1152–1158 *Nucleic Acids Research, 1999, Vol. 27, No. 4*

Telomerase protein rather than its RNA is the target of phosphorothioate-modified oligonucleotides

E. Matthes* and Ch. Lehmann

Max-Delbrück-Centrum für Molekulare Medizin, Robert-Rössle-Straße 10, D-13092 Berlin, Germany

Received September 21, 1998; Revised and Accepted December 22, 1998

ABSTRACT

Human telomerase is a ribonucleoprotein which uses its internal RNA moiety as a template for telomeric DNA synthesis. This enzyme is up-regulated in most malignant tumors and is therefore considered as a possible cancer target. Here we examined the effects of differently modified oligomers on telomerase activity from HL-60 cell extracts (TRAP-eze™ assay). Phosphorothioate-modified oligonucleotides (PS-ODNs) inhibited telomerase activity at subnanomolar concentrations and proved to be more efficient than peptide nucleic acids. In contrast to all the investigated oligomers, PS-ODNs were found to bind to the protein motif of telomerase called the primer binding site but poorly to its RNA. This is suggested by kinetic investigations demonstrating a competitive interaction of PS-ODNs and TS primer at the primer binding site. The K_m value of the TS primer was 10.8 nM, the K_i value of a 20mer PS-ODN was 1.6 nM. When the TS primer was PS-modified a striking increase in the telomerase activity was found which correlates with the number of phosphodiesters replaced. The K_m value of a completely PS-modified TS primer was 0.56 nM. Based on these results the design of chimeric ODNs is proposed consisting of a 5′-PS-modified part targeting the primer binding site and a 3′-terminus part targeting the telomerase RNA.

INTRODUCTION

Telomerase is a unique ribonucleoprotein polymerase utilizing its own RNA component as a template for the synthesis of multiples of telomeric repeats onto the end of replicating chromosomes. Originally identified in the ciliated protozoan *Tetrahymena thermophila* (1,2) it was subsequently detected in human cells (3). The extension mechanism of telomerase compensates for the loss of telomeric DNA associated with each round of DNA replication. However, most somatic cells lack telomerase, thus telomere length decline is thought to limit their proliferative capacity and to determine cellular senescence (4).

On the other hand, an activation of telomerase seems to be required for the sustained growth potential of malignant tumor cells, stem cells of renewable tissues and of germ cells (5). In 85–95% of advanced malignant human tumors telomerase activity was detected (6,7).

These findings have led to an increasing interest in developing selective inhibitors of telomerase to understand its biological role in cancer and to determine whether anti-telomerase therapy might present a new anticancer strategy (8–10).

One promising target might be the RNA molecule of telomerase which is an integral part of the enzyme. An 11 base sequence of it is used as template for the synthesis of the telomeric DNA repeats. Recently, the cloning of the complete sequence of human telomerase RNA (hTR) was described and used for the construction of a vector expressing antisense hTR. In HeLa cells this antisense hTR caused cell death after 23–26 cell doublings, supporting the idea that telomerase inhibition by eliminating the template function of its RNA might be a sufficient way to control tumor cell growth (11).

Unmodified antisense oligodeoxribonucleotides (ODNs) have been used earlier as tools for detecting the RNA as a functional part of the enzyme in different species (3,12–16).

However, high concentrations of long ODNs (18–40mers) were required to inhibit the enzyme (12,16) whereas shorter sequences (13mers and 15mers) were described to be ineffective (17).

More recently, peptide nucleic acids (PNAs) in which the negatively charged deoxyribosephosphate backbone of oligonucleotides was replaced by a pseudopeptide backbone [*N*-(2-aminoethyl)-glycine] proved very powerful and sequence-specific inhibitors of human telomerase when their sequences were complementary to the template as well as adjacent regions of telomerase RNA (17–19). In contrast, the inhibition of telomerase by phosphorothioate-modified antisense oligonucleotides (PS-ODNs) was described to be less potent and selective (17,19) or partially dependent on their ability to form G-quartets (20). Very recently, the hexameric PS-ODN of the telomeric sequence TTAGGG was found not only to inhibit the telomerase activity but also to decrease tumor growth *in vivo* (21).

Here we compare efficiency and selectivity of differently modified oligomers, including PNAs, as inhibitors of human telomerase directed to nine or 11 bases of the template region and the four subsequent upstream bases of telomerase RNA. Among the tested oligomers, PS-ODNs were found to display the highest efficiency but also the lowest selectivity. Thus, PS-ODNs, non-complementary to the RNA, produced nearly the same inhibitory activity as a complementary PS-ODN, suggesting a sequence-independent mode of action.

*To whom correspondence should be addressed. Tel: +49 30 9406 3409; Fax: +49 30 9406 3138; Email: emat@mdc-berlin.de

We demonstrate that not the RNA itself but the primer binding site of telomerase protein might be the target of PS-ODNs and we describe the length and sequence requirements of their efficiency. Based on our results we propose the design of chimeric ODNs consisting of a 5′-PS-modified part which is extended at the 3′-terminus by an oligomer forming a stable duplex with the subsequent template region of telomerase RNA. Such ODNs might combine both strong protein- and RNA-binding capacity and result in more efficient and selective inhibitors of telomerase. [Preliminary results were presented at the First Congress of Molecular Medicine, Berlin, May 3–5, 1997 (22).]

