Inhibition of human telomerase by oligonucleotide chimeras, composed of an antisense moiety and a chemically modified homo-oligonucleotide

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Abstract **Most tumor cells attain their immortality by reactivating telomerase. We report here the telomerase inhibitory potential of chimeric oligonucleotides composed of a 13mer antisense sequence targeting the telomerase RNA template region and a $(s^4dU)_n$ moiety at its 3′ or 5′-end. The increase of the thiolated chain length enhances the telomerase inhibitory potential, but decreases specificity, indicated by HIV reverse transcriptase inhibition. Chimeras with 5′ $(s^4dU)_n$s were more potent inhibitors than the antisense alone or the 3′ modified ones. Cy5-labeled $(s^4dU)_4$AS and $(s^4dU)_8$AS proved the internalization of the oligonucleotides, raising the possibility to be tested as cellular anti-telomerase agents.**

Keywords: Telomerase; Antisense; 4-Thio-deoxyuridylate

1. Introduction

Telomerase, a ribonucleoprotein complex that includes an RNA template (hTR) and a catalytic subunit (hTERT) with reverse transcriptase activity, is responsible for telomere protection and maintenance [1]. This enzyme binds the 3′ overhang of the telomeric DNA and extends it by copying its RNA-template that is complementary to the hexameric unit of the DNA telomeric repeat sequence (TTAGGG in vertebrates) [2]. Most of the human cancer cells exhibit measurable telomerase activity in contrast to normal human adult somatic cells [3] with a few exceptions like stem cells of renewable tissues and activated lymphocytes [4]. This relative specific expression pattern makes this ribonucleoprotein a rational target of antineoplastic chemotherapy, with considerations about the long lag phase before the onset of cell death as a result of critical telomere shortening. However, in some cases cell death occurs faster than one might predict based on the rate of telomere shortening, suggesting additional roles of telomerase in protecting cells against apoptosis [5].

There are several well-established classes of telomerase inhibiting compounds: small catalytic-inhibitor molecules, reverse transcriptase inhibitors, compounds that stabilize the folding of telomeric G-rich single-stranded DNA into G-quadruplex and oligonucleotide-type inhibitors [6].

hTR is a favorable target for antisense strategies because being a template, means exceptional accessibility. Previous studies have shown that anti-template phosphorothioate-modified oligonucleotides show competitive behavior regarding primer-substrates, what suggested their interaction with a protein motif, termed primer binding site, which normally binds to DNA to be elongated [7]. Matthes and Lehmann were the first, who proposed the evaluation of anti-telomerase chimeric oligonucleotides that contain a moiety targeting the primer binding site and a sequence against the RNA as well. These findings evoke our idea of the rational design of active molecules which contain, beside the well-established anti-template sequence, a chemically modified oligonucleotide moiety known to interact with reverse transcriptases, thus presumably also with the catalytic subunit of telomerase. Chemical modification of telomerase inhibiting oligonucleotides meant usually altered backbones like N3′ → P5′ phosphoramidate or thiophosphoramidate [8], phosphorothioate-modified DNA [9], peptide nucleic acid [10], 2′-*O*-alkyl RNA [11,12], and were mainly addressed to improve biological properties. To our knowledge, trials with base-modified oligonucleotide derivatives or base-modified chimeric molecules have not been made before. We report here the telomerase inhibiting activity of a novel type of chimeric oligonucleotides, which are composed of an antisense sequence, directed against the template region of hTR and a base-modified $(s^4dU)_n$ moiety. The aim of this trial was to evaluate if the anti-telomerase activity of the classical oligonucleotide can be enhanced by a modification serving the above-mentioned "dual target" strategy, without excluding the possibility of further chemical improvement using backbone modifications.

2. Materials and methods

2.1. Partial purification of human telomerase

The partially purified human telomerase was prepared from HL-60 cells with slight modifications of the previously described protocol [13]. Main steps of the protocol are: precipitation with 40% ammonium sulfate and anion-exchange chromatography on DEAE–Sepharose

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column (Pharmacia). After chromatography, active fractions were pooled, dialyzed and re-purified on DEAE–Sepharose column. Telomerase inhibition studies with the pooled extract after the first and second chromatography gave the same results.

