Stereopure oligonucleotide phosphorothioates as human telomerase substrates†‡

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To address the role of sulfur in the telomerase–primer recognition process, we investigated the effects of substitution of natural phosphodiester linked DNA telomerase substrates with phosphorothioate group-containing counterparts. Model dodecanucleotides with either stereorandom or stereopure all-Rp/Sp internucleoside phosphorothioate linkages were evaluated and the stereorandom models found to have slightly reduced affinity for the enzyme.

Human telomerase is a unique reverse transcriptase ribonucleoprotein, containing two essential structural domains: an RNA (hTR) and a protein component (hTERT).[1,2] One of the key cellular functions of this enzyme is telomere length maintenance at chromosomal ends in immortal cancer cells. Telomerase employs the 3′ single stranded overhang of the telomere as a primer and a segment of its RNA subunit as a template for *de novo* synthesis of d-$(TTAGGG)_n$ repeats at chromosomal DNA ends. Protective and *anti*-apoptotic roles of telomerase possibly involving mechanisms independent of the telomere have also been recently suggested.[3,4] Telomerase activity was detected in ~85% of all primary human tumors. At the same time normal somatic cells and tissues either lack telomerase activity or express only low or transient levels in certain stem cells or committed progenitor cell compartments.[5,6] Moreover, tumor cells typically have relatively short, but stable telomeres, while normal cells have long, but gradually shortening telomeres.[7,8]

These features mark telomerase as an attractive, potentially nearly universal and relatively safe anticancer target for rational drug development. Thus, the telomerase template antagonist oligonucleotide N3′ → P5′-thiophosphoramidate lipid conjugate GRN163L (Imetelstat sodium) was recently advanced into multiple (six) Phase I/II human clinical trials in patients with various cancer types. This development promoted a further search for a second generation of more potent and selective telomerase inhibitors. Investigation of the biochemical properties of telomerase also continues. For instance, phosphorothioate oligonucleotides with stereorandom (all-Rp/Sp) internucleoside groups were evaluated as telomerase inhibitors as well as primer substrates.[9] The authors observed an apparent enhancement in telomerase activity when the phosphorothioate primers were used instead of phosphodiester counterparts.

In this work we sought to begin evaluating the role of stereochemistry of internucleoside sulfur atoms in telomerase recognition properties of its primer substrates. Better understanding of this enzyme–DNA substrate interaction process and the effects of special orientation of sulfur and oxygen atoms might allow for rational design of more active and specific telomerase inhibitors.

In vitro telomerase recognizes and binds, with high affinity, to the single stranded d-$(TTAGGG)_n$ telomeric ends. The enzyme uses these telomeres as its primer substrates for further extension by TTAGGG hexanucleotide repeats. Several dodecanucleotide primers comprising the sequence $(TTAGGG)_2$ were designed and prepared for comparative evaluation as telomerase substrates. The sequences and exact chemical structures of these molecules, as well as their hybridization properties are summarized in Table 1. Among these compounds are: stereorandom all-Rp/Sp, stereopure all-Rp, and all-Sp phosphorothioate, stereorandom all-Rp/Sp N3′ → P5′-thiophosphoramidate oligonucleotides, as well as natural non-diastereomeric all-phosphodiester, and N3′ → P5′ phosphoramidate compounds (Fig. 1).

In selecting these molecules we hypothesize that the primer binding domain of telomerase might form specific contacts with the phosphate groups of the DNA substrate. Hence, a replacement of these phosphates by stereopure phosphorothioate groups might provide information about the preferred, (if any preferences in recognition exist at all), side or helical phase of interactions between the substrate's sugar–phosphate backbone and the telomerase protein domain. The stereopure phosphorothioate oligonucleotides were prepared as described.[10] To ascertain the properties of absorption on cationic surfaces of all-Rp and all-Sp oligonucleotides, these compounds were analyzed by ion exchange (IE) HPLC. The cationic surfaces of ion exchange media might be considered as a remote approximation of the positively charged surface of the DNA binding domain of hTERT. The isomeric all-Rp and all-Sp phosphorothioates demonstrated different absorption properties reflected by different retention times under the used IE HPLC conditions, (Fig. 2). Even under alkali conditions at pH 12, when all internucleoside groups and heterocyclic bases are largely ionized, these two isomers interact slightly differently

