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ARTICLES

Telomerase Targeted Oligonucleotide *thio*-Phosphoramidates in T24-luc Bladder Cancer Cells

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Abstract Bladder carcinoma is the second most common genitourinary malignancy. Treatment options for bladder cancer include surgery, combined with chemotherapy, radiation, and/or immunotherapy. The adjuvant chemotherapy and immunotherapy regimen have been widely used in locally invasive as well as metastatic disease. The evaluation of new active agents with improved tolerability has been the focus of investigations over the past decade with minimal overall improvements in outcomes. Telomerase activity has been found in ~85–90% of all human tumors, but not in the majority of adjacent normal tissues. This suggests that telomerase may be an attractive target for the development of novel anticancer therapeutic agents. GRN163L is a lipid conjugated oligonucleotide N3′ → P5′ *thio*-phosphoramidate, and is a potent telomerase RNA (*hTR*) template antagonist. In the present study, we show that the telomerase activity of T24-luc bladder cancer cells is inhibited by 1 μM GRN163L within 24 h of incubation. After two weeks of exposure to GRN163L, T24-luc cells became ''clustered'' whereas non-cancerous normal human uroepithelial cells were not morphologically affected. Moreover, in vitro GRN163L treated T24-luc bladder cancer cells entered G_0/G_1 arrest following 2 weeks of continuous exposure and stopped dividing. Mismatch control compound had no effect on normal bladder epithelial cells or T24-luc cells. Additionally, a new generation of *thio*-phosphoramidate oligonucleotides were designed and tested in T24-luc cells and compared with GRN163L. The obtained results warrant further in vivo evaluation of GRN163L as a potential treatment for bladder cancer. J. Cell. Biochem. 104: 444–452, 2008.

Key words: bladder cancer; telomerase; *thio*-phosphoramidate oligonucleotide; GRN163L

Human bladder cancer is the second most common malignant neoplasm of the urogenital system, and telomerase activity closely correlates with its occurrence, development, and prognosis [Orlando et al., 2001]. Recent evidence demonstrates that telomerase activity in urine has higher sensitivity and specificity compared with urine cytology and other biochemical markers for the early detection of bladder cancer [Erdem et al., 2003; Sanchini et al., 2004].

Treatment options for bladder cancer include transurethral resection (TUR), chemotherapy, immunotherapy, and irradiation depending on the stage and grade of the disease [Melquist et al., 2006]. Recurrent rates for superficial bladder cancer remains around 60–70% in the 5 years following surgery, so intravesical therapy is widely used as an adjunct to TUR. Intravesical Bacille Calmette-Guérin (BCG), an immunotherapeutic agent, is effective for limiting recurrences, reduces the progression of bladder cancer, and is believed to be superior to chemotherapy. Unfortunately, adverse effects of BCG limit its applicability and recurrences still develop in 20–30% of patients despite this immunotherapy. In those patients who progress or do not respond to intravesical therapies, cystectomy is usually considered. Therefore, new treatments are needed to improve the prognosis of patients with superficial, as well as locally advanced bladder cancer [Amling, 2001; Brassell and Kamat, 2006].

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Telomerase plays a critical role in cancer, and inhibition of telomerase activity in tumor cells may represent a promising anticancer strategy. Telomerase inhibition leads to telomere destabilization and consequently to growth inhibition and/or apoptotic cell death [Kraemer et al., 2003; Shay and Wright, 2005]. Telomerase is a ribonuculeoprotein complex consisting of a catalytic protein subunit (*hTERT*) and a functional (template) RNA (*hTR*) that base pairs with telomeres. The 11-base template region of telomerase RNA (*hTR*) is an attractive target for direct enzymatic inhibition of telomerase activity [Corey, 2000]. GRN163L is a 13-mer oligonucleotide *thio*-phosphoramidate carrying a palmitoyl (C16) group covalently attached to the 5′-*thio*-phosphate of the first nucleotide *via* an aminoglycerol linker. The oligonucleotide sequence of GRN163L—5′-Palm-TAGGGTTAGACAA-3′ is complementary to a 13-nucleotide-long segment partially overlapping and extending by four nucleotides beyond the 5′-boundary of the template region of *hTR*. This compound binds to the *hTR* active site and thus inhibits the enzyme activity [Gryaznov et al., 2001; Shay and Wright, 2006].