2.2. Oligonucleotides

Chemically modified oligonucleotides used for inhibition studies, Cy5-labeled molecules and primers used in TP-TRAP assay were synthesized by standard phosphoramidite chemistry and purified using anion-exchange chromatography as described previously [13,14]. $(s^4dU)_n$ and the chimeras were prepared by H_2S treatment (10 days at 55 °C) of the corresponding $(dC)_n$ or $(dC)_n$-extended AS/SCR [15]. The starting oligonucleotide of 3′-blocked $(s^4dU)_8$AS was synthesized using 3′-Spacer C3 CPG (Glen Research).

2.3. Telomerase activity measurement

Telomerase activity was measured using a modification of the TP-TRAP assay [13]. Briefly, inhibitor oligonucleotides (in various concentrations at a range between 2 nM and 4 μM, final concentrations) were preincubated at 25 °C with the partially purified telomerase enzyme, then the enzyme reaction was initiated by the addition of the MTS substrate primer (25 pmol) [AGCATCCGTCGAGCAGAGTT] as well as dATP, dCTP, dGTP (50 μM), dTTP (2 μM), 0.5 μCi [Me-^{3}H]dTTP (Amersham) and 2.25 U JumpStart Taq (Sigma) and reverse primers.

For telomerase-mediated extension of MTS primer, reaction mixtures were incubated at 30 °C for 10 min, heated to 95 °C for 2 min, followed by the PCR-amplification of the extension products. Labeled amplification products were collected by filtration and quantified by liquid scintillation counting. Measurements were performed at least as duplicates. Activity was plotted as a function of concentration of the added inhibitor using GraphPad Prism 2.01 software and IC_{50} parameters were determined graphically from these.

2.4. Reverse transcriptase-assay

Measurement of reverse transcriptase activity was performed as we described earlier [15]. Inhibitors were preincubated with 0.1 U HIV reverse transcriptase (Amersham) in a reaction mixture containing [Me-^{3}H]dTTP. Reverse transcriptase action was primed by adding the template primer, followed by incubation at 37 °C for 1 h. Radioactive products were collected and measured as in the TP-TRAP-assay.

2.5. Oligonucleotide uptake studies

A431 human planocellular carcinoma cells were allowed to adhere overnight, then transfected with Oligofectamine Reagent (Invitrogen) and Cy5-labeled oligonucleotides [Cy5-X_4AS and Cy5-X_8AS] at 200 nM concentration according to the manufacturer's directions. After 4 h of incubation cells were washed with PBS, fixed/permeabilized using 4% formaldehyde and 0.2% Triton X-100 in PBS, and incubated (15 min at room temperature) with 30 μg/ml Alexa Fluor 488-tagged 528 monoclonal antibodies against epidermal growth factor receptors (EGFR). Cells were then washed and stained with ethidium bromide (0.5 μg/ml). The cellular distribution of Cy5-labeled oligonucleotides was studied by confocal laser scanning microscopy (Zeiss LSM 510). The contours of the plasma membrane and the nucleus were visualized by fluorescent anti-EGFR antibodies (A431 cells are known to express EGFR in high number [16]) and ethidium bromide as described above. For the excitation of Alexa Fluor 488, the 488-nm line of an Ar ion laser, for ethidium, a 543-nm HeNe laser and for Cy5, a 633-nm HeNe laser were used. Fluorescence emission was detected through 500–550 nm and 560–610 nm bandpass and 650 nm longpass filters, respectively. With a Plan-Apochromat 63× oil immersion objective image stacks of 0.6 μm thick optical sections were collected.