MATERIALS AND METHODS

Preparation of cell extracts

The human promyelocytic leukemia cell line HL-60 and human liver carcinoma cells HuH-7 were maintained by serial passage (twice weekly) in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO_2/95% air. The cells were tested for mycoplasma and found to be free of contamination. Cells were washed twice with PBS and 1×10^5–1×10^6 cells were resuspended in 200 µl of lysis buffer provided with the TRAP-eze™ telomerase detection kit (Oncor, Gaithersburg) (0.5% 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate (CHAPS), 10 mM Tris–HCl, pH 7.5, 1 mM EGTA, 1 mM $MgCl_2$, 5 mM mercaptoethanol, 0.1 mM benzamidine, 10% glycerol). The suspension was kept on ice for 30 min, mixed several times and centrifuged at 12 000 *g* for 30 min at 4°C. The supernatants were diluted to give ~500 cell equiv./µl and were then aliquoted, frozen and stored at –80°C. Typical protein concentrations in the supernatants were 40–60 ng/2 µl cell extract determined by the method of Bradford using the Bio-Rad protein assay (Bio-Rad, Munich).

Telomerase assay

The activity of telomerase was estimated by the TRAP assay (telomeric repeat amplification protocol) using the TRAP-eze™ detection kit (Oncor). This PCR-based telomerase assay is a modification of the method described originally by Kim *et al.* (6). In a first step, telomerase activity from cell lysates adds a number of telomeric repeats to the 3′-end of a substrate oligonucleotide (TS primer) which is complementary to three bases of the template of telomerase RNA. In a second step, the extended products were amplified by PCR using TS and RP (reverse) primers, generating a ladder of products with six base increments starting at 50 nucleotides (i.e. 50, 56, 62, 68, etc.; Fig. 1). Additional to the telomerase products, a 36 bp internal control is co-amplified, facilitating both a quantitative evaluation of telomerase activity and the identification of oligonucleotides inihibiting the amplifying *Taq* polymerase rather than telomerase activity (below).

In a direct comparison, the TRAP-eze™ detection kit proved to be superior to the standard TRAP assay with respect to sensitivity, reliability and quantitative linearity (23), criteria which made it suitable for our investigation.

Corresponding to the instructions of the manufacturer the TRAP assay contained in a total volume of 50 µl: 20 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 50 µM dNTP, 2 µl 5′-^{32}P-phosphate-labeled TS primer (5′-AATCCGTCGAGCAGAGTT-3′), 1 µl of primer mix (RP, template and primer for the 36 bp internal control), 2 µl cell extract of HL-60 or HuH-7 cells (~1000 cell equiv., unless otherwise indicated) and the oligonucleotide to be tested. No preincubation of the ODNs with the telomerase extracts was required to obtain the described effects. In the case of PNAs such preincubation with the telomerase extract for 30 min at 25°C was absolutely essential (17) and might explain a lower reproducibility of the results obtained with PNAs. The TS primer was labeled with ^{32}P-phosphate according to the manufacturer's protocol with 0.2 µCi [γ-^{32}P]ATP (3000 Ci/mmol; NEN, Boston) and T4 polynucleotide kinase (Amersham Life Science, Cleveland, OH) for 20 min. The reaction was stopped at 85°C for 5 min and the product was added directly to the TRAP assay at given concentrations. In experiments in which the TS primer was applied at concentrations <200 nM or in which phosphorothioate-modified primers (TS/PS primers) were used during the telomerase reaction, ^{32}P-labeled TS primer was added to a final concentration of 200 nM before starting the PCR.

The tubes were incubated for 10 min at 30°C (in some experiments 25°C) for telomerase-mediated elongation of the TS primer. The reaction products were amplified in a thermal cycler for 30 rounds (30 s at 94°C and 30 s at 60°C). After amplification, 5 µl stop buffer (50 mM EDTA, 50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol) were added and 25 µl of the PCR products were electrophoresed in 0.5× Tris–borate, pH 8.3, 1 mM EDTA on 10% polyacrylamide non-denaturing gels at 180 V for 45 min followed by 280 V for 95 min. The gels were dried and phosphorimaged using a Bioimage Analyzer BAS 2000 (Fuji, Tokyo).

Quantitation of TRAP assays

After scanning of the complete gels, the intensities of individual lanes (measured as photostimulated luminescence, PSL) were quantitated using TINA 2.08 image software (Raytest, Straubenhardt). Two distinct areas were evaluated separately: the telomerase activity-related ladder bands and the 36 bp internal control band. The signals of the telomerase ladders of the lanes were normalized to the intensities of the internal controls. The intensities of the amplified telomeric repeats from HL-60 cells and HuH-7 cells were linear in the range 200–10 000 cells (unpublished data).

The oligonucleotides added as inhibitors of telomerase activity did not change appreciably the intensities of the internal control, indicating that they did not interfere with amplification of the internal control at concentrations inhibiting >80% of telomerase activity (Fig. 1, lanes 13 and 15).

The results were corrected for the background signal deriving from heated cell extract. Telomerase activity-related intensities remaining in the ODN-treated cell extracts were expressed as a percentage of the control. At least three independent dose–response curves were used to estimate the mean values of the concentrations required for a 50% inhibition of the telomerase activity (IC_{50}).

A series of control assays were included to ensure further the specificity of the telomerase-related banding pattern as shown in Figure 1 (lanes 1–3).

Oligonucleotides and PNAs

All oligonucleotides investigated were synthesized and HPLC purified by Eurogentec (Seraing) and BioTez (Berlin). Both PNAs were synthesized and purified by TIB MolBiol (Berlin) and

their identity confirmed by mass spectroscopy (PerSeptive Biosystems, Hamburg). Concentrations were checked by UV spectroscopy. The storage buffer was 10 mM Tris–HCl, 1 mM EDTA, pH 8.

