PAPER

抗hTERT siRNA及び抗hTR S-オリゴDNA-シグナルペプチドコンジュゲートによるヒト癌細胞中でのテロメラーゼ活性と増殖の抑制

Suppression of Telomerase Activity and Proliferation of Human Cancer Cells by anti-hTERT siRNAs and anti-hTR S-OligoDNA-Signal Peptide Conjugates

Masayuki Fujii[1)] Irmina Diala

和文要約：テロメラーゼ(hTERT)はヒト染色体のテロメアを伸長する逆転写酵素であり、癌細胞に特有に発現するため癌治療の格好の標的と目されている。テロメラーゼmRNAを標的とするsiRNAとテロメラーゼテンプレートRNA(hTR)を標的とするホスホロチオエートDNA(S-DNA)-シグナルペプチドコンジュゲートを用いて、ヒト癌細胞に発現するテロメラーゼ活性を阻害し、その増殖を抑制することに成功した。また、ホスホロチオエートDNA（S-DNA）と核局在化シグナル（NLS）ペプチドとのコンジュゲートは遺伝子導入剤を用いることなく細胞内に侵入し、標的であるテロメラーゼが存在している核内に局在化することを証明した。

Abstract: Telomerase activity has been regarded as a critical step in cellular immortalization and carcinogenesis and because of this, regulation of telomerase represents an attractive target for anti-tumor specific therapeutics. Recently, one avenue of cancer research focuses on antisense strategy to target the oncogenes or cancer causing genes, in a specific fashion to completely inhibit the expression of the target gene. The protein catalytic sub-unit, human telomerase reverse transcriptase, hTERT and the template RNA, hTR are essential for telomerase function, thus theoretically, inhibition of telomerase activity can be achieved by interfering with either the hTR or hTERT component of telomerase enzymatic complex.
This study made use of antisense strategy to compare effects of small interfering RNAs (siRNAs) that targets the telomerase reverse transcriptase hTERT mRNA with the effect of synthesized phosphorothioate oligonucleotide-peptide conjugates that targets the RNA component hTR in inhibiting telomerase activity. Based from the results of this study, both antisense approaches have successfully suppressed telomerase activity but of varying levels of inhibition. Several factors have been identified that may have caused such variation in the level of telomerase inhibition. For siRNA strategy, 6 siRNA sequences directed at different sites of the hTERT gene were screened using quantitative RT-PCR before choosing the best siRNA sequence that was to be use to determine effect on the inhibition of telomerase activity. While for the antisense approach that targets the hTR component of the enzyme, the covalent coupling of signal peptide with the oligonucleotide enhances the inhibitory effect of the conjugate to telomerase activity. Overall, the study was able to show the specificity of antisense strategy to inhibit telomerase activity.

Keywords：telomerase activity, small interfering RNA, hTERT, telomerase, s-DNA-peptide conjugate

Introduction

Genetic disease is often caused by genes, which are inappropriately transcribed − either too much or too little − or which are missing altogether. Such defects are especially common in cancers, which can occur when regulatory genes are deleted, inactivated or become constitutively active. Unlike some genetic diseases, in which a single defective gene is always responsible, cancers, which appear clinically similar, can be genetically heterogeneous involving dynamic changes in the genome. Cancer has become such a pervasive disease that the interest in studying its pathology and possible cures has heightened. Current research is focusing on the genetic level to eliminate the disease and most recently, the oligonucleotide-based antisense techniques represent the most common and, to date, the most successful approach to target the cancer causing genes in a specific fashion completely inhibiting the expression of the oncogenes. Theoretically, the antisense approach represents

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an elegant strategy, involving the targeting to and association of an antisense oligonucleotide sequence with a specific mRNA via base pairing.

The identification of molecular targets for use in cancer therapeutics has been part of the cancer drug discovery process. The current trend in research on anticancer drugs is to exploit particular traits or hallmarks unique to cancer cells. Although cancer display a great heterogeneity in clinical behavior, most if not all cancers have acquired the same set of functional capabilities that define the malignant state [1]. These acquired capabilities include self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, avoidance of programmed cell death (apoptosis), unlimited replicative potential, sustained angiogenesis, tissue invasion and metastasis [2]. Among these hallmarks, the acquisition of unlimited replicative potential is a key step to ensure expansive tumor growth. Several researches showed that high telomerase activity was correlated closely with tumor development [3, 4]. Telomerase activity has been regarded as an essential step for the immortalization of human cells, both in vitro and in tumor progression in vivo. Many reports in cancer genetics have shown that approximately 90% of all tumors exhibited telomerase activity [5]. At the same time telomerase activity is not detected in most mature somatic cells except for stem cells and germ line cells. Although altered telomerase activity is not a major cause of malignancy, a strong association between the presence of telomerase activity and malignancy makes telomerase an attractive molecular target toward which to direct cancer therapeutic agents.

Objectives of the Study

Inhibition of telomerase theoretically occurs by interfering with either the hTR or hTERT component of the enzymatic complex. The main focus of this study was the use of siRNA that targets the protein catalytic subunit of human telomerase (hTERT) and phosphorothioate-peptide conjugate that targets the template RNA (hTR) was designed and used to inhibit telomerase activity. More specifically, this study was able to

1. Measure the effect of the designed antisense oligonucleotides (siRNA for hTERT and Phosphorothioate-Peptide conjugate for hTR) on telomerase activity by Telomeric Repeat Amplification Protocol (TRAP) assay.
2. Detect quantitatively the amount of silencing effect of the siRNA that targets hTERT mRNA expression at the molecular level using Quantitative real time RT-PCR.
3. Investigate the cytotoxicity effect of the designed hTERT siRNA on the cell proliferation activity using cell proliferation assay.

Materials and Methods

Cell culture

Adherent cells (HeLa) and Suspension cells (K562 and Jurkat) were maintained in RPMI 1640 medium containing FBS and antibiotic and incubated at 37ºC, 5% CO_2 atmosphere in a humidified incubator. Cells were regularly passage to maintain exponential growth.

Selection of the target sequence of siRNA

A total of 6 siRNA sequences targeting different hTERT mRNA sequences were evaluated and screened to choose the most effective siRNA sequence that showed the highest inhibitory/silencing effect on the hTERT mRNA expression. All siRNAs were chemically synthesized, purified and annealed (Qiagen Co. Ltd, Japan) with a two-nucleotide overhang at the 3'-end. The list of siRNA sequences were as follows:

siRNA sequences targeting the hTERT gene

Seq No	GenBank Accession No.	Position hTERT	Sense sequence (5’-3)	Antisense sequence (5’-3’)
1	**NM_198255**	2291-2311	GGCCUUCAAGAGCCACGUCTT	GACGUGGCUCUUGAAGGCCTT
2		2321-2339	ACAGACCUCCAGCCGUACATT	UGUACGGCUGGAGGUCUGUTT
3		1800-1818	GGAGCAAGUUGCAAAGCAUTT	AUGCUUUGCAACUUGCUCCTT
4	**AB085628**	2127-2145	CAAGGUGGAUGUGACGGGCTT	GCCCGUCACAUCCACCUUGTT
5		2715-2734	AACAUGCGUCGCAAACUCUTT	AGAGUUUGCGACGCAUGUUTT
6		1163-1183	AAAUGCGGCCCCUGUUUCUGG	CCAGAAACAGGGGCCGCAUTT

Primer/Probe	Sequence
hTERT	
Forward primer	**5'- ACG GCG ACA TGG AGA ACAA-3'**
Reverse primer	**5'- CAC TGT CTT CCG CAA GTT CAC-3'**
hTERT probe	**5'-FAM d(CTC CTG CGT TTG GTG GAT GAT TTC TTG TTG)BHQ-1- 3'**

All siRNA sequences were submitted in a BLAST search of human EST libraries (http://www.ncbi.nlm.nih.gov/blast/) to confirm that only hTERT gene was targeted. A scrambled (non-silencing) siRNA was also purchased from Qiagen (Cat. No. 1022076) and used as the negative control.

siRNA Transfection

Adherent cells: One day before transfection, HeLa cells were plated in a 24-well flat-bottomed plate. Cells were seeded at a density of $1x10^5$ cells/well in a 0.5 ml of growth medium without antibiotics so that cells will be about 90-95% confluent at the time of transfection.

Suspension cells: On the day of transfection, k562 and Jurkat cells were separately seeded in 24-well flat-bottomed plates at a density of $4x10^5$ cells/well in 0.5 ml of growth medium without antibiotics.

*The cells were transfected with siRNA (200nM concentration) complexed with Lipofectamine™2000, according to the manufacturer's instructions (Invitrogen, Life Technologies Corporation, USA). Cells were then incubated at 37°C, 5% CO_2 atmosphere in a humidified incubator. Cells were harvested and assayed 24 hour after transfection.