3. Results

3.1. Effect of 3′ or 5′ attached $(s^4dU)_n$ on the telomerase inhibiting potential of a 13mer antisense oligonucleotide

The $(s^4dU)_{35}$ (abbreviated as X_{35}; X represents a 4-thio-deoxyuridylate unit, structure shown in Fig. 1) was a competitive inhibitor of HIV-RT and human telomerase with respect to the functioning RT template-primer and telomerase substrate primer [13,15]. The inhibitory features of X_{35} prompted us to develop chimera oligonucleotides, composed of a 13mer phosphodiesther antisense sequence (AS), designed to target hTR template region and a 3′ or 5′ X_n ($n = 4$, 8, 16 and 24) moiety, which is thought to interact with the protein subunit of human telomerase. First we determined the IC_{50} values for the inhibitors on partially purified telomerase using the TP-TRAP assay.

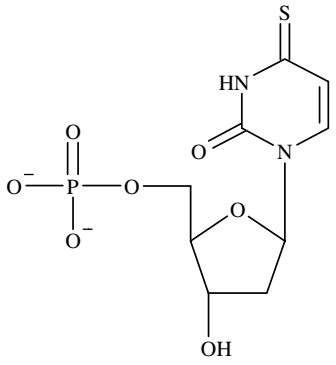

Fig. 1. Structure of the monomer unit of $(s^4dU)_n$ (4-thio-deoxyuridylate).

Since telomerase inhibiting potential of X_{35} was significantly affected by preincubation of the inhibitor with the enzyme [13], we tested the effect of various preincubation times (0–30 min) on inhibition (Fig. 2), and chose 10 min for the IC_{50} studies, because this period was measured to be obligatory and sufficient for the chimeras to exhibit maximal inhibitory potential. Considering the fact that oligonucleotides are relatively large molecules that need to hybridize to the target sequence to act as inhibitors this is not an unusual phenomenon, underlined also by the experimental layout for telomerase inhibition studies of other oligonucleotides [10,12].

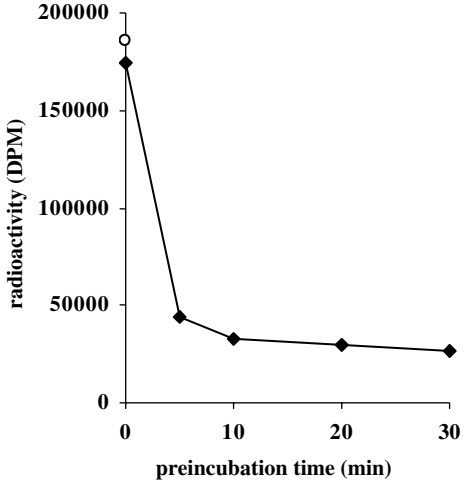

Fig. 2. Effect of preincubation time on the inhibitory activity of X_{16}AS. Black diamonds indicate the telomerase activity (represented by the DPM values) measured with TP-TRAP assay in the presence of 40 nM X_{16}AS. (This generates analyzable data in contrast to the utilization of a much weaker or a much stronger inhibitor.) Empty circle represents telomerase control without inhibitor.

The 13mer AS inhibited the partially purified telomerase with an IC_{50} of 174.2 nM (Table 1). Sequence specificity of AS-mediated inhibition was proven by scrambled control ($IC_{50\ [SCR]} > 4000$ nM; Table 1).

The chimeras were prepared by thiolation of the $(dC)_n$-extended AS. Although thiolation procedure was not expected to cause chemical modification [17], to ensure that the AS component remains unaltered, we thiolated a sample of AS, according to the standard procedure. Comparison of the UV-spectra and the inhibitory parameters, before or after the thiolation, gave the same results (data not shown).

Antisense oligonucleotides with 5′ $(s^4dU)_n$s were more potent inhibitors either than the antisense alone or the 3′-modified ones (Table 1 and Fig. 3 as an example of a dose–response curve). Increasing length of the thiolated moiety increased the telomerase inhibitory potential up to $IC_{50} = 24$ nM, when 16 or 24 modified bases were attached to the 5′-end. However, the inhibitory activity of the 3′ X_4 and X_8 modified oligonucleotides were less than that of the 13mer AS. A further increase of the length of the 3′ modified moiety increased the inhibition close to the same extent than the 5′ modified counterparts ($IC_{50\ [ASX24]} = 31.3$ nM).