RESULTS

Specificity and efficiency of telomerase inhibition by modified oligonucleotides and PNAs

The telomerase activity in extracts of human HL-60 cells was measured by the TRAP-eze™ assay and is shown by the ladder bands in Figure 1 (lane 4). Each of them represents an amplified telomerase product after addition of a six base telomeric repeat to a primer beginning at 50 bp (56, 62, 68 bp, etc.). Authenticity of the telomerase signals was demonstrated by a series of control assays (Materials and Methods).

The oligomers investigated as inhibitors of telomerase activity might interfere not only with the synthesis but also with the amplification of the telomerase products. However, none of the oligomers presented here interfered with the amplification of the 36 bp internal control at inhibiting concentrations, as shown for two PS-ODNs in Figure 1 (lanes 13 and 15).

The first series of oligonucleotides tested were complementary to 11 bases of the template region (T11) and to four adjacent upstream bases (U4) of the telomerase RNA following the sequence described by Feng *et al.* (11; Table 1). The inhibitory activity of these oligonucleotides (termed T11/U4) on telomerase activity from HL-60 cells proved to be dependent more on the backbone modification than on the base modification (Fig. 1, lanes 5–15).

The results are summarized in Table 1. The IC_{50} for the unmodified phosphodiester-linked oligomer (T11/U4/PO, IC_{50} = 460 nM) was not essentially changed by the replacement of four thymine and one cytosine bases by the corresponding C-5 propynyluracil and propynylcytosine (T11/U4/PO/Pr, IC_{50} = 430 nM), although these modified bases were described to enhance binding affinity to other RNA targets (24).

In contrast, a PS-ODN of the same sequence (T11/U4/PS) resulted in an IC_{50} value of 0.5 nM. Again, the replacement of the thymine and cytosine bases by C-5 propynyluracil and propynylcytosine (T11/U4/PS/Pr) did not change their efficacy.

Complete replacement of the phosphodiester backbone by methylphosphonates (T11/U4/MP) produced an IC_{50} in the same range (IC_{50} = 470 nM).

We compared the efficacy of the PS-ODN with a PNA in which the natural nucleobases were bound via carbonyl methylene linkages to the non-ionic *N*-(2-aminoethyl)glycine backbone (25). It covered only nine bases of the template and four adjacent upstream bases (T9/U4/PNA) because this sequence has been described recently as most efficient between a series of PNAs (17). We found an IC_{50} value of 6.5 nM (Table 1), whereas a 13mer PS-ODN covering exactly the same sequence (T9/U4/PS) was ~6-fold more effective (IC_{50} = 1 nM).

As shown further in Table 1, the corresponding non-complementary oligomers were less efficient. However, the differences between the IC_{50} values of non-complementary and complementary oligomers varied extremely. Thus, the selectivity index (IC_{50} value for non-complementary oligomers/IC_{50} value for complementary oligomers; Table 1) is the highest for PNA (>1000) followed by the methylphosphonate-modified ODN (22.3) and the PO and PO/Pr oligomers, respectively (7.4/6.4).

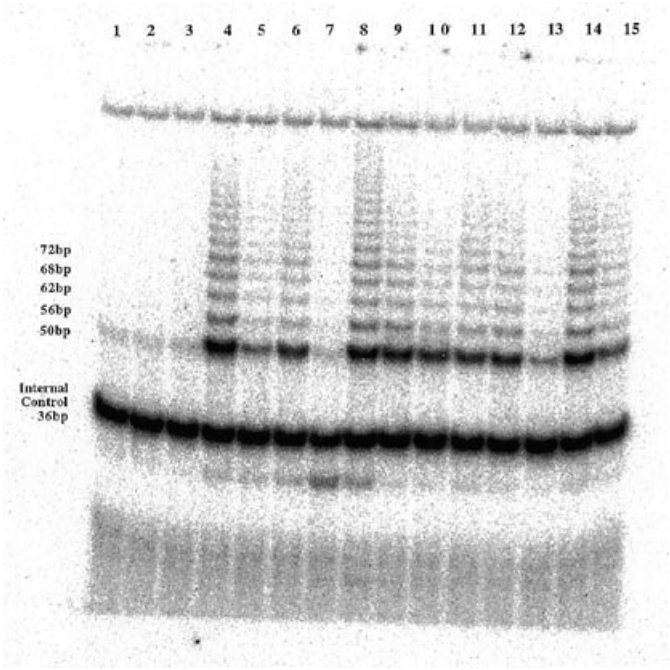

Figure 1. The inhibitory effect of differently modified oligonucleotides on telomerase activity. Telomerase reactions (TRAP assays) were performed as described in Materials and Methods with cell extracts of ~1000 HL-60 cells and 200 nM TS primer in the presence or absence of oligonucleotides covering 11 bases of template region and four adjacent upstream bases of telomerase RNA (T11/U4). The 36 bp internal PCR control is indicated. The first three lanes represent negative controls to demonstrate the telomerase specificity of the banding pattern. Lane 1, lysis buffer, without cell extract. Lane 2, heated cell extract. Lane 3, RNase (1 µg/µl) pretreated cell extract. Lane 4, telomerase activity of cell extract, positive control (=100%). The following lanes show the inhibition of telomerase activity caused by complementary (T11/U4) and non-complementary (15n) oligonucleotides (sequences and modifications in Table 1). Lane 5, +T11/U4/PO, 1 µM. Lane 6, +15n/PO, 3 µM. Lane 7, + T11/U4/PO/Pr, 1 µM. Lane 8, +15n/PO/Pr, 3 µM. Lanes 9 and 10, +T11/U4/MP, 0.2 and 0.8 µM, respectively. Lane 11, +15n/MP, 10 µM. Lanes 12 and 13, +T11/U4/PS, 1 and 10 nM, respectively. Lanes 14 and 15, +15n/PS, 0.5 and 5 nM, respectively.