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Table 1 Oligonucleotides evaluated as telomerase substrates, their relative affinity and melting temperature (T_m) values of their complexes

Experiment number	d-(Oligonucleotide)[a]	Type[b]	Duplex T_m/°C[c] DNA	RNA	G-quad. T_m/°C[d]	Rel. affinity[e]
1	TTAGGGTTAGGG, **1**	PO	49.5	37.0	36.5	1.0
2	TTAGGGTTAGGG, **2**	all-Rp/Sp PS	41.0	29.6	35.5; 51.2	0.4
3	TTAGGGTTAGGG, **3**	all-Rp PS	40.5	30.0	39.4; 54.4	0.9
4	TTAGGGTTAGGG, **4**	all-Sp PS	41.5	28.4	49.6	0.1
5	TTAGGGTTAGGG, **5**	NPS	59.8	60.0	65.6	n.d.
6	TTAGGGTTAGGG, **6**	NP	65.1	64.6	57.7	0.8

[a] Oligonucleotides **1–4** have a 3′-terminal hydroxyl group, and oligonucleotides **5**, **6** have a 3′-amino group. [b] Oligonucleotide structures are designated as in Fig. 1. [c] T_m of duplexes formed with complementary natural phosphodiester DNA: d-(CCCTAA)$_4$ or RNA: r-(UUUGUCUAACCCUAA) strands; sequence of the RNA strand corresponds to the template region of telomerase RNA component hTR; experiments were conducted in PBS, pH 7.4 at oligonucleotide strand concentrations of ~4 μM. [d] T_m of G-quadruplex formed by the oligonucleotide alone in 150 mM KCl, 10 mM sodium phosphate buffer, pH 7.4; UV thermal dissociation curves were recorded at 295 nm (see Fig. 3); two sets of T_m values correspond to biphasic transitions observed for compounds **2** and **3**. [e] Relative affinity of the tested oligonucleotides for telomerase; the values were derived from the enzyme–primer dissociation curves (Fig. 4A and B); affinity of natural phosphodiester substrate **1** considered to be 1.0; n.d.: not determined.

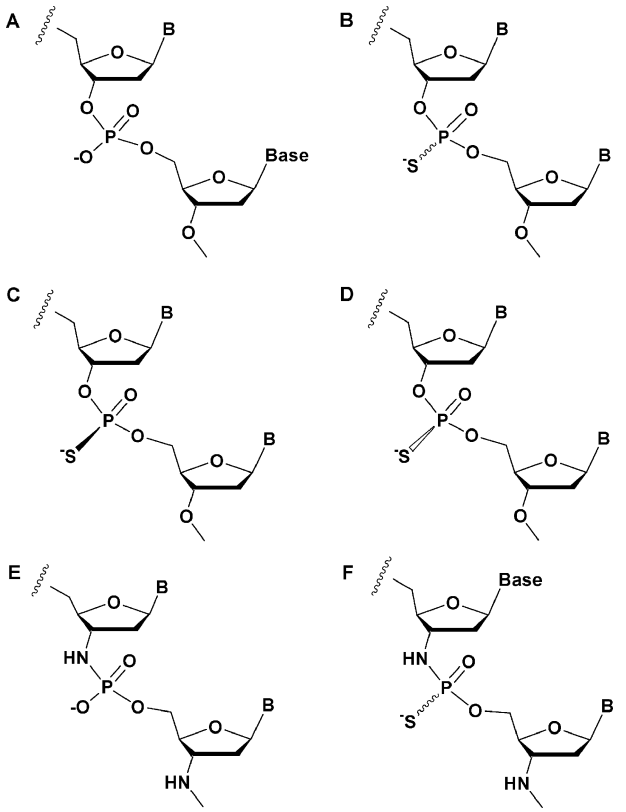

Fig. 1 General chemical structure of the oligonucleotides used in this study: (A) phosphodiester; (B) stereorandom Rp/Sp phosphorothioate; (C) stereopure Sp phosphorothioate; (D) stereopure Rp phosphorothioate; (E) N3′ → P5′ phosphoramidate; (F) N3′ → P5′-thiophosphoramidate.