Control experiments included oligonucleotides comprising just the chemically modified component [X_n], scrambled molecules with 5′ $(s^4dU)_n$s to prove sequence specifity [$(X)_n$SCR] and parent compounds for chemically modified chimeras [$(C)_n$AS] to evaluate the role of the thiolated moiety. X_n homo-oligomeres inhibited telomerase in chain length dependent manner, similarly as observed on HIV RT [15], where 25–40mers were shown to be active. Derivates containing scrambled sequences showed weak inhibition when the modified part was short, but the IC_{50} for X_{24}SCR reached the level of X_{24}AS. 5′ cytidilates almost abolished the anti-telomerase activity of the AS sequence. Surprisingly, C_{16}AS increased the telomerase activity substantially. As this augmentation was only present in the case of C_{16}AS, we suggest that it might be the result of an aptamer-like effect, underlined by the fact that base pairing between cytidilates and the G-rich AS-part is very likely.

Table 1
Inhibition of telomerase by oligonucleotides

Name	Sequence	IC50 ± SD (nM)
AS	AGTTAGGGTTAGA	174.2 ± 14.1
$(X)_4$AS	$(s^4dU)_4$AGTTAGGGTTAGA	134.2 ± 16.9
$(X)_8$AS	$(s^4dU)_8$AGTTAGGGTTAGA	38.2 ± 12.4
$(X)_{16}$AS	$(s^4dU)_{16}$AGTTAGGGTTAGA	24.0 ± 3.6
$(X)_{24}$AS	$(s^4dU)_{24}$AGTTAGGGTTAGA	24.3 ± 3.6
AS$(X)_4$	AGTTAGGGTTAGA$(s^4dU)_4$	292.4 ± 10.6
AS$(X)_8$	AGTTAGGGTTAGA$(s^4dU)_8$	308.1 ± 13.7
AS$(X)_{16}$	AGTTAGGGTTAGA$(s^4dU)_{16}$	112.4 ± 25.5
AS$(X)_{24}$	AGTTAGGGTTAGA$(s^4dU)_{24}$	31.3 ± 13.5
$(X)_4$	$(s^4dU)_4$	>4000
$(X)_8$	$(s^4dU)_8$	2974.1 ± 365.6
$(X)_{16}$	$(s^4dU)_{16}$	310.9 ± 76.0
$(X)_{24}$	$(s^4dU)_{24}$	116.6 ± 8.1
SCR	GTGATGATGATGA	>4000
SCR$(X)_4$	GTGATGATGATGA$(s^4dU)_4$	2127.1 ± 509.9
SCR$(X)_8$	GTGATGATGATGA$(s^4dU)_8$	154.4 ± 19.6
SCR$(X)_{16}$	GTGATGATGATGA$(s^4dU)_{16}$	65.7 ± 9.2
SCR$(X)_{24}$	GTGATGATGATGA$(s^4dU)_{24}$	25.3 ± 11.3
$(C)_4$AS	$(C)_4$AGTTAGGGTTAGA	2760.1 ± 617.4
$(C)_8$AS	$(C)_8$AGTTAGGGTTAGA	>4000
$(C)_{16}$AS	$(C)_{16}$AGTTAGGGTTAGA	–***
$(C)_{24}$AS	$(C)_{24}$AGTTAGGGTTAGA	417.1 ± 66.2
$(X)_8$ASsp	$(s^4dU)_8$AGTTAGGGTTAGA spacer	28.3 ± 5.1

Shown are synthesized chimeras with 3′ or 5′ $(s^4dU)_n$ and respective controls.
***telomerase activity was elevated by C_{16}AS.