In contrast, the complementary PS-ODNs were only slightly more active than the non-complementary PS-ODNs (Table 1). This was reflected in the fact that they had the smallest selectivity indices (2.6/2.3). These results suggest a nearly sequence-independent mode of action for the PS backbone-modified ODNs in contrast to other backbone or base modifications.

Length and sequence dependence of PS-ODN-induced inhibition of telomerase

In order to characterize further the requirements for their inhibitory activity, length- and further sequence-modified PS-ODNs were investigated. As demonstrated in Figure 2A, PS-ODNs extended successively by 5′-parts of the non-complementary 15mer sequence (Table 1) were more efficient, reaching the highest activity at a length of 30 bases (IC_{50} = 0.2 nM), whereas oligomers shorter than 15 residues proved to be less efficient. The limit seems to be a hexameric oligomer, since a 5mer was without any activity (Fig. 2A).

This limit of activity was found also for other hexameric PS-ODNs such as GAGTGT/PS ($IC_{50} \approx$ 1000 nM) and the

Table 1. Efficiency and selectivity of differently modified ODNs and PNAs, respectively, on telomerase of HL-60 cells

RNA sequence of human telomerase (hTR) targeted by oligomers

60 **Template site** Upstream site 30
3′....CGGGAAGAGU**CAAUCCCAAUC** UGUUUUUUACCGGUGGU....5′ hTR
5′-GTTAGGGTTAG ACAA-3′ T11/U4 complementary
5′-TAGGGTTAG ACAA-3′ T9/U4 sequences

5′-TCAGACATATACTGC-3′ 15n noncomplementary
5′-TCAGACATATACT-3′ 13n sequences

Complementary Seq./Modif.	IC_{50}; nM	Noncomplementary Seq./Modif.	IC_{50}; nM	Selectivity index IC_{50} N/ IC_{50} C
T11/U4/PO*	460	15n/PO	3400	7.4
T11/U4/PO/Pr	430	15n/PO/Pr	2750	6.4
T11/U4/PS	0.5	15n/PS	1.3	2.6
T11/U4/PS/Pr	0.6	15nPS/Pr	1.4	2.3
T11/U4/MP#	470	15n/MP	10500	22.3
T9/U4/PNA+	6.5	13n/PNA	> 10000	>1000

The upper part shows the targeted sequences of hTR. All oligonucleotides covered 11 bases of the template region and four of the subsequent upstream bases (T11/U4), whereas PNA covered nine bases of the template region and four of the subsequent upstream bases. The lower part gives IC_{50} values for inhibition of telomerase activity by complementary and non-complementary oligomers. The concentration of the TS primer was 200 nM.
*Abbreviations for oligomers: PO, phosphodiester-linked; PO/Pr, phosphodiester-linked, T replaced by 5-propynyluracil and C replaced by 5-propynylcytosine; PS, phosphorothioate-linked; PS/Pr, phosphorothioate-linked, T replaced by 5-propynyluracil and C replaced by 5-propynylcytosine; MP, methylphosphonate-linked; PNA, *N*-(2-aminoethyl)glycine-linked peptide nucleic acid.
#Methylphosphonate-modified ODNs were solved in 1% DMSO which had no influence on telomerase activity.
+PNA oligomers were preincubated with the cell extracts for 30 min at 25°C.

telomeric repeat sequence TTAGGG/PS (IC_{50} = 520 nM) which might be referred to the small number of PS linkages in the backbone. Increasing the chain length to 15 residues, the homooligomers of thymine and cytosine (dC15/PS and T15/PS) were found to have similar inhibitory activity (IC_{50} = 2.3 and 2.1 nM; Fig. 2B) as the non-complementary 15n/PS (IC_{50} = 1.3 nM). The corresponding purine oligomers dG15/PS and dA15/PS gave IC_{50} values of 4 and 57 nM, respectively (Fig. 2B). These and all other PS-modified ODNs mentioned above were also investigated at 25°C without any change in efficacy.

Multiples of the telomeric repeat sequence $(TTAGGG)_n$/PS (n = 2–4) inhibited telomerase activity at IC_{50} values between 2.7 and 4.0 nM, which is in a similar range as found for the corresponding oligomers with the same but mixed base compositions $(GAGTGT)_n$/PS (n = 2–4, IC_{50} = 1.0–5.3 nM; Fig. 2B).

To determine whether the PS-modified telomeric repeat sequences and their scrambled versions might be used by telomerase as competitive primers which might be elongated instead of the TS primer, we used TTAGGG/PS, $(TTAGGG)_2$/PS and $(GAGTGT)_2$/PS in which the last G and T, respectively, at the 3′-end were replaced by chain terminating 3′-deoxyguanosine (3′dG) or 3′-deoxyribothymidine (3′dT). However, these modifications did not essentially influence the activity of the oligomers (TTAGGG TTAGG-3′dG/PS, IC_{50} = 10.0 nM; GAGTGT GAGTG-3′dT/PS, IC_{50} = 10.2 nM; TTAGG-3′dG/PS, IC_{50} = 220 nM) (Fig. 2B). Therefore, we suggest that this alternative mechanism seems not to contribute substantially to the described inhibitory effects.

Besides the described single-stranded we investigated a double-stranded PS-ODN. The15n/PS-ODN (Table 1), annealed with an equimolar amount of complementary PS-modified strand of the same length, was nearly as efficient as the single strand (IC_{50} = 2 nM).

Competitive inhibitition of primer binding by PS-ODNs

The strong but largely sequence-non-specific inhibition of the investigated PS-ODNs suggested a mode of action other than complementary binding to the RNA template.

