

Role of Phosphoramidate Antisense in Specific Inhibition of K562 Leukemic Cell Line

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Abstract

Telomeres are specialized heterochromatin structures that act as protective caps at the ends of the chromosomes. Telomere maintenance in the majority of tumor cells is achieved by telomerase, a reverse transcriptase enzyme that catalyzes the synthesis of further telomeric DNA. Telomerase is detected in the majority of malignant cells. Thus, telomerase may represent a very good candidate for targeted therapy in cancer. To inhibit telomere maintenance by telomerase, approaches that directly target either telomerase and telomeres or the telomerase regulatory mechanisms have been used. Moreover, strategies targeting telomerase-positive cells as a means to directly kill the tumor cells have been tested. The aim of this study was to evaluate effects of antisense against the template region in the telomerase RNA (hTR). The K562 cells were cultured and to inhibit telomerase activity, a 19-mer antisense oligonucleotide, complementary to hTR was designed. The modification of this sequence was N3'→P5' phosphoramidate and FuGENE6 was used as transfection reagent. Telomerase activity was measured using TRAP assay. Telomerase activity decreased using both sense and antisense hTR after 3 days with 1 μM comparing to the scramble oligonucleotides. There was morphological changes observed in K562 cells and MTT assay demonstrated that treatment of these cells efficiently reduced cell viability. Our results clearly demonstrated that antisense phosphoramidate is a very good candidate for targeted therapy in K562 leukemic cell line. The applied antisense inhibits transcription of hTR efficiently and can be considered as a potential therapeutic approach for leukemia treatment.

Keywords: Telomerase; Antisense; Oligonucleotides; FuGENE6

Introduction

Telomeres are specialized heterochromatin structures that act as protective caps at the ends of chromosomes.

Human telomeres consist of tandem repeats of the hexanucleotide sequence TTAGGG in double strand, except for a terminal 30 G-rich overhang. In normal somatic cells, telomeres shorten with each round of cell

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division and, when they reach a critically short length, cells exit from the cell cycle and undergo a replicative senescence [1]. By contrast, immortal cells as well as germline cells adopt some mechanisms to bypass the senescence checkpoint. Telomere maintenance in 80-95% of tumor cells is achieved by telomerase, a reverse transcriptase enzyme that catalyzes the synthesis of further telomeric DNA repeats. The telomerase holoenzyme consists of the catalytic subunit reverse transcriptase protein hTERT the telomerase RNA template subunit, hTR and other associated proteins. Telomeric DNA and the core telomerase components of telomerase, hTR and hTERT, are definitely required for telomerase function and therefore they are good targets for anti-telomerase strategy. Telomerase activity is typically absent from most normal human cells, but is expressed in nearly all human cancer cells. All these findings suggest that telomerase might represent a very good candidate for targeted therapy in leukemia. To inhibit telomere maintenance by telomerase, approaches that directly target either telomerase and telomeres or the telomerase-associated regulatory has been applied [2,3].

N3'→P5' phosphoramidate oligonucleotides have been designed, synthesized and evaluated as telomerase inhibitors. The oligonucleotides were targeted at a segment of hTR downstream from the hTR template region. It behaves like classical active-site enzyme inhibitors, *i.e.* 'template antagonists'. The oligonucleotides were employed because they form very stable duplexes with single-stranded RNA, are resistant to nucleases and display a high affinity for nucleic acids, but not proteins. The same group then tested the anti-telomerase effect of N3'→P5' phosphoramidate oligonucleotides. Telomerase inhibition occurred 24 h after transfection of an immortalized human breast epithelial cell line at submolar concentrations [4,5]. Long-term treatment with NP oligonucleotides resulted in gradual telomere shortening followed by cellular senescence and apoptosis, while the scramble control compound had no effect on cell proliferation [6,7]. Optimization of the sequence, length and bioavailability resulted in the selection of the novel telomerase template antagonist [5,8]. Although antisense against hTERT has widely been studied, but there are only a few study on antisense against hTR, application of antisense against hTR in cell line with high telomerase activity has not been studied. The aim of this study was to evaluate a relatively long antisense (19-mer) on a cell line with high telomerase activity (K562). To the best of our knowledge it is the first study, using the mentioned settings.