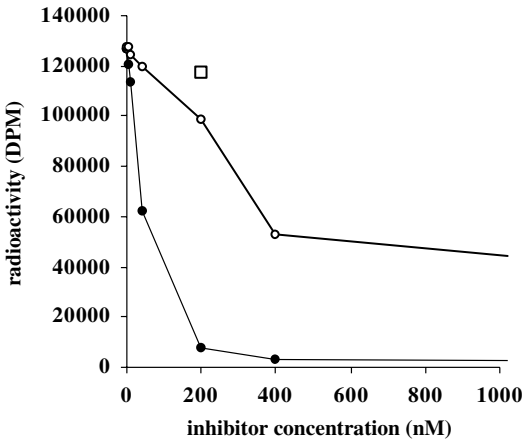

Fig. 3. Dose–response curve for inhibition of telomerase with $(s^4dU)_8$AS (filled circles) or AS$(s^4dU)_8$ (empty circles). After preincubation of the inhibitors at different concentrations with the partially purified telomerase, enzyme activity (represented by the DPM values) was measured by TP-TRAP assay. The square represents the results of the control experiments for the PCR-step using $(s^4dU)_8$AS or AS$(s^4dU)_8$ at 200 nM. Inhibitors added after the telomerase reaction but before the PCR amplification proved that measured effect is due to telomerase inhibition.

3.2. Effect of blocking the 3′-OH of X_nAS

The $(X)_n$AS oligonucleotide inhibitors carry telomeric sequences at their 3′-end, therefore they can be utilized as telomerase substrates elongated by telomeric repeats. To evaluate if the extension of the inhibitors has a role in their function, we used oligonucleotides carrying 3′ propyl-group as blocker of the 3′-function [$(X)_8$ASsp]. As demonstrated in Table 1, we did not see a difference between the IC_{50} of $(X)_8$AS and $(X)_8$ASsp ($IC_{50[X8AS]}$: 38.2 nM and $IC_{50\ [X8ASsp]}$: 28.3 nM ± 5.1).

3.3. Specificity of chimeric oligonucleotides for telomerase

To exclude the possibility that oligonucleotides affect the Taq-polymerase, control experiments were run with 200 nM of the appropriate inhibitors, which were added after the telomerase reaction, but before the PCR. Amplification was barely affected by the presence of the inhibitors (Fig. 3 and data not shown), indicating that the effects observed in the TP-TRAP-assay were due to the inhibition of telomerase.

Specificity of the oligonucleotides was also proven on HIV reverse transcriptase, which shares common motifs with hTERT [18]. We tested all synthesized oligonucleotides in reverse transcriptase assays using them at concentrations that corresponded to the IC_{90}s for telomerase. Chimeras with long modified moieties turned out not to be specific for telomerase (Fig. 4), as suggested by the 90% inhibition of RT at their IC_{90}

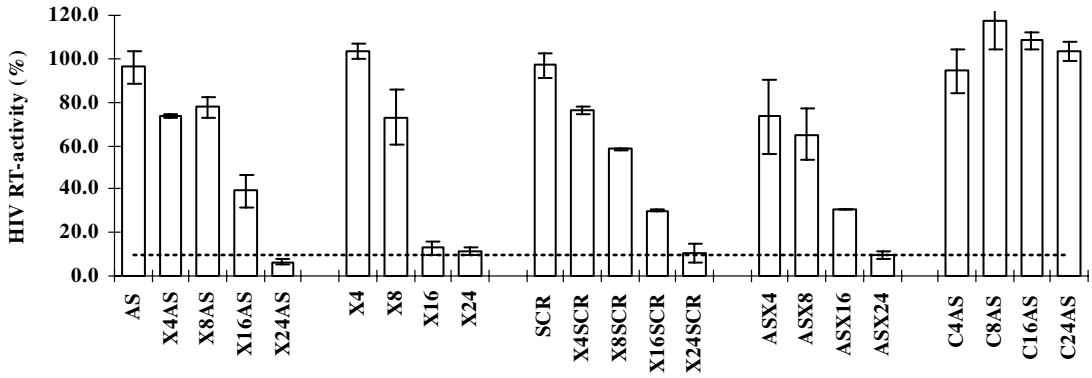

Fig. 4. Inhibition of HIV reverse transcriptase by anti-telomerase oligonucleotides. Applied concentrations correspond to the IC_{90} (dashed line indicates 10% activity) of the inhibitor for partially purified telomerase or 4 μM, the maximal tested concentration in the TP-TRAP assay (in cases when calculated IC_{90} would have been higher than this concentration). RT-activity assays were performed in duplicate, reported values are averages, error bars correspond calculated standard deviations (±).

for telomerase. In contrast, AS and AS with 4 or 8 (X)s were weak inhibitors of the viral reverse transcriptase.