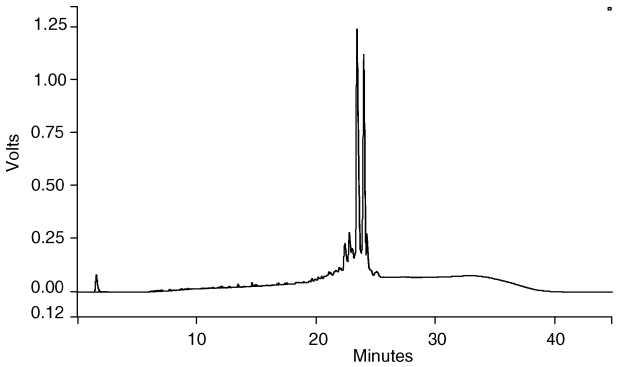

Fig. 2 IE HPLC analysis profile of co-injected all-Rp and all-Sp isosequential dodecanucleotide phosphorothioates d-(TTAGGGTTAGGG). The retention times are 23.41 min and 23.97 min for all-Rp and all-Sp isomers, respectively. The analysis was performed on a Dionex NucleoPak 200, 4.6 × 250 mm ion-exchange column using a linear gradient of buffer B, 1.5% min^{-1} increase rate, from 0% to 45%; flow rate 0.75 ml min^{-1}; temperature 35 °C; Buffer A: 10 mM NaOH, Buffer B: 2 M LiBr in 10 mM NaOH, pH 12; both buffers contain 20% v/v of acetonitrile.

with the IE cationic surface, reflecting the effects of sulfur stereochemistry.

The ability of these telomerase substrates to form duplexes with complementary DNA and RNA strands, as well as G-quadruplex structures was also evaluated using thermal denaturation experiments. The results are summarized in Table 1. The thermal stabilities of the duplexes formed by phosphorothioate oligonucleotides **2–4** were very similar for all three compounds, and lower than that for their phosphodiester counterpart **1**. Oligonucleotide thiophosphoramidate **5** and phosphoramidate **6** formed the most stable duplexes. Significant differences were observed in the thermal stabilities of the G-quadruplex structures formed by all compounds. Also, the shapes and hypochromicity of the thermal dissociation curves (recorded at 295 nm, the wavelength characteristic for detection of guanosine quartet dissociation detection) were also different, (Fig. 3). While the introduction of internucleoside sulfur atoms had little effect on the duplexes, they led to stabilization of the G-quadruplex structures, (*cf.* expts. 1 to 2–4, and 5 to 6, Table 1). In addition, tertiary structures and/or stoichiometries of the G-quadruplexes formed by all-Rp and all-Sp molecules appear to be different, as was judged from the thermal dissociation curves. All-Sp isomer **4** demonstrated a monophasic transition with a T_m of 49.6 °C, with relatively low hypochromicity. At the same time all-Rp

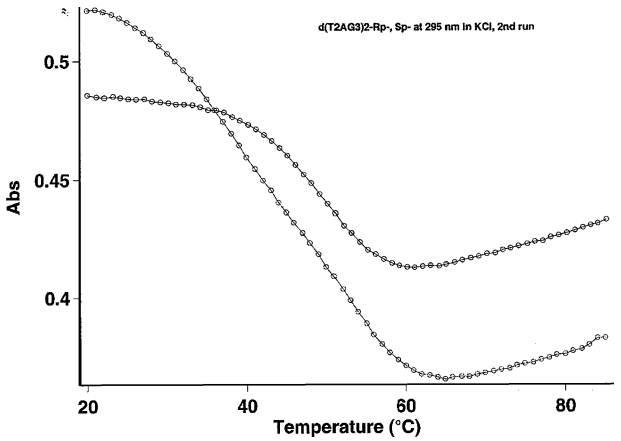

Fig. 3 Thermal dissociation curves for G-quadruplex structures formed by stereopure all-Rp and all-Sp 12-mers **3** and **4**, Table 1, respectively; temperature gradient 1 °C min^{-1}, 150 mM KCl in 10 mM sodium phosphate buffer, pH 7.4. The upper curve (starting at 20 °C) with the larger hypochromic effect corresponds to all-Rp compound **3**, and the lower curve with the smaller hypochromic effect to all-Sp compound **4**.

isosequential compound **3** showed a biphasic curve with estimated T_m values of 39.4 °C and 54.4 °C, with higher hypochromicity, similar to the behavior of the stereorandom all-Rp/Sp counterpart **3**, (Fig. 3). Further experiments, including high resolution NMR or X-ray crystallography studies, should be done to better understand the exact tertiary structures of the formed G-quadruplexes.