Corresponding to the two-site binding model proposed for telomerase, the primer is thought to bind with its 3′-end to the template RNA while the primer 5′-end is suggested to be attached to a specific anchor site on a telomerase protein which is called the primer binding site or lagging site, as shown in Figure 3 (26,27; Discussion).

To examine the affinity of a non-complementary 20mer PS-ODN for the primer binding site, we investigated its inhibitory

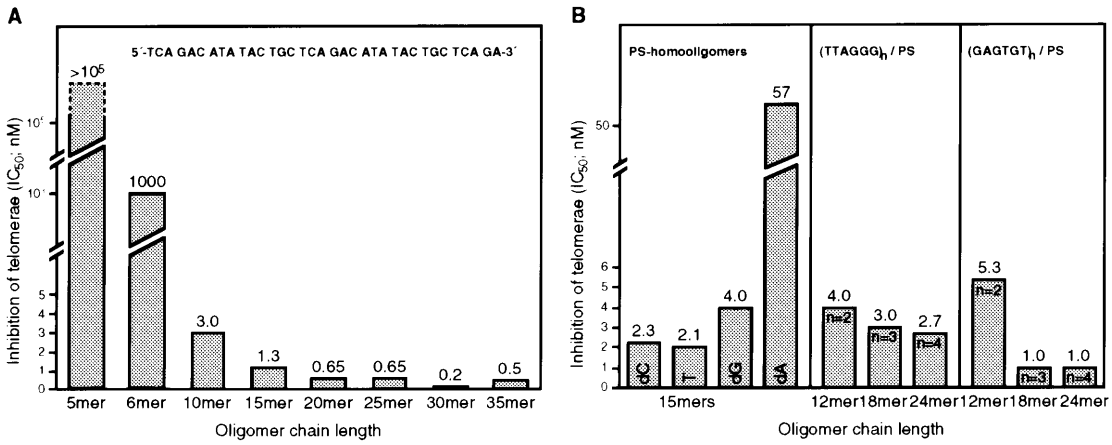

Figure 2. Effect of length and sequence modifications of PS-ODNs on telomerase activity. (**A**) Influence of phosphorothioate linkage number on inhibition of telomerase activity. The given 35mer non-complementary sequence or corresponding upstream parts of it were used at different concentrations in the TRAP assay to estimate the IC_{50} values. (**B**) Influence of PS-modified homooligomers of dC15, T15, dG15 and dA15 and of telomeric repeat sequences $(TTAGGG)_n$/PS (n = 2–4) and the corresponding scrambled variants $(GAGTGT)_n$/PS (n = 2–4) on telomerase activity given as IC_{50} values.

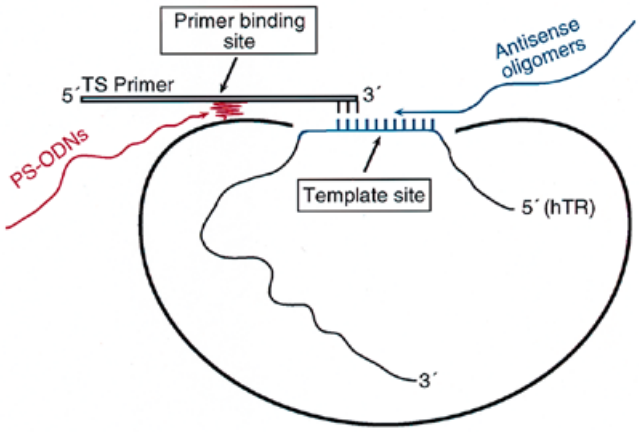

Figure 3. Two site model for primer interaction with human telomerase. As shown for the TS primer used, three bases of the 3′-terminus anneal with the corresponding template bases of hTR whereas the upstream region is thought to be fixed at the enzyme's primer binding site. Antisense oligomers complementary to the RNA template are suggested to anneal with the corresponding RNA sequence thus suppressing its template function for telomeric repeat synthesis. In contrast, PS-ODNs were proposed to bind avidly in a widely sequence-non-specific manner to the primer binding site at the catalytic telomerase subunit (hTERT) displacing the primer from this binding site. This displacement mechanism of PS-ODNs produced a more powerful inhibition of telomerase than found for antisense oligomers.

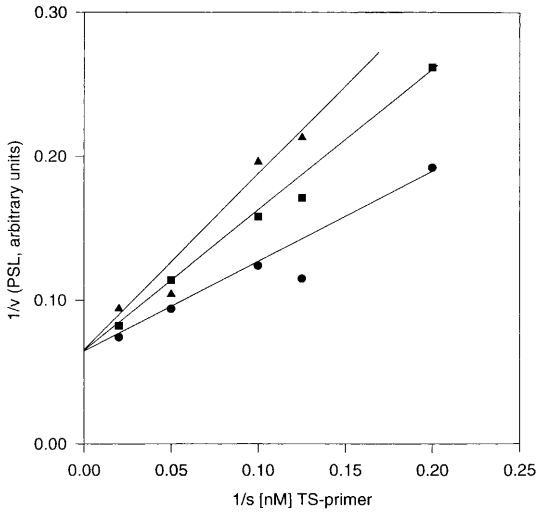

Figure 4. Kinetics of inhibition of human telomerase by the non-complementary 20mer PS-ODN (5′-TCAGACATATACTGCTCAGA-3′/PS). Double reciprocal plot of TS primer-dependent reaction velocities without inhibitor (●) and in the presence of 1 (■) and 2 nM 20mer PS-ODN (▲) The intensities of the individual lanes were measured as PSL and quantitated as described in Materials and Methods.

activity in the presence of increasing concentrations of the TS primer (5–30 nM). The kinetic data were evaluated as described and analyzed using a Lineweaver–Burk plot.