Material and Methods

Cell Culture Conditions

Cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), streptomycin and penicillin at 37°C in a humidified atmosphere containing 5% CO₂ as previously described [1,5].

Antisense Oligonucleotides

The sense, antisense and scramble oligonucleotides were synthesized by Faza Pajoh Company and were purified by HPLC. They were designed to have 19-mer lengths and to be complementary to the template (5'-CUAACCCUAA-3') region of the telomerase RNA. To increase instability, these oligonucleotides were modified with phosphoramidate groups. The sequence of antisense oligonucleotide was (5'-CTCAGTTAGGTTAGACAA-3').

Oligonucleotide Transfection Protocol

Before transfection, cells were plated on 6-well plates and were washed with FCS-free medium RPMI-1640. Then 900 µl FCS-free RPMI-1640 were added in to each well prior to transfection, for the preparation of the complex of antisense oligomers/transfactor, the FuGENE6 transfection reagent (Roche, Germany) was diluted with serum-free medium and incubated for 5 min at room temperature. Then, the desired amount of oligomer was added into the FuGENE6 for 15 min at room temperature. The final volume of the complex in each sample was 100 µl. These complexes were added drop wise to the cell culture containing 900 µl FCS-free medium. A control containing only FuGENE6 reagent was also included. The cultures were incubated for 4 h at 37°C; 1 ml of 20% FCS in RPMI-1640 was added into each well, to a final FCS concentration of 10%.

Effect of Oligonucleotides on Number and Viability of K562 Cells

K562 cells were suspended at a concentration of 5×10⁴/ml. Then 200 µl of the cell suspension was placed in each well of a triplicate 96-well microtiter plate. Different concentrations (1 µM and 8 µM) of phosphoramidate oligonucleotides together with FuGENE6 were transfected to the cells. MTT assay was performed after 48 h of growth, by adding 40 µl of 5 mg/ml of MTT to each well and incubation for 4 h at 37°C. The Formazan crystals were dissolved in 200 µl DMSO and optical density value (OD) was measured at 450 nm wavelength by ELISA. These assays were

performed three times and the average results were calculated [3,4].

Telomerase Activity with Telomerase Repeat Amplification Protocol (TRAP) Assay

Cultured cells in logarithmic growth were suspended at a concentration of 2×10^4 /ml, and then 5 ml was placed into a cell culture flask. Cells were harvested after 12 h, 24 h, and 36 h, washed once with wash buffer and homogenized in 150 μ l cell lysis buffer, on ice for 30 min. The homogenates were then centrifuged at $12000 \times g$ for 20 min at 4°C . The supernatants were snap-frozen in liquid nitrogen and stored at -80°C . Telomerase activity was then measured on samples using TRAP-PCR-ELISA assay. In brief, 2 μ l of tissue extract and 48 μ l TRAP reaction mixture were placed into tubes. PCR was performed at 94°C for 120 s (1 cycle) followed by 35 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 90 s. The PCR products (20 μ l) were hybridized to a digoxigenin (DIG)-labelled telomeric repeat specific detection probe. The PCR products immobilized via the biotin-labelled primer to a streptavidin-coated microtiter plate. The immobilized PCR products detected with a peroxidase-conjugated anti-DIG antibody and visualized following addition of the stop reagent. Microtitre plate was assessed on an enzyme-linked immunosorbent assay (ELISA) plate reader at 960 and 540 nm wavelength [8,10].

Effect of Oligonucleotides on K562 Cell Morphological Changes

Morphology of the cells were observed using light microscopy and compared to the control cells in different time intervals (24 h, 48 h and 72 h) after transfection.

Results

Inhibitory Effect of N3'→P5' Phosphoramidate Oligonucleotides on Telomerase Activity

Differences between absorbance in 540 nm and 960 nm were used as indication of telomerase activity. Using 1 μM oligonucleotides, it was observed that telomerase activity was 1.8 in control group, 1.3 in scramble (non-significant decrease), 0.6 in antisense and 0.7 in sense oligonucleotides (significant decrease in both). In summary, telomerase activity decreased ($p < 0.05$) with N3'→P5' phosphoramidate hTR sense and antisense after 3 day with 1 μM compared to scramble oligonucleotides and control group (Fig. 1).