We evaluated the affinity of the phosphorothioate oligonucleotides for telomerase using competition with a standard 24 nucleotide long primer, which has previously been described in ref. 11. In brief, the tested oligonucleotide primers were allowed to bind to partially purified enzyme and then the formed complex was challenged with 200-fold excess of d-$(TTAGGG)_4$ phosphodiester substrate, which has an extremely high affinity for telomerase.[11] At various time points nucleoside triphosphates dTTP and ^{32}P-dATP were added to extend the primer that is bound to the enzyme. By comparing the extension of the original test primer (*i.e.* 12-mer primer converted into 15-mer product *via* addition of 3′-TT^{32}pA nucleotides) with that of the competitive primer extension product, (*i.e.* 27-mer d-$(TTAGGG)_4$-TT^{32}pA oligonucleotide product obtained *via* extension of the 24-mer d-$(TTAGGG)_4$ challenger) allows one to derive the relative affinities (K_{off}) of the primers for telomerase. Thus, natural phosphodiester 12-mer **1** has a demonstrated half-life ($\tau_{1/2}$) of 27.8 min for the telomerase complex. The isosequential stereorandom phosphorothioate **2** showed lower affinity, with a $\tau_{1/2}$ of 10.4 min, (Fig. 4A). This observation is somewhat different from the previously reported findings of higher affinity (by ~6.7-fold) of stereorandom phosphorothioate primer for telomerase relative to isosequential phosphodiester compound.[9] This difference in relative affinities is likely related to the following factors: (a) the assays used—a PCR based TRAP assay in ref. 9 *vs.* a direct assay for our experiments, and importantly (b) primer lengths and their nucleotide sequences. In the prior work the authors used 20-mer primers with a 5′-TCAGACATATACTGCTCAAGA non-telomeric sequence,[9] whereas telomere-derived 12-mers d-$(TTAGGG)_2$ were used for this study. It was shown before that telomere derived oligonucleotides d-$(TTAGGG)_n$, which are structurally similar/identical to the "native" telomerase substrates, have significantly higher affinity for telomerase than other oligonucleotides have. This affinity is increased even further by the presence of a -GGG-3′ terminus in these primers.[11] Hence, for our studies we used human telomere sequence based oligonucleotides, which are as close as possible to natural telomerase substrates. Furthermore, the previous study used crude cellular lysate instead of purified enzyme, which has been observed to increase the apparent K_m of 18-mer telomeric primers by at least 10-fold (R. Pruzan, unpublished observation). A difference in hydrolytic stability of the phosphodiester *vs.* phosphorothioate primers in the crude cellular lysate may also be a contributing factor.

Interestingly, stereopure all-Rp primer **3** had significantly higher telomerase affinity than its all-Sp counterpart **4**, with complex $\tau_{1/2}$ values of 25.0 min and 2.9 min, respectively, (Fig. 4B). The apparent affinity of the all-Rp isomer was noticeably (~2.4-fold) higher than that for stereorandom phosphorothioate **2**, ($\tau_{1/2}$ 25.0 *vs.* 10.4 min), and close to that for natural phosphodiester **1**, ($\tau_{1/2}$ 25.0 *vs.* 27.8 min). The relative affinities of these substrates for telomerase are summarized in Table 1.