As shown in Figure 4, a 20mer PS-ODN inhibited telomerase activity competitively with respect to the phosphodiester-linked TS primer. Therefore, the same binding site for both the TS primer and the PS-ODN can be suggested. The binding constant of the TS primer (K_m) was estimated to be 10.8 nM, the value of the inhibition constant (K_i) of the 20mer PS-ODN was 1.6 nM. The K_m/K_i ratio indicates an ~6.7-fold higher affinity of the PS-ODN for the primer binding site than of the phosphodiester-linked TS primer. Similar results were obtained with PS-ODNs of other non-complementary sequences (data not shown).

Increase in telomerase activity by PS-modified primers

The high affinity of the PS-ODNs for the primer binding site has pompted us to estimate the telomerase activity with TS primers in which the phosphodiester bonds were successively replaced by PS linkages.

Thus, we applied TS primers in which 6, 10, 14 or all 17 phosphodiester bonds of the TS primer beginning from the 5′-end were replaced by phosphorothioates.

We found that all investigated PS-modified TS primers (TS/PS primers) produced higher telomerase activity when compared

with the unmodified TS primer. The highest stimulation of telomerase activity was reached with the completely PS-modified TS primer (TS/PS17 primer), as shown in Figure 5.

From kinetic data evaluated by double reciprocal plots the K_m values for the PS-modified TS primer were calculated. A replacement of all phosphodiester linkages by phosphorothioates (TS/PS17) gave a K_m value of 0.56 nM, in contrast to the K_m value of the unmodified TS primer of 10.8 nM as described above.

The K_m values for the TS/PS6, TS/PS10 and TS/PS14 primers containing 6, 10 and 14 PS linkages were estimated to be 2.11, 1.79 and 0.79 nM. Thus these data demonstrate a close correlation between the number of PS linkages in a TS primer and its affinity for the primer binding site.

DISCUSSION

Telomerase elongates a single-stranded primer along an RNA template which is associated tightly with the protein components. Besides this alignment with the template RNA, the primer is thought to be fixed by its 5′-end at a separate protein site which is called the primer binding site, anchor site or lagging site and seems to be necessary for processivity of telomeric DNA synthesis (Fig. 3).

While the 5′-end of the primer remains attached, the elongated 3′-end can translocate on the template for additional rounds of repeat synthesis. Between both binding sites the elongated strand is looped out and can be released only when both primer ends dissociate simultaneously from telomerase. Thus the processivity of the enzyme reaction is mainly determined by the interaction of the primer with the protein site (3,26,27).

In the present report we describe that PS-linked ODNs, in contrast to otherwise modified oligomers, bind tightly in a sequence-independent manner to the primer binding site. This mode of action makes them more efficient inhibitors than antisense oligomers targeting telomerase RNA.

First we showed that a 15mer PS-ODN covering 11 bases of the template and four of the subsequent upstream bases of the telomerase RNA (T11/U4/PS) proved to be nearly as effective as a non-complementary 15n/PS-ODN (IC_{50} = 0.5/1.3 nM). In contrast, the corresponding phosphodiester-linked (T11/U4/PO) or methylphosphonate-modified (T11/U4/MP) ODNs or the PNA derivative (T9/U4/PNA) showed a higher sequence selectivity, as demonstrated by their selectivity indices (e.g. 2.6 for the PS-ODN, but >1000 for the PNA derivative; Table 1).

These differences in selectivity between PS and PNA oligomers are in agreement with earlier findings of Norton *et al.* (17,19). In contrast to these authors, however, we found that not the PNA oligomers but the PS-ODNs are most efficient inhibitors of telomerase and that phosphodiester-linked ODNs are not generally inactive, as shown here for (T11/U4/PO).

Norton *et al.* (17) indicate IC_{50} values of 0.9 and 5 nM (at 25 and 37°C) for PNA(XII) (= T9/U4/PNA) which is in the range of our findings with an IC_{50} value of 6.5 nM at 30°C. However, for PS(III) (= T9/U4/PS) they described IC_{50} values of 50 and 200 nM (at 25 and 37°C), which is substantially higher than we found in HL-60 cell extracts for the same sequence (IC_{50} = 1 nM, at 25 and 30°C).

Our further characterization of the PS-ODN-induced inhibition of telomerase confirmed that modification of length rather than of sequence influenced the efficiency of PS-ODNs. Between them a 30mer non-complementary sequence was found to be most efficient with an IC_{50} value of 0.2 nM, which increased to 1.3 nM for a 15mer PS-ODN and to 3 nM for a 10mer PS-ODN, whereas PS-ODNs below 10 base residues lost most of the activity (Fig. 2A).