Note: The transfected cells were first screened for QRT-PCR to detect the effect of the different siRNA sequences in silencing the hTERT mRNA gene expression. The best sequence with the highest inhibitory effect to the hTERT mRNA gene expression was further evaluated for TRAP assay and Proliferation assay

Quantitative Reverse Transcriptase - Polymerase Chain Reaction (QRT-PCR)

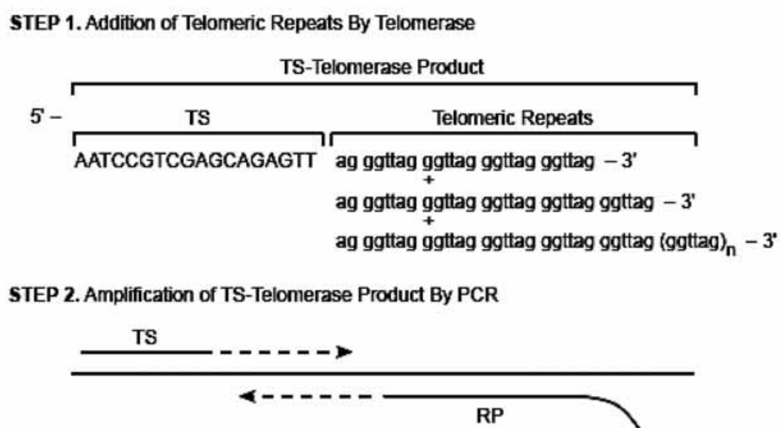
hTERT mRNA level was quantified by Real-Time, two-step reverse transcriptase-PCR (QRT-PCR) method. Briefly, total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen). Two-step RT-PCR was done using SuperScript™ III Platinum® 2-Step qRT-PCR kit (Invitrogen, Life Technologies Corporation, USA) according to the manufacturer's instructions. The conditions of 2-step RT-PCR were as follows: 2 minutes at 50°C, 2 min at 95°C and then 45 cycles of amplification for 30 sec at 95°C and 1 min at 62°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to ensure accuracy. The assay used an instrument capable of measuring fluorescence in real time (MX3005P™, Stratagene). The table below shows the sequences for the TaqMan probe and primers used.

Telomerase activity detection assay

The TRAPeze® telomerase detection kit (Chemicon International, USA) was used according to the manufacturer's protocol with a minor modification. Briefly, cells were harvested and washed once with chilled PBS and then homogenized in 200ul 1x CHAPS lysis buffer on ice for 30 min and centrifuged at 12,000X g for 20 min at 4°C. The supernatant liquid was collected and BCA protein assay kit (Pierce Biotechnology, USA) was used to measure protein concentration. Sets of diluted protein aliquots were tested for each sample in the TRAP assay to establish the linearity of the assay. Telomerase reaction was carried out at 30°C for 30 minutes followed by a 3-step PCR amplification (94°C, 30 sec, 59°C, 30sec and 72°C, 1 minute for 33 cycles). The amplified products were electrophoresed on a 12.5% non-denaturing polyacrylamide gel. The gel was stained with SYBR® green nucleic acid stain (Molecular Probes, USA) and analyzed with Doc-ItLS UVP ver.5.5.4 (Life Science Software, USA). Telomerase activity was quantified from all assays within the linear range by normalizing the total amount of reaction products (telomeric bands) in each lane to the signal obtained from the internal telomerase assay standard present in the same lane, as described in the kit's protocol. Levels of telomerase activity were obtained from a minimum of three assays of at least two independent prepared extracts from each sample.

The methodology utilized in the TRAPEZE® Gel-Based

Schematic Diagram of *TRAPEZE® Gel-Based Telomerase Detection kit* Assay



Telomerase Detection kit is based on an improved version of the original method described by Kim *et al* [71]. This technique is highly sensitive in vitro assay system utilizing the polymerase chain reaction (PCR). In the first step of the reaction, telomerase adds a number of telomeric repeats (GGTTAG) onto the 3'end of a substrate oligonucleotide (TS). In the second step, the extended products are amplified by PCR using the TS and RP (reverse) primers, generating a ladder of products with 6 base increments starting at 50 nucleotides: 50,56,62,68,etc. This kit reduces amplification artifacts and permits better estimation of telomerase processivity.

Synthesis of phosphorothioate oligonucleotide-peptide conjugates

Phosphorothioate Preparation for hTR

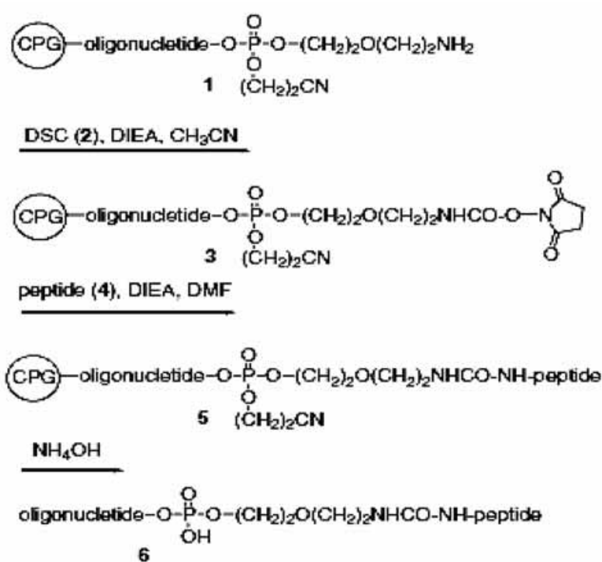
Solid phase synthesis of peptide fragments:

Peptides were prepared by a standard fmoc-chemistry using 100mg of Wang resin (novabiochem, 100-200 mesh, 0.50-1.30mmol/g). Each peptide is modified with lysine, which is initially protected by tert-butyloxycarbonyl (boc) group and finally deprotected after the cleavage, at the second terminal position of carboxyl terminus to put a free reactive amino group on peptides. The obtained peptide fragments were fully characterized by RP-HPLC and MALDI-TOF-MS to give satisfactory results.

Synthesis of s-DNA-peptide conjugates by SPFC:

The syntheses of the conjugates involve a solid phase fragment condensation (SPFC) as shown in Scheme 1.

Using the commercially available phosphoramidite (Glen Research 5'-Amino Modifier5) a phosphorothioate oligonucleotide assembled in 1 mmol scale on CPG (PROLIGO, 500 Å, 30-40 mol/g) support was modified at the 5'-end of the oligonucleotides [72]. Next a bifunctional linker molecule, di(N-succinimidyl)carbonate (DSC) [73], was reacted with the terminal amino group on solid phase, and then partially protected peptide fragment [74] bearing a single free reactive amino group, independently synthesized and purified, was reacted with CPG-linked phosphorothioate oligonucleotides [75] to give phosphorothioate oligonucleotide-peptide conjugates still attached to CPG [76]. In the peptide fragments, trifluoroacetyl (tfa) group protected the e-amino groups of lysine (K) except for a reactive site, thiols of



cysteine (C) and hydroxyls of serine (S) and threonine (T) are protected by acetyl (ac) group, and guanidyl and carboxyl group are already deprotected before the condensation. Finally, CPG-linked products are treated with concentrated aqueous ammonia at 55°C for 4 hours to give fully deprotected product. Reversed phase HPLC purification gives a single peak pure product in 3-13% over-all yields in >95% purities and all the products are fully characterized by MALDI-TOF-MS to give satisfactory results. The phosphorothioate − peptide conjugate was transfected to Jurkat cells and later evaluated the effect on telomerase activity by TRAP assay.

Oligonucleotide and Transfection Experiment

There were 4 phosphorothioate oligonucleotide-peptide conjugates used in the experiment namely: S-oligo-SV-40LT-ant NLS(**6a**); S-oligo-HIV-1 tat NLS (**6b**) ; S-oligo-HIV-1 rev NES (**6c**) and S-oligo-LRAL-4(**6d**). Two concentrations of phosphorothioate oligonucleotide-peptide conjugates (1uM and 5uM) were examined and compared.

Jurkat cells were seeded in 24-well flat-bottomed plates at a density of $2x10^5$ cells/well in a 0.5ml of growth medium without antibiotic. Then cells were transfected once with the conjugates diluted in a serum-free OptiMEM (Invitrogen, Life Technologies Corporation, USA). Jurkat cells were then incubated at 37°C humidified atmosphere containing 5% CO_2. Telomerase activity was examined 24 and 48-hr after the end of transfection.