3.4. Cellular uptake of oligonucleotide inhibitors

The efficiency of antisense strategies is highly dependent on the cellular delivery of oligonucleotide agents. It was found that X_{35} does not penetrate into the cell (Horvath, A., Aradi, J., unpublished data), suggesting that we may face similar problem with X-extended anti-telomerase oligonucleotides. Therefore we examined the cellular uptake of the inhibitors with confocal laser scanning microscopy using Cy5-labeled oligonucleotides. Chosen candidates were X_4AS and X_8AS, since their inhibition characteristics seem to be the most attractive for anti-telomerase strategies in cellular systems. We treated A431 cells with 200 nM Cy5-X_4AS or Cy5-X_8AS and Oligofectamine, a lipid-type carrier. Inhibitor-transfected cultures showed no morphological changes and no signs of acute cytotoxicity with light microscopy compared with the control samples treated only with Oligofectamine.

Pictures obtained with the confocal microscope after staining the plasma membrane and nuclei, clearly showed the internalization of the chemically modified oligonucleotides (Fig. 5). Labeled inhibitors were found in the cytosol and in the nucleus as well, with a characteristic perinuclear enhancement. Cell line 293T was also examined (pictures not shown) and showed similar intracellular oligonucleotide distribution.

4. Discussion

The inhibitory potential of the above-presented chimeric oligonucleotides seem to support the dual targeting hypothesis, namely that AS sequence of X_nAS inhibitors forms double helix with the template region of hTR, while X_n interacts with the protein part (Fig. 6) and the two interactions enhance each other. However, structural proves will need detailed experiments. Evaluation of primer-enzyme interactions proved that beside the interaction with the 3′ of the primer at the catalytic

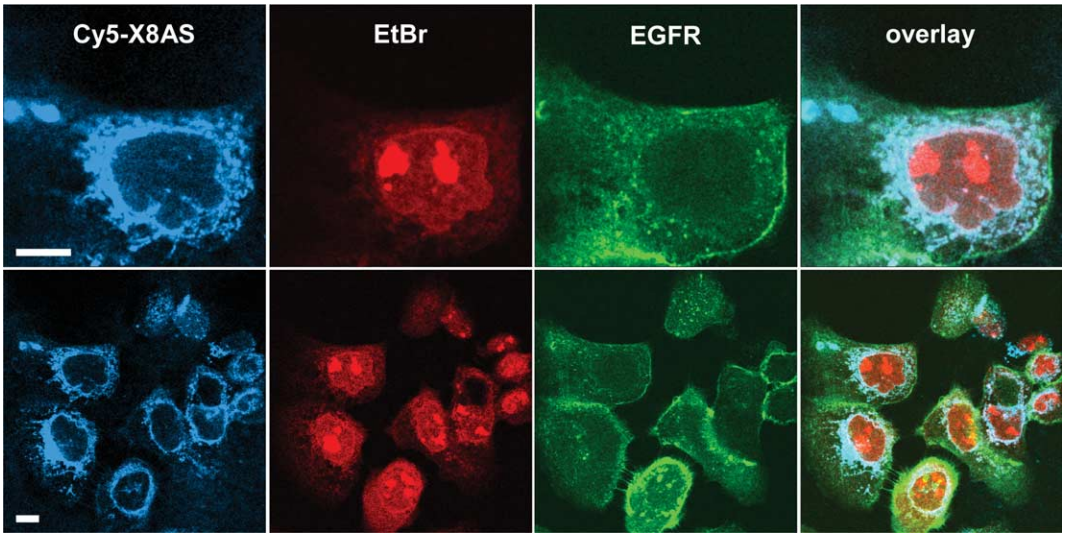

Fig. 5. Optical sections of A431 cells treated with Cy5-tagged X_8AS. Oligonucleotides (Cy5-X8AS, blue) are present in the cytoplasm and the nucleus as well, showing a perinuclear enrichment. The nucleus was stained by ethidium bromide (EtBr, red). Epidermal growth factor receptors (EGFR) were labeled by Alexa Fluor 488-tagged 528 monoclonal antibodies to visualize the plasma membrane (green). Scale bar: 10 μm.