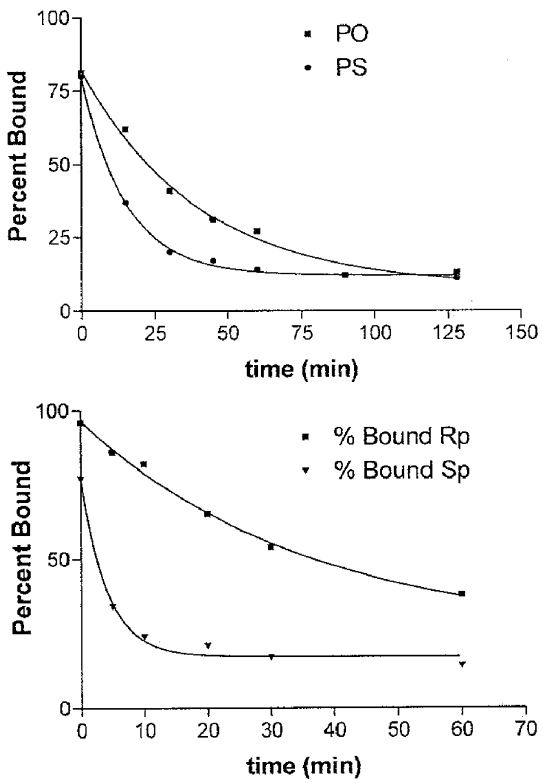

Fig. 4 Dissociation curves for the enzyme–primer substrate complexes formed by: (A) phosphodiester **1** and stereorandom phosphorothioate **2** substrates with telomerase; the complex half life ($\tau_{1/2}$) was 27.8 min and 10.4 min, respectively; (B) stereopure oligonucleotide phosphorothioates all-Rp **3** and all-Sp **4**; the complex half life ($\tau_{1/2}$) was 25.0 min and 2.9 min, respectively. The oligonucleotide sequences are in Table 1.

The observed difference in affinity of all-Rp and all-Sp isomers likely stems from the different interactions formed by the sulfur atoms with the DNA–primer binding site of telomerase protein component hTERT, since the thermal stabilities of the duplexes formed by these molecules with hTR are essentially the same, (Table 1). Moreover, the estimated telomerase affinity of ionic non-chiral sulfur-less oligonucleotide N3′ → P5′ phosphoramidate **6** was slightly lower than that for phosphodiester counterpart **1**: $\tau_{1/2}$ 23.0 min *vs.* 27.8 min, despite the highest duplex thermal stability with hTR, 64.6 °C and 37.0 °C, respectively, (Table 1). The data indicate that these 12-mer d-$(TTAGGG)_2$ based substrates interact primarily with the DNA binding site of telomerase hTERT, and not with hTR (otherwise the affinity of phosphoramidate **6** for telomerase would have been noticeably higher than that for phosphodiester **1** or phosphorothioate **3**; also see ref. 11). The results also suggest that Sp-oriented sulfur atoms disrupt or destabilize potential contacts formed by internucleoside phosphate groups with hTERT within the DNA primer binding site. These contacts could be of either electrostatic or steric origin.

It was previously noted that interaction of oligonucleotide phosphorothioates with other proteins, *i.e.* either basic fibroblast growth factor, or with laminin and fibronectin are independent of the thiophosphate linkage chirality.[12] We would like to suggest that phosphorus chirality probably may play a role when the protein of interest forms specific and direct contacts with the substrate/inhibitor internucleoside phosphate groups, such as telomerase.

Additionally, oligonucleotides **1–4** were evaluated as telomerase primers. The results of the assay are summarized in Fig. 5. There was no apparent difference in the ability of these oligonucleotides to a serve as telomerase primers, regardless of their phosphorus stereochemistry. All four oligonucleotides were able to generate telomerase specific six nucleotide long ladder, as was judged from PAGE analysis. Both the enzyme processivity and efficiency of the primer elongation were not noticeably affected by the oligonucleotide chirality, or by the presence of sulfur atoms in the sugar–phosphate backbone. It was previously reported that 18-mer phosphorothioate group-containing primers, (based on so-called TS primers, used for PCR based TRAP assays), were more efficient than their phosphodiester counterpart.[9] The apparent difference between the prior report and our finding is likely determined by the difference in the length and the sequence of the primers: telomere derived 12-mer's d-$(TTAGGG)_2$ *vs.* relatively low affinity non-telomeric 18-mer d-(AATCCGTCGAGCAGAGTT).[11]

In summary, stereopure oligonucleotide phosphorothioates with all-Rp or all-Sp internucleoside linkages were evaluated as human telomerase substrates. It was shown that these compounds exhibit different affinity for the enzyme, with all-Sp isomer forming noticeably less stable complexes. These molecules also form G-quadruplex structures with different thermal stabilities and structures. Duplexes formed by the stereopure and stereorandom phosphorothioates with DNA and RNA exhibit a similar stability, however. The obtained results warrant further and detailed investigation of the exact tertiary structure of G-quadruplexes formed by stereopure phosphorothiates, and evaluation of this molecule type for potential telomerase inhibitors.