Cell Proliferation assay

Cellular proliferation was assayed using Cell Proliferation Reagent WST-1 (4-[3-(4-Iodophenyl)-2(4-nitrophenyl)2H-5 tetrazolio]-1,3-benzene disolfunate (Roche Applied Science and Diagnostics, Germany). It is a colorimetric assay system for the quantification of cell proliferation and cell viability based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase in viable cells. In brief, $1x10^4$ cells/well were incubated in a 96-well plate, in the presence or absence of siRNA, at 37°C, 5% CO_2 atmosphere in a humidified incubator. About 10ul of WST-1 reagent was added to each well. After 4 hours, absorption at 450nm was determined on a Wallac 1420 ARVOmx/Light spectrophotometer reader (Perkin Elmer, USA). The proliferation inhibition rate is calculated as follows: Inhibitory rate= Abs control − Abs sample / Abs control − Abs blank X 100, where Abs control is the absorbance value of cells without treatment, Abs sample is the absorbance value of cells treated with antisense agent, Abs blank is the absorbance value of media (RPMI) as blank control.

Results

Use of siRNA as an agent for down-regulation of telomerase activity

Screening for effective siRNA sequence to silence hTERT mRNA expression

In order to detect whether siRNA could inhibit hTERT gene expression, the mRNA level of hTERT was determined by Quantitative RT-PCR. As shown in Figure A, three cancer cell lines namely HeLa, Jurkat and K562 cells were treated all at the same time with several siRNAs. The 6 siRNA sequences used, targets different mRNA sequence of the hTERT gene. All 6 siRNAs were 21 nucleotide (21 nt) in length and were given at the concentration of 200nM.

The result of the quantitative RT-PCR showed varying rates of inhibition of the 6 siRNAs to the hTERT mRNA expression compared with cells treated with control siRNA. Of all the siRNAs tested, only siRNA-3 (seq-3) appeared to be able to reduce mRNA levels for all 3 cancer cell lines. An approximately 85% reduction of hTERT mRNA was observed in HeLa cells using siRNA-3 and 60% and 50% reduction of hTERT mRNA for K562 and Jurkat cells respectively were also observed.

Compared to the control cells, the cells treated with siRNA-3 (seq-3) exhibited different degrees of reduction in the hTERT mRNA expression. As shown in the data (Figure B) HeLa cells showed favorable response to the siRNA treatment as compared to the reaction elicited by K562 and Jurkat after 24 hour of transfection.

The result of the Quantitative RT-PCR have shown that siRNAs targeting different hTERT sequences have significantly various inhibitory effects on hTERT gene expression. Quantitative RT-PCR was suitable for screening high-effect siRNA targeting site for specific gene. siRNA-3 exhibited favorable effects of inhibiting hTERT mRNA. Thus, further biological assays used in this study such as TRAP and

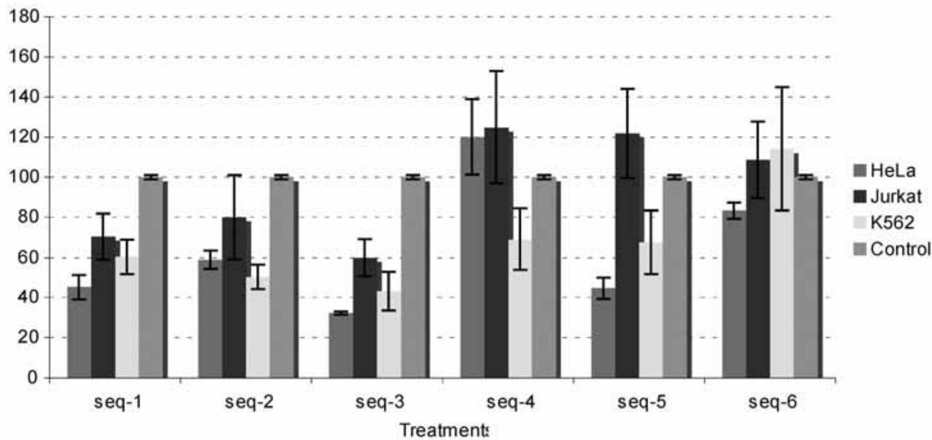


Figure A: Inhibition effects of different siRNA targeting hTERT mRNA expression in HeLa, Jurkat and K562 cancer cell lines after 24-hour transfection with 200nM siRNA concentration. Among the six siRNA sequences used, siRNA sequence-3 showed the highest percentage of silencing in the three cancer cell lines used. The results were expressed as mean± SD from three determinants.

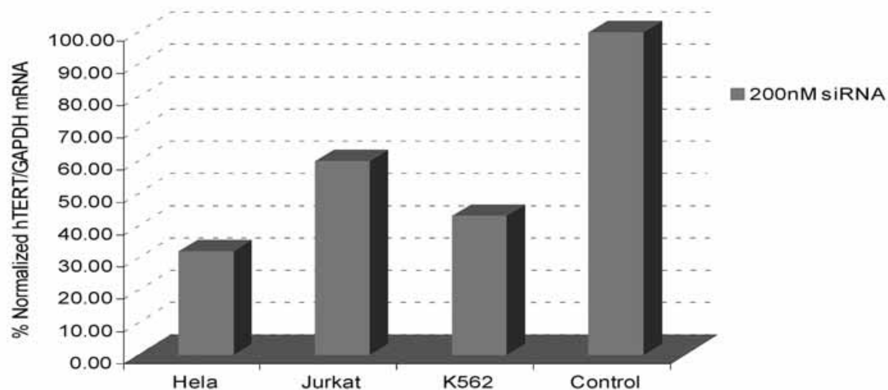


Figure B: Silencing efficiency of siRNA- 3 (sequence-3) in HeLa, Jurkat and K562 cancer cell lines after 24-hour transfection using 200nM siRNA concentration complexed with Lipofectamine™2000. Normalized to the levels of GAPDH, the relative inhibitory rate of siRNA-3 varies in the 3 cancer cell lines used. Among the 3 cancer cell lines, HeLa, which is an adherent cell showed the highest reduction of the hTERT mRNA level. Meanwhile between the 2 suspension cells, K562 showed approximately 60% reduction in its hTERT mRNA level as compared to Jurkat.

Proliferation assay made used of siRNA-3.

The effect of siRNA treatment on telomerase activity

The effect of the hTERT siRNA on telomerase activity was evaluated by telomeric repeat amplification protocol (TRAP) assay. Gel electrophoresis (Figure C) showed that cancer cells treated with 200nM concentration of hTERT siRNA after 24 hours of transfection had reduced their telomerase activity as compared to the untreated cells (positive control). The positive control showed a distinct and clear 6bp ladder pattern compared to the treated cells. The levels of telomerase activity for K562, Jurkat and HeLa were obviously decreased.

The ladder pattern of telomerase activity observed after polyacrylamide gel electrophoresis was semi-quantitatively analyzed with Doc-ItLS UVP ver.5.5.4 software. Based on the data (Figure D), the treated cells showed a decreased in their telomeric repeat banding pattern in which case suggests down-regulation of their telomerase activity. HeLa cells

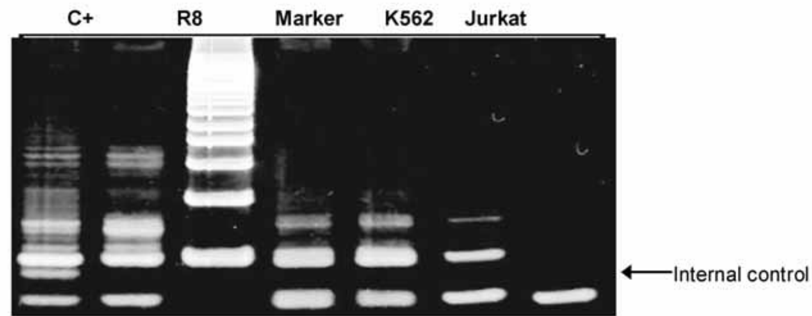


Figure C: Image of the telomerase activity of cells transfected with siRNA-3 (one representative experiment). The TRAP assay products were resolved in a 12.5% non-denaturing polyacrylamide gel and stained with SYBR® green gel stain. Lane 1 is the positive control (untreated cells); Lane 2 (R8) is the quantitation control standard provided in the assay kit. A 50-bp Molecular Weight Marker was used for this experiment. Following are the lanes for K562, Jurkat and HeLa cells treated with 200nM siRNA-3. Note the marked decrease in intensity of the telomerase ladder signal in the treated cells in comparison from the positive control and R8 quantitation standard.

showed the least number of telomeric repeats among the treated cancer cell lines.

The decreased in the TPG units of the treated cells showed that telomerase activity was affected by the action of the siRNA targeted at the protein subunit of the telomerase holoenzyme.

Antiproliferative effect of siRNA treatment

The results of the proliferation assay (Figure E) showed that the proliferation activities of the cells decreased when treated with hTERT siRNA (200nM). Inhibition of hTERT might have caused a rapid decrease in cell proliferation as observed in the data.

Among the 3 cancer cell lines treated with hTERT siRNA, HeLa showed a marked decrease in its cell viability. The bar graph shows that proliferation rate was suppressed in treated samples.