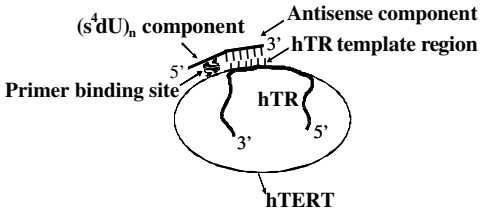

Fig. 6. Putative mechanism of X_nAS inhibitors. AS forms a double helix with the template region of hTR, and X_n interacts with the protein part responsible for primer (telomerase substrate) binding.

site, the upstream region of the DNA also has a sequence-specific contact with the protein, which seems to influence binding affinity as well as rate of polymerization [19]. It seems to be possible that the protein surface that normally interacts with the 5′ of its primer-DNA has a high affinity for thiolated oligonucleotides too, and their binding impairs the catalytic activity or inhibits the interaction of the telomerase with the natural DNA-substrate. The fact that blocking of the 3′ end has no effect on inhibition suggests that extension of the chimeric molecules has no crucial role in the mechanism, although we cannot exclude that non-blocked inhibitors might be elongated.

In case of ASX_n type of inhibitors, the hybridization to the RNA and the binding of the chemically modified part to the protein (presumably to other sites than 5′-extended ones) is also possible. The ''antisense''-contact to the RNA is slightly weakened when short thiolated moiety is attached to the 3′-end, indicating that they may disturb the interaction of the AS with the target sequence. The similar inhibitory activity of X_{24} modified antisenses (3′ or 5′) indicates that these long inhibitors may not follow the proposed mode of action. This conclusion was also supported by the potent inhibitory potential of the $(X)_{24}$SCR which is not able to form base pairs with the hTR template sequence (IC_{50}s of $(X)_{24}$AS, $AS(X)_{24}$ and $(X)_{24}$SCR did not differ essentially). Moreover, these derivates are not specific, since they inhibited HIV RT with similar parameters. These findings can be explained by the property of the chemically modified oligonucleotides to inhibit DNA-polymerases in a sequence nonspecific manner, since 37mer chimeras (X_{24} modified antisenses and scramble) reach the length reported to have maximal inhibitory activity (30–40mers [15]). In contrast, X_{24} (as shown by its IC_{50} in Table 1) is too short to exert full sequence nonspecific inhibition.

Considering is the physico-chemical nature of the interaction of thiolated oligonucleotides with the protein, there are two likely modes: (a) hydrophobic interaction, due to the hydrophobic character of the 4-thiono group, or (b) disulfide bond formation between properly localized cysteinyl side chains on hTERT surface and reactive –SH, formed on the (s^4dU) base due to tautomeric conversion. The latter, covalent interaction between proteins and (s^4dU) containing oligonucleotides is chemically feasible and was proven (Horvath, A., Aradi, J., unpublished data).

Since telomerase exerts its function as a DNA-polymerase obligatory in the nucleus, the observed nuclear uptake of our chimeric oligonucleotides seems to be promising. The perinuclear portion might correspond to the dissociating lipid-oligonucleotide complexes [20]. Experiments to evaluate hTERT subcellular localization in immortal cell lines showed that hTERT is immunologically detectable not only in nuclei but also in the cytoplasm [21]. Strong Cy5 signals detected in the cytoplasm may also correspond, at least to some degree, to oligonucleotides bound to the cytoplasmic telomerase.

In conclusion, the properties of the novel family of chimeric oligonucleotides, described here for the first time, indicate that development of such molecules might be a favorable anti-telomerase strategy since further improvement in inhibitory parameters and intracellular properties are possible (for e.g., by introducing various backbone modifications). Moreover, following the idea of dual interaction, using the presented chemically modified chimeras as lead compounds, could provide new data in the biology of telomerase inhibition.

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