	$^{5'}$Oligonucleotide[a]	IC_{50}, nM
1	163	TAGGGTTAGACAA	0.026–0.2
2	776	TAGGGTTAGACA	0.2–0.5
3	777	TAGGGTTAGAC	0.6–1.1
4	940	CAGTTAGGGTTAG	0.044–0.1
5	164	GTTAGGGTTAG	0.2–0.6
6	227	TAGG*TG*TA*AG*CAA	53–112
7	228	GTT*GA*G*TG*TAG	88–274
8	321	TTTTTTTTTT	>1000
9	924	TTGTCTAACCCTA	1000
10	925	TAGGGTTAGACAA ATCCCAATCTGTT	6
11	401	TTAGGG	1200
12	930	TAGGGTTAGACAA[b]	0.4–0.8
13	973	TAGG*TG*TA*AG*CAA[b]	>1000
14	868	TAGGGTTAGACAA[c]	62[d]

[a]All oligonucleotides contain 3′-NHP(O)(S^-)O-5′ *thio*-phosphoramidate linkages and 3′-terminal aminogroup, except compounds in Expts 12, 13 and 14.
[b]Contains 3′-NHP(O)(O^-)O-5′ phosphoramidate linkages.
[c]Contains 3′-OP(O)(S^-)O-5′ phosphorothioate linkages.
[d]Value derived from a TRAP assay.

hTERT, which takes place upon duplex formation with hTR. Importantly, mismatched, a "sense" oriented control, or NPS decathymidylate oligonucleotides were significantly less active having IC_{50} values ~100–1000-fold higher, than fully complementary oligonucleotides (Table 1). Also, GRN163 did not inhibit DNA polymerase from E.coli, T3 RNA polymerase, HIV-I reverse transcriptase or topoisomerase I from calf thymus at concentrations up to 30 μM (data not shown), indicating its high specificity towards telomerase.

Next, we studied the effects of GRN163 on telomerase activity and telomere extension in vitro in cells. Several immortal cell lines of various cancer origins, having different levels of telomerase activity and telomeric lengths were tested. Telomerase activity was measured as a function of the oligonucleotide concentration, in the presence or absence of several lipid-based cellular uptake enhancers 24–72 hours after treatment using a cell-based TRAP assay. The results are summarized in Table 2. GRN163 was able to inhibit telomerase activity in cells even without the presence of cellular uptake enhancers with IC_{50} values of ~0.3 to 1 μM (Table 2). This is in contrast to the parental NP compound GRN930 (as in expt 12, Table 1), where IC_{50} values were >20 μM without lipid carriers, and ~0.1–0.3 μM with lipid formulations (data not shown and ref. 4). Mismatched or sense-oriented control oligonucleotides, in general, had no noticeable effect on telomerase activity in the cells at concentrations up to 30 μM.

GRN163, when formulated with lipids or peptide-based cellular up-take enhancers, was even more efficacious as a telomerase inhibitor: IC_{50} values were observed

Table 2. Inhibition of Telomerase in vitro (IC_{50} values) by oligonucleotide N3′ → P5′-*thio*-phosphoramidate GRN163.

Cell Type/Origin	TRF, kb	IC_{50}, μM Carriers[a]		p53 Status[c]
		(−)	(+)	
HME50-5E/breast epith.	4.5	0.5	0.0008	*mu*
Caki-1/ renal carcinoma	2.3	0.3	<0.001; 0.01[b]	*wt*
DU145/ prostate adenocarc.	2.8	0.5	n.d.	*mu*
A431/ epidermoid	4.5	1.0	<0.001	*mu*
HT-3/cervix carcinoma	3.0	1.0	n.d.	*wt*
ACHN/renal carcinoma	2.1	0.3	0.001	*wt*
A549 /lung carcinoma	6.5	0.5	n.d.	*wt*
SW620 /colon carcinoma	2.6	0.5	n.d.	*mu*
786-O/kidney	3.4	0.5	n.d.	*mu*
K562 /leukemia	7.3	1.0	n.d.	*mu*

Carriers are: [a]Lipofectamine, or Lipofectamine 2000, or FuGene6.
[b]MPG Peptide Carrier; MPG is a "cage-forming" 27- amino acid long peptide used for cellular delivery of plasmid DNA and oligonucleotides (see Ref.[5]).
[c]*wt* and *mu* correspond to wild type and mutant p53, respectively.

in the range of 1–10 nM, Table 2. This result strongly suggests that one of the possible ways for the further improvement of the anti-telomerase activity of NPS compounds in cells is through optimized formulations. Inhibition of telomerase activity in cancer cells (with relatively long telomeres) during long-term (more than 3 days) treatment with GRN163 was accompanied by gradual shortening of telomeres, as determined by telomere restriction fragment (TRF) analysis. The observed telomere length reduction was reversible upon cessation of treatment with GRN163. Withdrawal of the oligonucleotide resulted in apparent restoration of telomeres in A431 and K562 cells.

Importantly, the viability of normal non-cancerous cells, such as human fibroblast lines WI-38 or BJ, both lacking detectable telomerase activity, was not affected by treatment with up to 100 μM of GRN163 for 72 hours. These data underline the specificity of anti-telomerase GRN163 against cancer, but not normal cells.

In summary, oligonucleotide N3′ → P5′ thio-phosphoramidates were prepared and studied as telomerase inhibitors. The activity of these compounds is primarily dependent on their ability to form stable duplexes with the telomerase RNA component.

The oligonucleotide thio-phosphoramidates exerted sequence specific telomerase inhibitory activity with IC_{50} values in the pico-molar concentration range in biochemical assays, while in cell culture IC_{50} values were in the high nM- to low-μM concentration range without uptake enhancers. Treatment of cancer cells with the 13-mer thio-phosphoramidate telomerase inhibitor GRN163 resulted in telomere shortening followed by cellular apoptosis. These findings provide rationale for the development of the telomerase template antagonist GRN163 as a novel and specifically designed anticancer chemotherapeutic agent with increased tumor selectivity.

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