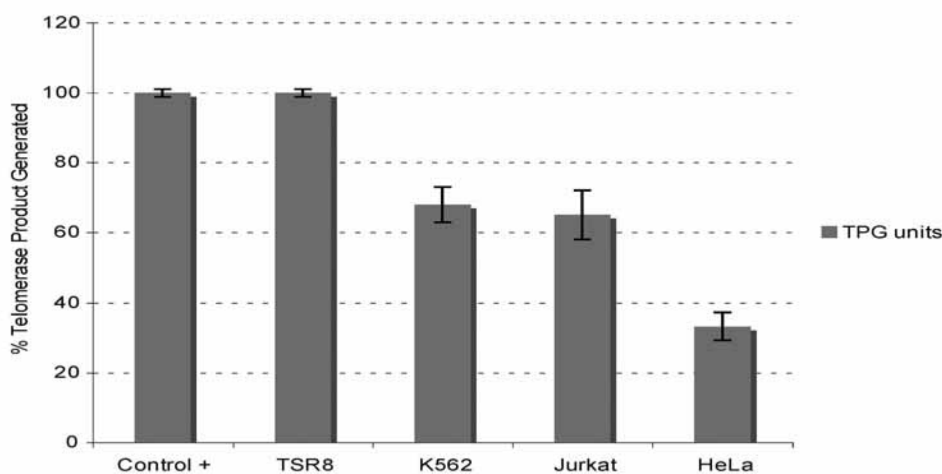


Figure D: Telomerase enzymatic activities were calculated as total product generated (TPG) units and normalized to a value of 100%. The bar graph represents the telomerase product generated after 24 hours transfection of the siRNA to the cells. The results were expressed as mean± SD from three independent experiments.

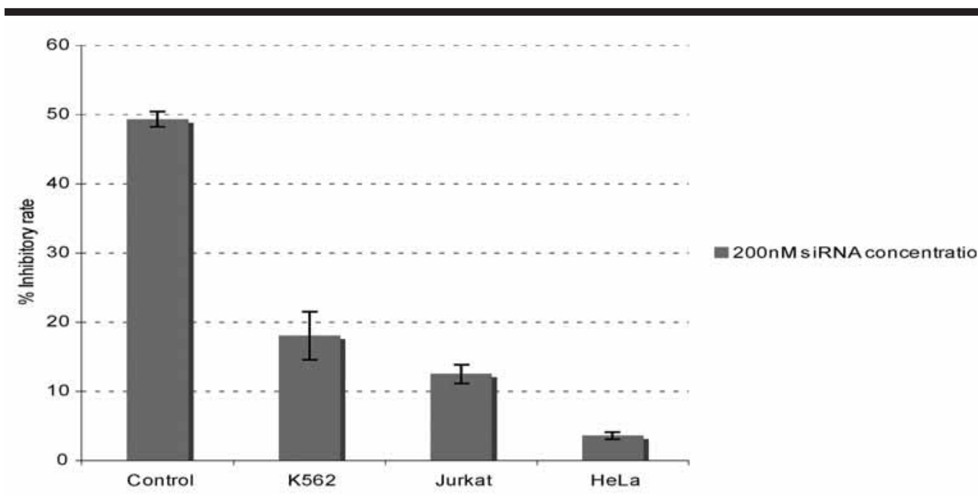


Figure E: Inhibitory effect of siRNA on the proliferation rate of the treated cancer cell lines. Cell Proliferation Reagent WST-1 was used to measure for cell viability after 24-hour transfection of siRNA. hTERT siRNA (200nM) treatment resulted in a marked inhibition of cell proliferation among the cancer cell lines used as compared to the untreated cells (control). The results were expressed as mean± SD from three determinants.

Use of phosphorothioate-oligonucleotide conjugate as agent for down-regulation of telomerase activity

Synthesis of oligonucleotide-peptide conjugate

Solid phase fragment coupling of peptides with oligonucleotide involves the synthesis of the oligonucleotide and peptides on separate solid support and later be reacted and linked with each other. Table 1 shows the synthesized peptides with the corresponding percentage yield and the correct mass as found in MALDI-TOF-MS.

An advantage of the fragment − coupling route is that both components can be purified by reverse phase HPLC before conjugation, making it easier to identify the conjugation product in the event of a low yield. As shown in Table 2 conjugates were successfully purified by reversed phase HPLC and the correct mass is shown.

Effect of phosphorothioate oligonucleotide peptide conjugate treatment on telomerase activity

The antisense inhibition effect of the synthesized phosphorothioate oligonucleotide-peptide conjugate that targets the hTR component of the human telomerase was evaluated in Jurkat cells using telomeric repeat amplification protocol (TRAP) assay (Figure F1-2).

Table 1: Synthesized peptides

Peptide	Sequence (origin)	Yield[a] (%)	MALDI-TOF-MS (found/calcd)
4a	Ac-GPKKKRKVKG-OH (SV40LT-ant NLS)	5.6	1552.69/1551.56
4b	Ac-GRKKRRQRRRPPGKG-OH (HIV-1 tat NLS)	4.3	2196.11/2194.36
4c	Ac-LPPLERLTLKG-OH (HIV-1 rev NES)	11.8	1278.45/1278.59
4d	Ac-LRALLRALLRALLRALKG-OH (designed)	4.1	2063.18/2058.64

K; ε-amino group is protected by trifluoroacetyl (tfa) group.
[a] Isolated yields.

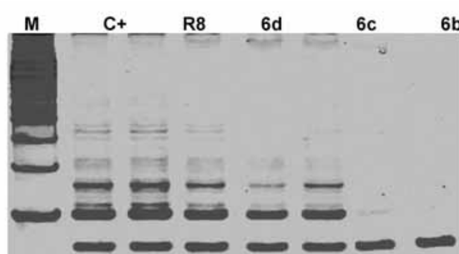
4 peptides have been successfully synthesized and purified using reverse phase HPLC and further characterized by MALDI-TOF-MS to give satisfactory results.

Table 2: Phosphorothioate oligonucleotidea-peptide conjugates

Conjugate	Peptide	Yield[b] (%)	MALDI–TOF–MS (found/calcd)
6a	**4a**	8.5	5582.24/5581.96
6b	**4b**	2.8	6420.67/6418.85
6c	**4c**	13.4	5695.16/5694.06
6d	**4d**	8.7	6475.67/6475.12

[a] Oligonucleotide sequence 5′-s(CAGTTAGGGTTAG)-3′.
[b] Isolated yields determined on A_{260}.

Phosphorothioate-oligonucleotide-peptide conjugate products as characterized by reverse phase -HPLC and MALDI-TOF-MS.



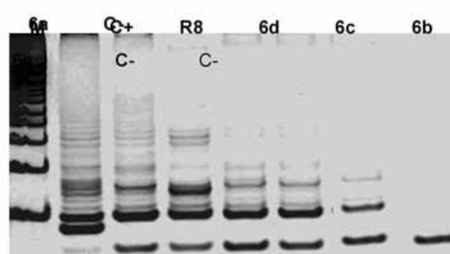


Fig. F-1: (Telomerase activity after 24hr) **Fig.F-2:** (Telomerase activity after 48-hr)

Figure F: Telomerase activity of Jurkat cells treated with 5uM concentration of various phosphorothioate oligonucleotide-peptide conjugates after 24-hr (**F-1**) and 48-hr (**F-2**) of transfection (one representative experiment). Lane 1 is the 50bp Molecular Weight Marker, Lane 2 is the positive control, next is the R8 (quantitation standard). The following wells are the Jurkat cells treated with 5uM concentration of the different phosphorothioate oligonucleotide-peptide conjugates. Telomerase activity was decrease as evident by reduced intensity of the 6bp increments in the ladder formation of the treated samples.

The result clearly shows that there was a decreased in telomerase activity of Jurkat cells after being treated with 5uM concentration of each of the conjugates as compared to the intensity of bands seen in the positive control and the R8 standard. However, as shown in Figure F-1, the phosphorothioate oligonucleotide-NLS conjugates, **6a** and **6b** showed a much higher inhibitory effect after 24 hours of transfection as evident by the reduced number of telomeric ladder pattern as compared to the other treatments. After 48-hr the inhibitory effect of the conjugates were slightly reduced (Figure F-2).

The relative telomerase activity for each sample is presented as percentage of the telomeric product generated (average of 3 assays). The results obtained revealed that there was a decrease in the telomerase activity of the Jurkat cells after being treated with 5uM concentration of the phosphorothioate oligonucleotide-peptide conjugates (Figure G). Measurement taken after 24-hr of transfection showed that phosphorothioate oligonucleotide-NLS conjugates, **6a** and **6b** effectively inhibited telomerase activity (approximately 96% and 80% respectively) as compared to the phosphorothioate oligonucleotide-NES conjugates, **6c** and **6d** (approximately 20% and 50% respectively). After 48-hr, the measured telomerase activity shows that the effect of the S-oligo-peptide conjugates lessen as there was an increase number of bands with higher intensity which denotes up-regulation of telomerase activity in the cells.