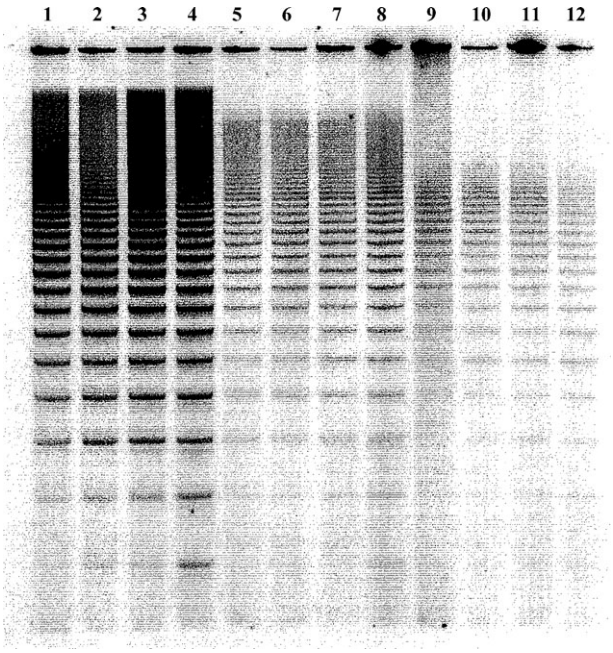

Fig. 5 Gel electrophoresis analysis (PAGE) of DNA products obtained by extension of phosphodiester (PO) and phosphorothioate (PS) primers by the purified telomerase. Lanes 1, 5 and 9: all-Sp PS primer **4** after 60 min, 20 min and 7 min, respectively; lanes 2, 6 and 10: all-Rp PS primer **3** after 60 min, 20 min and 7 min, respectively; lanes 3, 7 and 11: stereorandom all-Rp/Sp PS primer **2** after 60 min, 20 min and 7 min, respectively; lanes 4, 8 and 12: PO primer **1** after 60 min, 20 min and 7 min, respectively. Analysis was conducted in 12% denaturing polyacrylamide gel, and each band corresponds to the primer extension by six nucleotides d-(TTAGGG).

Experimental section

Natural phosphodiester and stereorandom Rp/Sp phosphorothioate d$(TTAGGG)_{2,4}$ oligonucleotides were purchased from TriLink Inc., (San Diego, CA). Stereopure Rp and Sp d-$(TTAGGG)_2$ phosphorothioates were prepared as described before.[10] Oligonucleotide thermal dissociation experiments were conducted using a Cary 1E UV/VIS spectrophotometer. A temperature gradient of 1 °C per minute and oligonucleotide strand concentrations of ~4 μM for duplexes or ~10 μM for G-quadruplex stability studies were used. Human telomerase was isolated and partially purified (approximately 10 000 fold) as described in ref. 11. The affinity of the tested oligonucleotides for the partially purified human telomerase (K_{off} and $\tau_{1/2}$) was determined using a competition assay, which has previously been described in ref. 11. In brief, any tested oligonucleotide primer/substrate was allowed to bind to the enzyme at 30 °C, and then the formed complex was challenged with a 200-fold molar excess of high-affinity d$(TTAGGG)_4$ native phosphodiester telomeric substrate. At various time points aliquots were removed and enzyme-associated primer/substrate was identified by extending and labeling for three minutes with nucleoside triphosphates dTTP and ^{32}P-dATP. The resulting products of enzymatic extension of the test

primers or the competitor by three nucleosides (-TT-^{32}P-A) were analyzed using 18% denaturing PAGE (containing 7 M urea) and quantified using PhosphorImager (Molecular Dynamics). K_{off} and $\tau_{1/2}$ were derived using the Prizm graphical software package (GraphPad Sofware Inc.)

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