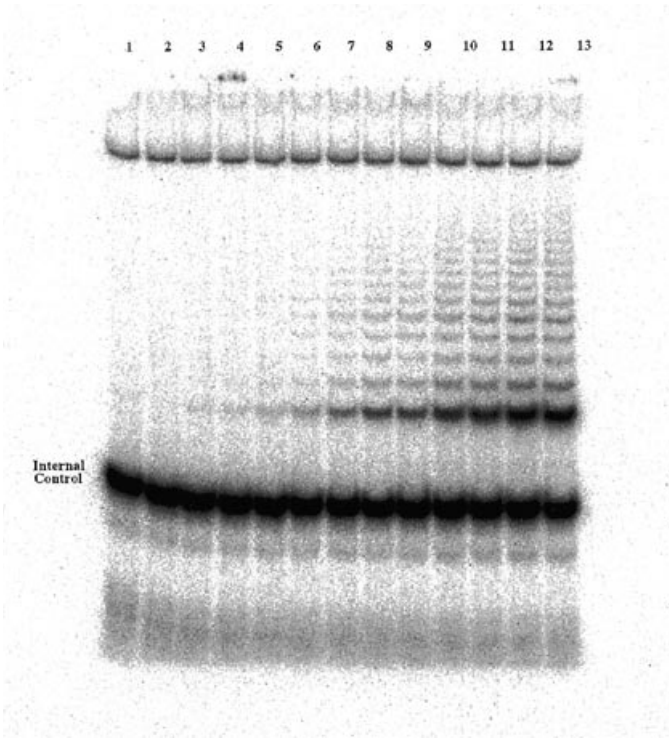

Figure 5. Effect of PS-modified TS primer on telomerase activity. Increasing concentrations of the TS primer or of its completely PS-modified derivative (TS/PS17 primer) were applied in the TRAP assay and the telomerase activity measured as described. Lane 1, lysis buffer, without cell extract. Lane 2, heated cell extract. Lanes 3–8, TS primer, 0.125, 0.25, 0.5, 1, 2 and 5 nM, respectively. Lanes 9–13, TS/PS17 primer, 0.125, 0.25, 0.5, 2 and 5 nM, respectively.

This length dependence was also found for the telomeric repeat sequences $(TTAGGG)_n$/PS and their scrambled variants $(GAGTGT)_n$/PS, when n = 2–4 (IC_{50} = 2.7–4.0 and 1.0–5.3 nM; Fig. 2B). Also, homopolymers such as T15/PS, dC15/PS and dG15/PS (IC_{50} = 2.1–4.0; Fig. 2B) did not differ considerably from the non-complementary sequence 15n/PS. Even a double-stranded oligodeoxynucleotide formed by 15n/PS-ODN and its complementary PS-modified ODN proved to be nearly as efficient as the single-stranded ODN (IC_{50} = 2 nM).

Therefore, we assume that secondary structures, at least such as G-quartets formed by G-rich ODNs, seem not to be required for the inhibitory activity of PS-ODNs on human telomerase as suggested by Sharma *et al.* (20). On the other hand, it remains to be understood why the homopurine oligomer dA15/PS was at least 10-fold less efficient than all other PS-ODNs of the same length.

In agreement with the described length requirements, the hexameric repeat TTAGGG/PS and the non-complementary variants GAGTGT/PS and TCAGAT/PS were observed to be much less effective. The differences between the IC_{50} values (TTAGGG/PS, 520 nM; nonsense oligomers, 1000 nM) might be caused by the sequence more than by the few PS-linkages.

The PS-modified telomeric repeat TTAGGG/PS has recently been described to be responsible for growth-inhibiting effects on

human Burkitts lymphoma cells *in vitro* and *in vivo* (21). The authors assume that the telomeric repeat, but not the scrambled motif (TGTGAG/PS), may act as a competitive substrate for telomerase. However, we found that a modification of the oligomers TTAGGG/PS and $(TTAGGG)_2$/PS at the 3′-end by the chain terminator 3′-deoxyguanosine did not essentially change their IC_{50} values, thus it seems unlikely that this mechanism might contribute to the described inhibitory activity.

In contrast, we present evidence that a tight binding of PS-ODNs to the primer binding site of telomerase might be the main mode of action. First, we have demonstrated that a 20mer PS-ODN of a sequence non-complementary to the template RNA is able to interact with the binding of the TS primer at the primer binding site. Kinetic investigations showed that increasing concentrations of TS primer are sufficient to abolish the PS-ODN-induced inhibition of telomerase in a manner which fulfills the criteria of a competitive interaction of TS primer and PS-ODN at the primer binding site of telomerase (Fig. 4).

The binding constant (K_m) of the TS primer on the telomerase was estimated to be 10.8 nM, which is in agreement with the apparent K_{mapp} of 8 nM as reported by Norton *et al.* (17). The inhibition constant K_i of human telomerase for the 20mer PS-ODN was 1.6 nM, demonstrating an ~6-fold higher affinity of the PS-ODN for the primer binding site.

We therefore asked whether the 18mer TS primer of the original sequence in which the PO linkages were replaced by 6, 10, 14 or 17 PS bonds might influence the telomerase activity.

Our data showed that human telomerase can use TS/PS primers more efficiently than the unmodified TS primer for elongation (Fig. 5). This increase in primer efficiency is clearly correlated with the number of PS linkages. The K_m value obtained for completely PS-modified TS primer was 0.56 nM, demonstrating an ~19-fold higher affinity for the primer binding site compared with the unmodified TS primer. As a result, the same but more intense banding pattern was seen. However, no additional bands could be detected, representing the synthesis of longer telomeric repeats, which might be predicted as a consequence of stronger interaction of the upstream site of the primer with the primer binding site (28).

Therefore, more appropriate experiments are required to establish whether the PS-modified primers are able to affect the processivity of the telomerase reaction.

Apart from this, the increased telomerase activity found with TS/PS primers points out further the essential role played by the protein binding of a primer for telomerase activity.

While the sequence-non-specific binding of PS-ODNs to the primer binding site of human telomerase is described here for the first time, there is an increasing body of evidence that some of the biological effects described for antisense phosphorothioates are not due to an antisense mechanism but seem to be caused by non-sequence-specific interactions with proteins. Such phosphorothioate–protein complexes can be formed particularly with such proteins binding the negatively charged glycosaminoglycan heparin. Examples of heparin-binding proteins and receptors are basic fibroblast growth factor, platelet-derived growth factor, vascular endothelial growth factor and its receptor and the epidermal growth factor receptor (reviewed in 29).