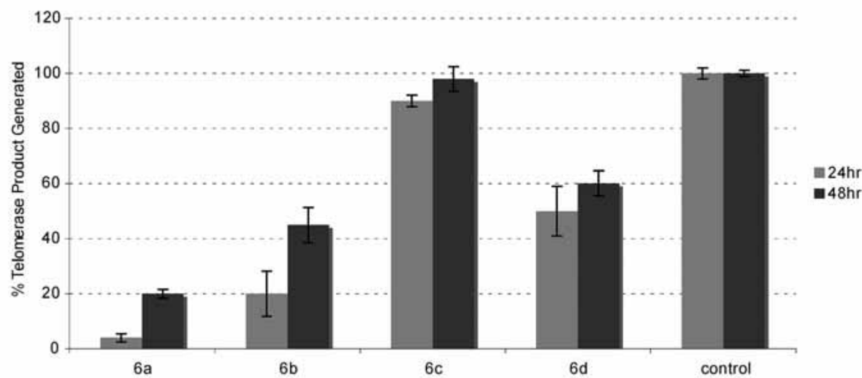
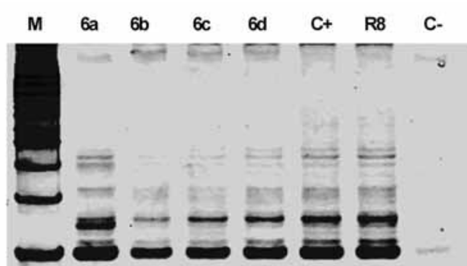


Figure G: The telomerase enzymatic activities of Jurkat cells were calculated as total product generated (TPG) units and normalized to a value of 100%. The bar graph represents the difference in the calculated TPG units after 24-hr and 48-hr transfection of 5uM concentration of the phosphorothioate oligonucleotide-peptide conjugates to the cells. The results were expressed as mean± SD from three independent experiments.



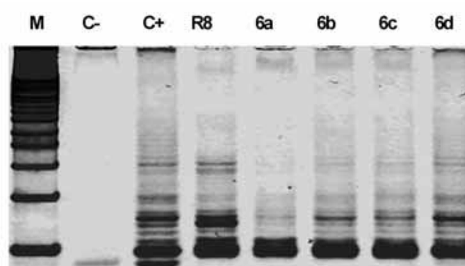


Figure H-1: (Telomerase activity after 24hr) **Figure H-2:** (Telomerase activity after 48hr)

Figure H: Telomerase activity levels of Jurkat cells (one representative experiment) treated with 1uM concentration of phosphorothioate oligonucleotide-peptide conjugates as determined by telomeric repeat amplification protocol (TRAP) assay after 24-hr of transfection (Fig.H-1) and 48-hr of transfection (Fig.H-2).

The enzymatic activity of telomerase in Jurkat cells treated with 1uM concentration of each of the 4 conjugates showed a slight down-regulation of telomerase activity (Figure H). There was a very slight difference as to the banding pattern observed in samples measured after 24-hr (Fig. H-1) and from samples measured after 48-hr as shown in Fig.H-2.

Based from the data gathered as for the effect of the designed phosphorothioate oligonucleotide-peptide conjugates on the inhibition of telomerase activity in Jurkat cells, there was repression of the telomerase activity. However, lower concentration (1uM) of the conjugate showed slight decrease in the telomerase activity (Figure I), showing some dose-dependent mechanism in its effectivity to inhibit telomerase activity.

Discussion

Numerous published studies have demonstrated the significant relationship between cancer and telomerase. The ability of cancer cells to divide indefinitely is attributable to the activation of telomerase. The increased level and frequency of telomerase activity in cancers when compared with normal cells makes telomerase an extremely attractive target for anti-cancer strategies.

The current picture of telomerase regulation is complex. It has been suggested in several reports that the expression of the protein catalytic sub-unit hTERT and the RNA template component hTR is important in telomerase

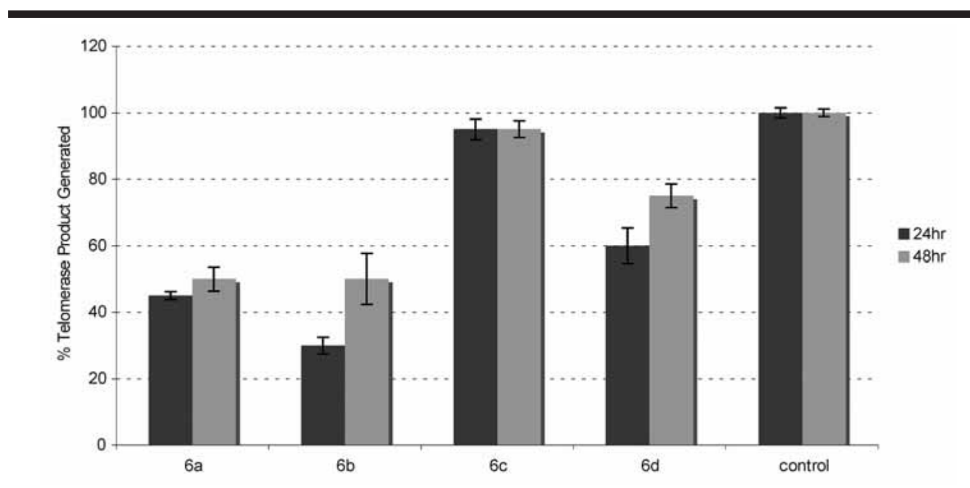


Figure I: Measured telomerase activity in Jurkat cells treated with 1uM concentration of phosphorothioate oligonucleotide-peptide conjugates after 24-hr and 48-hr transfection. The results were expressed as mean± SD from three independent experiments.

activity [77] but the question of whether telomerase activity is controlled by hTERT alone or/and hTR remains to be answered.

The present study made use of antisense strategy to compare the effects of small interfering RNAs (siRNAs) that targets the telomerase reverse transcriptase hTERT mRNA with the effect of antisense oligomers that targets the RNA component hTR in inhibiting telomerase expression.

Use of siRNA hTERT as an agent for down-regulation of telomerase activity

Screening for effective siRNA sequence to silence hTERT mRNA expression

RNA interference (RNAi) is a sequence-specific post transcriptional gene silencing process, which is triggered by double stranded RNA (dsRNA), causing degradation of mRNAs homologous in sequence to the dsRNA that subsequently leads to effectively inhibiting the specific gene expression [78,79]. As reported by Santoyo *et al* [80], the effectiveness of the chemically synthesized short double stranded siRNA is likely to be determined by the accessibility of its target sequence in the intended substrate. They further suggest that there are no reliable ways, however of predicting or identifying the "ideal" sequence for a siRNA and the selection of siRNAs sequences are largely empirical. Site selection is one of the confronting challenges in nucleic acid-base gene inactivation strategies. Like that of antisense DNA and ribozymes approaches, the use of various siRNAs directed at different sites of target gene exhibit different suppression effect [81].

In this study, 6 different 21bp siRNA sequences that target different sites of hTERT gene were screened (Please refer to the Materials and Methods section for the sequences). There were two GenBank entries namely NM_198255 and AB085628 for hTERT gene mRNA transcript that were considered for the site selection of siRNAs sequences. These two GenBank entries gave the complete cds (coding segment) of *Homo sapiens* mRNA for telomerase reverse transcriptase (hTERT). The complete coding segment is important because it describes the gene's open reading frame (ORF). The siRNAs were designed using bioinformatics algorithm software programs developed by Thomas Tuschl and Fran Lewitter of Whitehead Institute (http://www.jura.wi. mit.edu/siRNA). The website provided tools to blast-search in GenBank the designed siRNA to confirm that only hTERT gene was targeted.

In this study the siRNA transfection was carefully optimized for both adherent cells (HeLa) and suspension cells (K562 and Jurkat). The 6 siRNA sequences were transfected into cells using cationic lipid (Lipofectamine™2000) at a concentration of 200nM; this was chosen because it was the highest concentration that could be used to inhibit telomerase without causing most of the cells to die, as reported in the work of Natarajan *et al* [82]. The transfected cells were allowed to grow for 24-hours. Then, they were harvested for quantitative RT-PCR to screen the effective siRNA sequence that efficiently silences the hTERT mRNA gene expression.