Effects of Oligonucleotides on Cell Proliferation and Cell Number

K562 cells were treated with different concentrations of sense and antisense oligonucleotides for 3 days. The applied antisense and sense oligonucleotides reduced the cell number (Fig. 2). The result of MTT assay, which was used to measure proliferation and viability in K562 cells, is shown in Figure 3. The applied nucleotides significantly ($p < 0.05$) inhibited these leukemic cell proliferations in 8 μM concentration 3 days after transfection (Fig. 3).

Morphological Changes in K562 Cells

The results of light microscopy demonstrated that the cells became round after treated with antisense for 24 h. After 48 h, the cells were crumpled and floated. The cells also had big nucleoli and aberrant nuclei and the cell membranes were also disrupted (Fig. 4).

Discussion

In this investigation we applied antisense and sense oligonucleotides against hTR of a leukemic cell line, K562. We applied a long, 19-mer, N3'→P5' phosphoramidated oligonucleotide, on a leukemic cell line with a very high telomerase activity. To the best of our knowledge it is the first report of application of antisense technology with modification, length, cell line, time and concentration of treatment that we used. Our results clearly demonstrated that telomerase activity in K562 cells was efficiently decreased with N3'→P5' phosphoramidate hTR sense and antisense oligonucleotides after 3 day with 1 μM compared to scramble oligonucleotides. Application of these antisense and sense oligonucleotides reduced the cell number. The antisense was complementary to the telomeric RNA (hTR) and could suppress reverse transcriptase enzyme. The sense oligonucleotides are complementary to the telomeric repeats of the end of chromosomes and therefore inhibited increase in the length of telomeres. Another effect of treatment of the cells with anti-hTR oligonucleotides was reduction in cell viability in 3 days post transfection. This result is similar to the results obtained by Kondo *et al.* 2000 [9] although the modification of the oligonucleotides and the time of treatment were different between their study and ours. Similar anti-tumor effects following treatment with N3'→P5' phosphoramidate anti-hTR were observed in other telomerase-positive tumors, such as malignant glioma, ovarian, bladder and cervical cancer cells [10-12]. Interestingly, our different settings with different cell type showed more or less the same results. Light

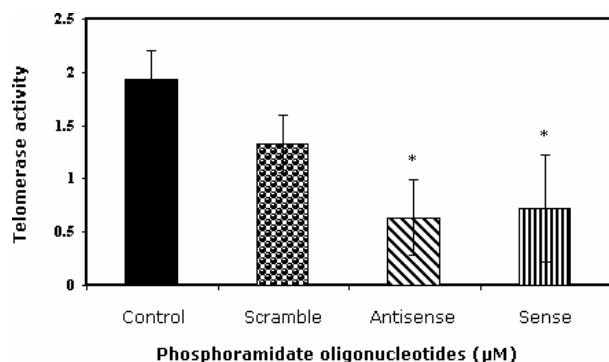


Figure 1. ELISA-TRAP assay demonstrating acquisition of telomerase activity in K562 cells transfected after 3 days with phosphoramidate oligonucleotides (formulated with FuGENE6). The concentrations of all nucleotides were 1 µM. Telomerase activity decreased significantly in both antisense and sense, but not in scramble compared with control group.

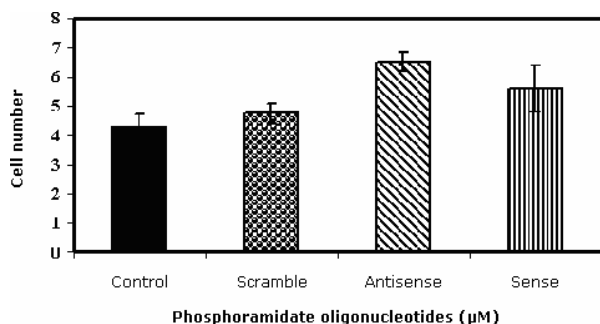


Figure 2. Effect of antisense, sense and scramble phosphoramidate Oligonucleotides (formulated with FuGENE6) cell number 24 h post transfection. The concentrations of all oligonucleotides were 1 µM. There were no significant differences between groups.