These proteins tend not only to bind PS-ODNs with much lower dissociation constants than unmodified ODNs but may perturb their function (29). Similarly, enzymes such as HIV-RT or HIV-RT-associated RNase H can be inhibited by PS-ODNs in a sequence-independent manner (30,31).

Such sequence-non-specific effects might be considered as a disadvantage in the application of phosphorothioate-modified antisense oligonucleotides. However, the unique structure and function of telomerase might allow the application of oligomers consisting of a PS-ODN extended at the 3′-terminus by an oligomer hybridizing effectively with the subsequent template region of RNA.

Such chimeric ODNs could join optimum protein- and RNA-binding properties thus retaining the high efficacy of PS-ODNs but providing the required specificity by an antisense partner.

The design and study of such chimeric ODNs as selective inhibitors of telomerase activity are under way.

REFERENCES

1 Greider,C.W. and Blackburn,E.H. (1985) *Cell*, **43**, 405–413.
2 Blackburn,E.H. and Greider,C.W. (1995) *Telomeres*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
3 Morin,G.B. (1989) *Cell*, **59**, 521–529.
4 Harley,C.B. (1991) *Mutat. Res.*, **256**, 271–282.
5 Shay,J.W. and Wright,W.E. (1996) *Curr. Opin. Oncol.*, **8**, 66–71.
6 Kim,N.W., Piatyszek,M.A., Prowse,K.R., Harley,C.B., West,M.D., Ho,P.L., Coviello,G.M., Wright,W.E., Weinrich,S.L. and Shay,J.W. (1994) *Science*, **266**, 2011–2015.
7 Shay,J.W. and Bacchetti,S. (1997) *Eur. J. Cancer*, **33**, 787–791.
8 Corey,D.R. (1996) *Chem. Biol.*, **3**, 863–867.
9 Raymond,E., Sun,D., Chen,S.-F., Windle,B. and Von Hoff,D.D. (1996) *Curr. Opin. Biotechnol.*, **7**, 583–591.
10 Sharma,S., Raymond,E., Soda,H., Sun,D., Hilsenbeck,S.G., Sharma,A., Izbicka,E., Windle,B. and Von Hoff,D.D. (1997) *Ann. Oncol.*, **8**, 1063–1074.
11 Feng,J., Funk,W.D., Wang,S.S., Weinrich,S.L., Avilion,A.A., Chiu,C.P., Adams,R.R., Chang,E., Allsopp,R.C., Yu,J. *et al.* (1995) *Science*, **269**, 1236–1241.
12 Shippen-Lentz,D. and Blackburn,E.H. (1990) *Science*, **247**, 546–552.
13 Zahler,A.M., Williamson,J.R., Cech,T.R. and Prescott,D.M. (1991) *Nature*, **350**, 718–720.
14 Counter,C.M., Avilion,A.A., LeFeuvre,C.E., Stewart,N.A., Greider,C.W., Harley,C.B. and Bacchetti,S. (1992) *EMBO J.*, **11**, 1921–1929.
15 Melek,M., Davis,B.T. and Shippen,D.E. (1994) *Mol. Cell. Biol.*, **14**, 7827–7838.
16 Collins,K., Kobayashi,R. and Greider,C.W. (1995) *Cell*, **81**, 677–686.
17 Norton,J.C., Piatyszek,M.A., Wright,W.E., Shay,J.W. and Corey,D.R. (1996) *Nature Biotechnol.*, **14**, 615–618.
18 Hamilton,S.E., Pitts,A.E., Katipally,R.R., Jia,X., Rutter,J.P., Davies,B.A., Shay,J.W., Wright,W.E. and Corey,D.R. (1997) *Biochemistry*, **36**, 11873–11880.
19 Zaffaroni,N., Villa,R., Folini,M., Daidone,M.G. and Silvestrini,R. (1998) *Ann. Oncol.*, **9** (suppl. 2), 140.
20 Sharma,H.W., Hsiao,R. and Narayanan,R. (1996) *Antisense Nucleic Acid Drug Dev.*, **6**, 3–7.
21 Mata,J.E., Joshi,S.S., Palen,B., Pirruccello,S.J., Jackson,J.D., Elias,N., Page,T.J., Medlin,K.L. and Iversen,P.L. (1997) *Toxicol. Appl. Pharmacol.*, **144**, 189–197.
22 Matthes,E., Lehmann,C. and von Janta-Lipinski,M. (1997) *J. Mol. Med.*, **75**, B115.
23 Holt,S.E., Norton,J.C., Wright,W.E. and Shay,J.W. (1996) *Methods Cell Sci.*, **18**, 237–248.
24 Wagner,R.W., Matteucci,M.D., Lewis,J.G., Gutierrez,A.J., Moulds,C. and Froehler,B.C. (1993) *Science*, **260**, 1510–1513.
25 Nielsen,P.E., Egholm,M., Berg,R.M. and Buchardt,O. (1991) *Science*, **254**, 1497–1500.
26 Morin,G.B. (1991) *Nature*, **353**, 454–456.
27 Harrington,L.A. and Greider,C.W. (1991) *Nature*, **353**, 451–454.
28 Morin,G.B. (1996) *Dev. Biol.*, **7**, 5–13.
29 Stein,C.A. (1996) *Chem. Biol.*, **3**, 319–323.
30 Majumdar,C., Stein,C.A., Cohen,J.S., Broder,S. and Wilson,S.H. (1989) *Biochemistry*, **28**, 1340–1346.
31 Cheng,Y.-C., Gao,W.-Y. and Han,F.-S. (1991) *Nucleosides Nucleotides*, **10**, 155–166.