Based on the collected data for QRT-PCR (Figure A), the 6 siRNAs that targets various sites of the hTERT gene showed different inhibitory effects to the mRNA level of the target gene. This data confirms what has been recently reported that siRNA efficiency is highly dependent upon target position, this maybe an effect of the secondary structure of the target RNA that is an important determinant of activity for siRNA [83)]. From the data, it showed that siRNA-3 (sequence 3) effectively down-regulated the expression of the hTERT gene for all the cell lines used in the experiment. As shown in Figures A and B, there was a significant decrease of approximately 85% on the hTERT mRNA expression in HeLa cells and of moderate inhibition for K562 and Jurkat (approximately 60% and 50% respectively). siRNA sequences 4 to 6 showed off-target effects as can be observed from the aberrant change in the normalized level of hTERT and the internal control, GAPDH. The use of Real Time RT-PCR assay based on TaqMan methodology in this study, makes the detection and quantitation of hTERT mRNA gene expression more precise and reproducible, being based on Ct values established in the early exponential phase of the PCR reaction (when none of the reagents is rate limiting) rather than end point measurement of the amount of accumulated PCR product. After quantitative RT-PCR evaluation of the effect of the siRNAs on hTERT gene expression, the selected siRNA was further evaluated for its effect on telomerase activity to determine whether there is association in the effect of inhibiting the hTERT gene to the down regulation of telomerase activity.

Effect of hTERT siRNA treatment on telomerase activity

After silencing hTERT gene expression, telomerase activity was measured using telomeric repeat amplification protocol (TRAP) assay. The gel electrophoresis image (Figure C) is one representative experiment for TRAP on the effect of siRNA-3 on the treated cells after 24-hour transfection. There was a down regulation of telomerase activity as evident by the lesser number of telomeric ladder observed in the treated samples as compared to the positive control and the R8 quantitation standard. It is interesting to note that the result of the QRT-PCR have significant association with the results obtained from TRAP assay. Based from the data, the silencing of hTERT gene is highest in HeLa cells as compared to its effect in K562 and Jurkat; the same evaluation was observed in the telomerase activities of the cells. The graph in figure D shows the reduced telomerase generated product unit (TPG) of HeLa cells. This suggests that telomerase activity was down regulated. The results of this study agree with those reported in the literature that after silencing hTERT gene expression, telomerase decreased correspondingly. Hahn *et al* [84)] have suggested that the expression pattern of hTERT gene is rate-limiting determinant of the enzymatic activity of human telomerase. In addition, many reports have demonstrated that specific hTERT siRNA could successfully inhibit telomerase activity in several cancer cell lines [85,86)].

Effect of siRNA on cell proliferation activity

Rapid and accurate assessment of viable cell number and cell proliferation is an important requirement in many experimental situations involving in vitro and in vivo studies. Accurate assessment of cell proliferation is useful especially in the determination of the cytostatic potential of anti-cancer compound.

Several laboratories have reported that inhibition of hTERT expression causes immediate anti-proliferative effects [87,88)]. In the present study, the effect of hTERT siRNA on cell proliferation was also evaluated. The results of the proliferative assay (Figure E) of the 3 cancer cell lines showed that the proliferation activities were dramatically decreased when treated with 200nM concentration of hTERT siRNA. As mentioned earlier, the said concentration was used in this study because it has been reported that 200nM concentration of siRNA is the highest concentration that could be used to confer inhibition without causing most of the cells to die.

Metabolism in viable cells produces "reducing equivalents" such as NADH or NADPH [89)], thus metabolic activity can be assayed as an indication of cell viability. In recent years, different tetrazolium salts like MTT, XTT, TTS and WST-1 have been described which can be used for measurement of cell proliferation and viability [90)]. Usually metabolic activity is measured in population of cells by incubating the cells with tetrazolium salt that is cleaved into colored formazan product by metabolic activity. An expansion in the number of viable cells results in an increase in the over-all activity of mitochondrial dehydrogenase in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture.

In this study, the quantification of the formazan dye produced by metabolically active cells was measured using spectrophotometer at an absorbance wavelength of 450nm.

The result of this study agree with the report of Kraemer *et al* [91] that inhibition of telomerase activity by targeting hTERT also caused significant inhibition of proliferation and induction of apoptosis in cancer cells. They suggested that this rapid loss of cell viability could be attributable to the cells having at least some very short telomeres or to a rapid telomere loss mechanism after telomerase enzyme activity has been down regulated. Thus, telomerase inhibitors by themselves will result in telomere shortening.

It is therefore an important challenge for basic and preclinical work to determine combination therapies to best enhance the erosion of telomeres to cause a more rapid decrease in cancer cell proliferation without affecting normal cell telomeres.

Use of antisense hTR as an agent for down-regulation of telomerase activity

For the past few years, the correlation between telomerase and tumor cells has fascinated the scientific community. In the recently reported crucial proof-of-principle studies, the treatment of various tumor-derived cell lines with oligonucleotide or protein based telomerase inhibitors resulted in cellular senescence [92,93]. The onset of cellular senescence was observed to be in direct correlation with the length of telomeres. The RNA component of telomerase hTR represents an attractive target for oligonucleotide- based inhibitors. Among other functionally and structurally important genes [94], it contains a crucial eleven nucleotides long "template region", part of which serves as the template for telomere elongation by TTAGGG repeats. Another part of this region may also be involved in chromosomal end recognition by telomerase [95].

In previous studies, anti-hTR 2'methoxyethyl oligonucleotides were able to block telomerase activity in Jurkat cells without the need to add lipid to enable efficient cell uptake [96,97]. One explanation given for the ability of anti-hTR oligomers to inhibit telomerase activity in the absence of lipid is that the oligonucleotides may associate with the outer cell membrane and be released to bind telomerase after cell lysis [98]. This statement that lipid is not needed for the introduction of anti-hTR oligomers into cells is remarkable because principle in the antisense oligonucleotide field holds that lipid is required for efficient oligonucleotide uptake in almost all cells [99].

The present study also looked into the potential of anti-hTR oligonucleotide in inhibiting telomerase activity. Phosphorothioate oligonucleotide-peptide conjugates were synthesized using Solid Phase Fragment Condensation (SPFC). The phosphorothioate hTR oligonucleotide was covalently linked to peptides. The peptides incorporated into the conjugates include 2 nuclear localization signal (NLS) sequences, namely, 4a peptide which is derived from SV40 large T-antigen [100] and 4b peptide from HIV-tat-1 protein [101]. Peptide 4c originate from HIV-REV protein; it's a nuclear exporting signal sequence [102] and one designed peptide with a repeated LRAL sequence [103,104]. The conjugates were transfected into Jurkat cells without the use of cationic lipid as transfecting agent at a concentration of 5uM and 1uM respectively. These concentration were chosen because based on the report of Chen *et al*, 5uM was the concentration that produced maximal inhibition of telomerase by anti-hTR oligonucleotide in their work. While several papers reported that the IC_{50} values for inhibition of telomerase in various cell lines were measured to be as low as 0.5uM [105]. Cells were harvested, one batch after 24-hours while another batch after 48-hours. Based on the results for 5uM concentration (Figures F and G), telomerase activity was greatly inhibited with a 96% and 80% inhibitory rate for conjugates 6a and 6b respectively. The data shows that conjugates with NLS peptide signals gave a more positive effect in inhibiting telomerase activity as compared to the NES signal peptide and the designed peptide. An explanation maybe is that binding to hTR using these peptides are efficiently carried-out. Data for 1uM concentration of conjugates (Figures H and I) showed a slight inhibition of telomerase activity. This may suggest that dose-dependent mechanism is one factor for such reaction.

Over-all the findings of this study is important because it is consistent with those reported in other literatures that the telomerase RNA template hTR and the protein sub-unit hTERT are an exceptionally promising target for oligonucleotide-mediated inhibition.

References

1) Hanahan D. and Weinberg R.A. (2000) Hallmarks of cancer. Cell 100: 57-70
2) Olaussen KA, Dubrana K, Domont J, Spano JP, Sabatier L and Soria JC (2006) Telomeres and telomerase as targets for anticancer drug development. Crit. Rev Oncol/Haemal 57:191-214
3) Hayflick L. (1997) Mortality and immortality at the cellular level. A review. Biochemistry 62: 1180-90