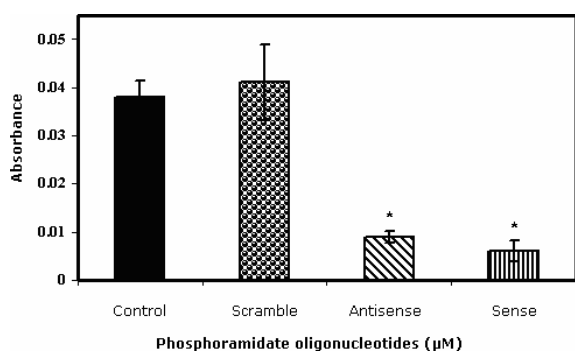


Figure 3. Effect of antisense, sense and scramble phosphoramidate oligonucleotides on K562 cells ELISA-based MTT assay demonstrating number of the cell in 8 µM concentration. Optical density decreased significantly in both antisense and sense, but not in scramble compared with control group.

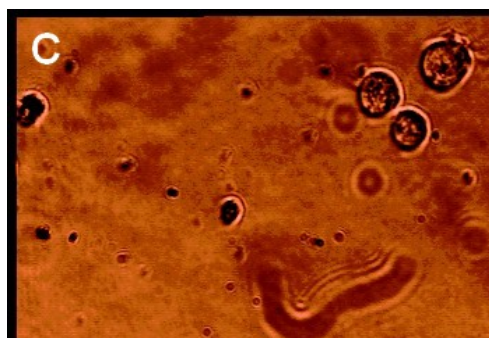
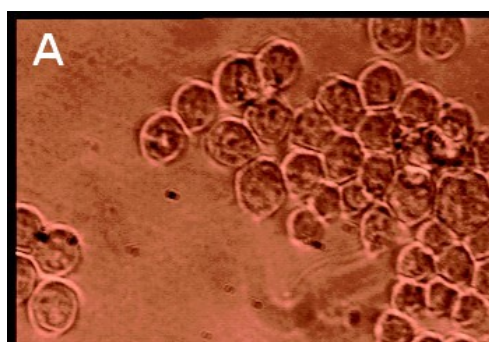


Figure 4. The morphological changes in the K562 cell line after the transfection with hTR antisense formulated with FuGENE6 in 1 µM concentration. The pre-apoptotic appearance of the treated cells can be observed in cells. The magnitude of the figures are 400× A) control group transfected with only FuGENE6. B, C and D) treated with 1 µM antisense 1, 2 and 3 days after transfection, respectively.

microscopy experiments on our cells demonstrated that the morphology of the cells changed greatly as effect of both antisense and sense oligonucleotides treatment, so that cells were very similar to pre-apoptotic cells. These results are in accordance with the findings of others that telomere shortening and senescence can promote apoptosis [13] and the cells are probably arrested in G2/M. Interestingly it is demonstrated that induced apoptosis is related to decreased telomerase activity [14]. The role of Bcl-2 inhibition in cell apoptosis is shown by other investigators and it is also shown that telomeres shortening and Bcl-2 reduced expression are related to apoptosis. Therefore the effects of our treatment might be through Bcl-2 inhibition. Although there are some evidences to support this idea, but more investigations is necessary to prove this idea [13,14].

Our results also demonstrated that Phosphoramidate oligonucleotides can be considered as an efficient modification in antisense technology. Other modifications have also been used by others [13], but our results demonstrated that application of Phosphoramidate modified oligonucleotides is a very powerful approach in antisense technology [15].

In conclusion, our results as well as others in different settings clearly demonstrated that these modified oligonucleotides could effectively cause telomerase inhibition in human malignant cell lines. Therefore it may provide important insights into therapeutic approach against leukemia. We suggest that this technology could be considered as a strong approach in treatment of leukemic cells in human.

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