4) Wright WE, Pereira-Smith OM, Shay JW. (1989) Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. Mol Cell Biol 9: 3088-92
5) Nakamura TM, Morin GB, Chapman KB, and Weinrich SL. (1997) Telomerase catalytic subunit homologs from fission yeast and human. Science 277: 955-959
6) Paterson BM, Roberts BE, Kuff EL (1977) Structural gene identification and mapping by DNA − mRNA hybrid-arrested cell-free translation. Proc Natl Acad Sci USA 74: 4370-72
7) Zamecnik PC and Stephenson ML (1978) Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc Natl Acad Sci USA 75: 280-83
8) Simons RW and Kleckner N (1983) Translational control of IS10 transposition. Cell 34: 683-85
9) Mizuno T, Chou MY, and Inouye M (1984) A unique mechanism regulating gene expression: Translational inhibition by a complementary RNA transcript (micRNA). Proc Natl Acad Sci USA 81: 1966-69
10) Crooke S. (2000) Potential roles of antisense technology in cancer chemotherapy. Oncogene 19:6651-59
11) Monia BP, Lesnik EA, Gonzales C, Lima WF, McGee D, Guinosso CJ, Kawasaki AM, Cook PD and Freier SM. (1993) Evaluation of 2' modified oligonucleotides containing 2'-deoxy gaps as antisense inhibitors of gene expression. J Biol Chem 268: 14514-22
12) Dias N, Dheur S, Nielsen PE, Gryaznov S, Van-Aerschot A, Herdewijn P, Helene C and Saison-Behmoaras TE. (1999) Antisense PNA tridecamers targeted to the coding region of H-ras mRNA arrest polypeptide chain elongation. J Mol Biol 294: 403-16
13) Arzumanov A, Walsh AP, Rajwanshi VK, Kumar R, Wengel J and Gait MJ. (2001) Inhibition of HIV-1 Tat-dependent trans activation by steric block chimeric 2'-O-methyl/LNA oligoribonucleotides. Biochemistry 40: 14645-54
14) Dias N, Senamaud-Beaufort C, Forestier-El E, Auvin C, Helene C and Ester-Saison-Behmoaras T. (2002) RNA hairpin invasion and ribosome elongation arrest by mixed base PNA oligomers. J Mol Biol 320: 489-501
15) Baker BF, Lot SS, Condon TP, Cheng-Flournoy S, Lesnik EA, Sasmor HM and Bennett CF. (1997) 2'-O-(2-Methoxy)ethyl-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells. J Biol Chem 272:11994-2000
16) Kole R and Mercatante D. (2001) in Antisense Technology: Principles, Strategies and Applications (Crooke, ST., ed) pp. 517-539, Marcel Dekker, Inc., New York
17) Vickers TA, Wyatt JR, Burckin T, Bennett CF and Freier SM. (2001) Fully modified 2'-MOE oligonucleotides redirect polyadenylation. Nucleic Acid Res 29: 1293-99
18) Crooke ST (1996) Progress in antisense therapeutics. Med Res Rev 16: 319-44
19) Boiziau C, Thuong NT and Toulme JJ. (1992) Mechanisms of the inhibition of reverse transcription by antisense oligonucleotides. Proc Natl Acad Sci USA 89: 768-72
20) Crooke ST, Lemonidis KM, Nielson L, Griffey R, Lesnik EA and Molina BP. (1995) Kinetic characteristics of Escherichia coli RNAse H1: cleavage of various antisense oligonucleotide-RNA duplexes. Biochem J 312: 599-608
21) Veal GJ, Agrawal S and Byrn RA. (1998) Sequence-specific RNAse H cleavage of gag mRNA from HIV-1 infected cells by an antisense oligonucleotide in vitro. Nucleic Acids Res 26: 5670-75
22) Wu H, Macleod AR, Lima WF and Crooke ST. (1998) Identification and partial purification of human double strand RNase activity. A novel terminating mechanism of oligoribonucleotide antisense drugs. J Biol Chem 273: 2532-42
23) Zamore PD, Tuschl T, Sharp PA and Bartel DP. (2000) RNAi: double stranded RNA directs the ATP-dependent cleavage of mRNA at 21 and 23 nucleotide intervals. Cell 101: 25-33
24) Conrad C and Rauhut R. (2002) Ribonuclease III: new sense from nuisance. Int J Biochem Cell Biol 34: 116-129
25) Dias N and Stein CA. (2002) Antisense Oligonucleotides: Basic concept and mechanisms. Mol Can Therap 1: 347-55
26) Stein CA. (2001) The experimental use of antisense oligonucleotides: a guide for the perplexed. J Clin Invest 108: 641- 44
27) Miller PS, Yano J, Yano E, Carroll C, Jayaraman K and Ts'o PO. (1979) Nonionic nucleic acid analogues. Synthesis and characterization of dideoxyribonucleoside methylphosphonates. Biochemistry 18: 5134-43
28) Agrawal S and Goodchild J. (1987) Oligodeoxynucleoside methylphosphonates: Synthesis and enzymatic degradation. Tetrahedron Letts 28: 3539-42
29) Chen TL, Miller PS, Ts'o PO and Colvin OM. (1990) Disposition and metabolism of oligonucleoside methylphosphonate following single iv injection in

mice. Drug Metab Dipos 18: 815-18
30) Cormier JF and Pannunzio T. (1991) An improvement in the stereospecific synthesis of dinucleoside methylphosphonates. Tetrahedron Lett 32: 7161-64
31) Bhan P, Cushman CD, Kean JM, Levis JT and Miller PS. (1991) Antisense oligonucleoside methylphosphonates and their derivatives. Nucleosides and Nucleotides 10:37-46
32) Eckstein F. (1967a) A dinucleoside phosphorothioate. Tetrahedron Lett 1157-1160
33) Campbell JM, Bacon TA and Wickstrom E. (1990) Oligodeoxynucleoside phosphorothioate stability in subcellular extracts, culture media, sera and cerebrospinal fluid. J Biochem Biophy Methods 20: 259-267
34) Matsukura M, Shinozuka K, Zon G, Mitsuya H, Reitz M, Cohen JS and Broder S. (1987) Phosphorothioate analogs of oligodeoxynucleotides: Inhibitors of replication and cytopathic effects of human immunodeficiency virus. Proc Natl Acad Sci USA 84: 7706-10
35) Jansen B, Wacheck V, Heere-Ress E, Schlagbauer-Wadl H, Hoeller C , Lucas T, Hoermann M, Hollenstein U, Wolff K and Pehamberger H. (2000) Chemosensitization of malignant melanoma by BCL-2 antisense therapy. Lancet 356: 1728- 33
36) The Vitravene Study Group. (2002) A randomized controlled clinical trial of intravitreous formivirsen for treatment of newly diagnosed peripheral cytomegalovirus retinitis in patients with AIDS. Am J Opthalmol 133: 467-74
37) Fatta E and Bochot A. (2006) Antisense oligonucleotides, Aptamers and siRNA: promises for the treatment of ocular diseases. Arch Soc Esp Oftalmol 81: 3-6
38) Guvakova MA, Yakubov LA, Vlodavsky I, Tonkinson JL and Stein CA. (1995) Phosphorothioate oligonucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix. J Biol Chem 270: 2620: 27
39) Brown-Driver V, Eto T, Lesnik E, Anderson KP and Hanecak, RC. (1999) Inhibition of translation of hepatitis C virus RNa by 2-modified antisense oligonucleotides. Antisense Nucleic Acid Drug Dev 9: 145-54
40) Monia BP, Johnston JF, Geiger T, Muller M and Fabbro D. (1996) Antitumor activity of phosphorothioate antisense oligonucleotide targeted against C-raf kinase. Nature Med 2: 668-675
41) Schulz RG and Gryaznov SM. (1996) Oligo-2'-fluoro-2'-deoxynucleotide N3'-P5' phosphoramidates: synthesis and properties. Nucleic Acids Res 24:2966-73
42) Nielson PE and Haaima G. (1997) Peptide Nucleic acid (PNA). A DNA mimic with a pseudopeptide backbone. Chem Soc Rev 26: 73-78
43) Sorensen JJ, Nielsen JT and Petersen M. (2004) Solution structure of a dsDNA:LNA tiplex. Nucleic Acids Res 32: 6078-85
44) Yakubov LA, Deeva EA, Zarytova VF,Ivanova EM, Ryte AS, Yurchenko LV and Vlassov VV. (1989) Mechanism of oligonucleotide uptake by cells: involvement of specific receptors? Proc Natl Acad Sci USA 86: 6454-58
45) Vlassov VV, Balakireva LA and Yakubov LA. (1994) Transport of oligonucleotides across natural and model membranes. Biochim Biophys Acta 1197: 95-108
46) Loke SL, Stein CA, Zhang XH, Mori K, Nakanishi M, Subasinghe C, Cohen JS and Neckers LM. (1989) Characterization of oligonucleotide transport into living cells. Proc Natl Acad Sci USA 86: 3474- 78
47) De Diesbach P, Berens C, N-Kuli F, Monsigny M, Sonveaux E, Wattiez R and Courtoy PJ. (2000) Identification, purification and partial characterization of an oligonucleotide receptor in membranes of HepG2 cells. Nucleic Acids Res 28: 868-74
48) Spiller DG, Giles RV, Grzybowski J, Tidd DM and Clark RE. (1998) Improving the intracellular delivery and molecular efficacy of antisense oligonucleotides in CML cells: a comparison of streptolysin-O permeabilization, electroporation, and lipophilic conjugation. Blood 91: 4738-46
49) Spiller DG and Tidd DM. (1995) Nuclear delivery of antisense oligodeoxynucleotides through reversible permeabilization of human leukemia cells with streptolysin-O. Antisense Res Dev 5: 13-21
50) Fisher TL, Terhorst T, Cao X and Wagner RW. (1993) Intracellular disposition and metabolism of fluorescently-labeled unmodified and modified oligonucleotides microinjected into mammalian cells. Nucleic Acids Res 21: 3857- 65
51) Zelphati O and Szoka FC. (1996) Mechanism of oligonucleotide release from cationic liposomes. Proc Natl Acad Sci USA (3: 11493-98
52) Derossi, D and Chassaing G. (1998) Trojan peptides: the penetratin system of intracellular delivery. Trends Cell Biol 8: 84-87
53) Kubo T, Bakalova R, Zhelev Z, Ohba H and Fujii M. (2005) Highly sensitive inhibition of hTERT mRNA expression and telomerase activity by DNA-signal peptide conjugates. Nucleic Acid Symp Series 49: 337-38
54) Stetsenko DA, William D and Gait M (2001) Synthesis

of peptide-oligonucleotides: Application to basic peptides. Nucleic Acids Res Suppl 1: 153-54

55) Blackburn EH and Greider CW. (1985) Identification of a specific telomeric terminal transferase in Tetrahymena extract. Cell 43: 405-13

56) Nakamura TM and Cech TR. (1998) Reversing time: Origin of Telomerase. Cell 92: 587-90

57) Weinrich SL, Pruzan MR, Oullette M and Holt SE. (1997) Reconstitution of human telomerase with the catalytic protein subunit hTERT. Nature Genet 17: 498-502

58) Meyerson M. (2000) Role of telomerase in normal and cancer cells. J Clin Oncol 18: 2626-34

59) Lin SY and Elledge SJ. (2003) Multiple tumor suppressor pathways negatively regulate telomerase. Cell 113: 881-9

60) Cong YS, Woodring EW and Shay JW. (2002) Human telomerase and its regulation. Microbiol Mol Biol Rev 66: 407-25

61) Collins K (2000) Mammalian Telomeres and Telomerase. Curr.Opin.Cell Biol 12: 373-83

62) Hsu Y and Lin J. (2005) Telomere and Telomerase as targets for anti-cancer and regeneration therapies. Acta Pharmacol Sinica 5: 513-18

63) Feng J and Chiu CP. (1995) The RNA component of human telomerase. Science 269: 1236-41

64) Zou L, Zhang P, Luo C and Tu Z. (2006). shRNA-targeted hTERT suppress cell proliferation of bladder cancer by inhibiting telomerase activity. Cancer Chemother Pharmacol 57: 328-34

65) Herbert BS, Pitts AE, Wright WE and Shay JW. (1999) Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. Proc Natl Acad Sci USA 96: 14276-81

66) Neidle S and Parkinson G. (2002) Telomere maintenance as a target for anticancer drug discovery. Nat Rev Drug Discov 1: 383-93

67) Yokoyama Y, Takahashi Y, Shinohara A and Niwa K. (1998) Attenuation of telomerase activity by a hammerhead ribozymes targeting the template region of telomerase RNA in endometrial cancer cells. Cancer Res 58: 5406-10

68) Rezler EM and Hurley LH. (2002) Telomeres and telomerases as drug targets. Curr Opin Pharmacol 2: 415-23

69) Damm K Hemman U and Prepke H. (2001) A highly selective telomerase inhibitor limiting human cancer cell proliferation. EMBO 20: 6958-68

70) Ohyashiki JH, Hisatomi K and Nagao K (2005) Quantitative relationship between functionally active telomerase and major telomerase component (hTERT and hTR) in acute leukemia cells. British J Can 1-6

71) Kim NW, Piatyszek KR, Harley MD et al (1994) Specific association of human telomerase activity with immortal cells and cancer. Science 266: 2011-15

72) Crooke ST. (2004) Progress in antisense technology. Annu. Rev.Med 55:61-95

73) Crooke ST (1998) An overview of progress in antisense technology. Antisense & Nucl Acid Drug Develop 8: 115-18

74) Wilson C and Keefe AD. (2006) Building oligonucleotide therapeutics using non-natural chemistries. Curr Opin Chem Biol 10:607-09

75) Kurreck J. (2003) Antisense Technologies: Improvements through novel chemical modifications. Eur J Biochem 270: 1628-44

76) Katesan N and Kim BH. (2006) Peptide conjugates of oligonucleotides: synthesis and applications. Chem Rev 106: 3712-61

77) Zhang X, Mar V et al (1999) Telomere shortening and apoptosis in telomerase – inhibited human tumor cells. Genes Dev 13: 2388-89

78) Fire A, Montgomery MK, Mello CC and et al (1998) Potent and specific genetic interference by dsRNA in C. elegans. Nature 391: 806-11

79) Hannon GJ. (2002) RNA interference. Nature 481: 244-51

80) Santoyo J, Vaquerizas JM and Dopazo J. (2005) Highly specific and accurate selection of siRNAs for high throughput functional assays. Bioinformatics 21: 1376-82

81) Reynolds A, Leake D and et al (2004) Rational siRNA design for RNA interference. Nat Biotechnol 22: 326-330

82) Natarajan S, Chen Z, Monia BP and et al (2004) hTERT mRNA and hTR as Targets for down regulation of telomerase activity. Oligonucleotides 14: 263-273

83) Xia Y, Lin RX, Zheng SJ and et al (2005) Effective siRNA targets screening for hTERT. World J Gastroenterol 11: 2497-2501

84) Hahn WC, Stewart SA and et al (1999) Inhibition of telomerase limits the growth of human cancer cells. Nat Med 5: 1164-70

85) Matsutomi K, Yu EY and et al (2003) Telomerase maintains telomere structure in normal human cells. Cell 114: 241-53

86) Mergny JL, Riou JF and et al (2002) Natural and pharmacological regulation of telomerase. Nucleic Acids Res 30: 839-65

87) Kraemer K, Fuessel S and et al (2003) Antisense-mediated hTERT inhibition specifically reduces the growth of human bladder cancer cells. Clin Cancer Res 9: 3794-3800

88) Yuan Z and Mei HD. (2002) Inhibition of telomerase activity with hTERT antisense increases the effect of CDDP-induced apoptosis in myeloid leukemia. Hematol J 3: 201-05
89) Stanley PE. (1986) Extraction of adenosine triphosphate from microbial and somatic cells. Methods Enzymol 133: 14-22
90) Gonzalez RJ and Tarloff JB. (2001) Evaluation of hepatic subcellular fractions for alamar blue and MTT reductase activity. Toxicology in vitro 15: 257-9
91) Shay JW. (2003) Telomerase Therapeutics: Telomeres Recognized as a DNA damage signal. Clin Cancer Res 9: 3521-25
92) Herbert BS, Pongracz K and et al (2002) Oligonucleotide N3'-P5' phosphoramidates as efficient telomerase inhibitors. Oncogene 21: 638-42
93) Drayton S. (2000) Immortalization and transformation revisited. Curr Opin Genet Dev 12: 98-104
94) von Zglinicki T. (2002) Oxidative stress shortens telomeres. Trends Biochem Sci 27: 339-44
95) Yi X and Shay JW. (2001) Quantitation of telomerase components and hTERT mRNA splicing pattern in immortal human cells. Nucleic Acids Res 29: 201-09
96) Chen Z, Koeneman KS and Corey DR. (2003) Consequences of telomerase inhibition and combination treatments for the proliferation of cancer cells. Cancer Res 63: 5917-25
97) Elayadi A.N and Corey D.R. (2002) Implications of high affinity LNA for inhibition of telomerase. Biochemistry 41: 9973-81
98) Chen Z , Monia BP and Corey DR. (2002) Telomerase inhibition , telomere shortening and decreased cell proliferation by cell permeable 2'-O-methoxyethyl oligonucleotides. J. Med Chem 45: 5423-25
99) Corey DR. (2002) telomerase inhibition, oligonucleotides and clinical trials. Oncogene 21: 631-37
100) Goldfarb DS and et al (1986) Synthetic peptides as nuclear localization signals Nature 322:641-44
101) Dingwell C and Laskey, R.A. (1986) Protein import into the cell nucleus Annu Rev.Cell Biol 2: 367-90
102) Fujii M; Yokoyama K, Kubo K, Ueki R, Abe S, Goto K, Niidome, T, Aoyagi H, Iwakuma K, Ando S and Ono S. (2000) Design, Synthesis and characterization of DNA binding- peptides. Peptide Sci 2001:109-12
103) Kubo T and Fujii M (2001) Specific binding and stabilization of DNA and phosphorothioate DNA by amphiphilic a-helical peptides. Nucleosides Nucleotides Nucleic Acids 20: 1313-16
104) Kubo T, Morikawa M, Ohba H and Fujii M. (2003) Synthesis of DNA-peptide conjugates by Solid phase fragment condensation. Org Lett 5:2623-26
105) Grayznov S and et al (2001) Telomerase inhibitors as potential therapeutic agents. Nucleosides Nucleotides Nucleic Acids 20: 401-10