We have previously shown that GRN163L causes telomerase inhibition, telomere shortening and cancer cell death without significantly affecting the growth of normal cells [Gellert et al., 2006]. Furthermore, the potent anti-metastatic activity of GRN163L has been shown in xenograft models of human lung and breast carcinoma in nude mice [Dikmen et al., 2005; Hochreiter et al., 2006]. Additionally, in vivo biodistribution experiments reveal that the highest concentration of ^{35}S labeled GRN163L was found in liver, kidney, and bladder 72 h after administration of the radio-labeled GRN163L through the tail vein [Dikmen et al., 2005]. Thus, the bladder environment containing tumor cells should be a preferred targeted area for GRN163L therapy.

In the present study, we tested GRN163L in T24-luc bladder cancer cells and SV40 immortalized human uroepithelial non-cancerous cells in vitro. Additionally, using T24-luc cells as model system, several GRN163L analogues with modifications of the sugar-phosphate backbone, oligonucleotide sequence, chemical structure, attachment side of lipid groups, and linkers joining the oligonucleotide with the lipid groups were investigated and compared with GRN163L.

MATERIALS AND METHODS

Cell Culture and Morphological Analysis

T24-luciferase expressing (T24-luc) cells and SV40 immortalized non-cancerous uroepithelial epithelial cells (SVHUC) were obtained from the Department of Urology at the University of Texas Southwestern Medical Center (Dallas, TX). The cells were grown in X media (4:1 DMEM/Medium 199 + 10% Cosmic Calf Serum) (HyClone, Logan, UT) at 37°C under 5% CO_2.

The effects of GRN163L on T24-luc cells and cellular morphology were tested by short-term and long-term experiments. For morphological analyses, (1×10^5/2 ml media) were seeded into 6 well cell culture dishes and GRN163L or the mismatch oligonucleotide (both at 1 μM) were added directly to the media prior to cell attachment. Phase-contrast micrographs were taken using an inverted Zeiss Axiovert 200 M at 20× magnification.

For short-term treatment, T24-luc cells (2.5×10^4/2 ml media) were plated into 6-well cell culture dishes and 1 μM of GRN163L (5′-Palm-TAGGGTTAGACAA-NH_2-3′) or Mismatch (MM) control oligonucleotide (5′-Palm-TAGGTGTAAGCAA-NH_2-3′, mismatch nucleosides are underlined), were added to the media after cell attachment. Samples were collected for TRAP (Telomeric Repeat Amplification Protocol) assay after 6, 12, 18, and 24 h of incubation.

For the long-term treatment experiments, T24-luc cells were plated and (1×10^5/10 cm dish) and GRN163L was added to the media immediately. The cells were fed with GRN163L (1 μM) containing medium every three days, trypsinized and re-plated every week in the presence of freshly added drug.

Oligonucleotide Chemistries

All oligonucleotides were synthesized by Geron Corporation (Menlo Park, CA). Oligonucleotides N3′ → P5′ *thio*-phosphoramidate (NPS) were prepared using ABI 394 or Akta 100 synthesizers as previously described [Asai et al., 2003], except that step-wise sulfurization during synthesis of NPS compounds was done with 0.1 M phenylacetyl disulfide (PADS) in CH3CN/2,6-lutidine (1/1, v/v), 5 min, after the coupling step. Additionally, acetic anhydride was replaced by *iso*-butyric anhydride in the capping reagent. Oligonucleotide N3′ → P5′ phosphoramidates (NP) were assembled

similarly to NPS counterparts using *tert*-butylhydrogen peroxide (0.4 M solution in dichloromethane, 5 min, as an oxidizing agent replacing PADS). Oligonucleotide compounds were analyzed and purified, if needed, by reversed phase (RP) HPLC, and then desalted by either gel filtration on NAP-10 columns (Pharmacia), or by precipitation with ice cold ethanol (5–7 volumes) from 0.5–1.0 M NaCl solutions. The isolated compounds were characterized by ^{31}P NMR, mass spectrometry, polyacrylamide gel electrophoresis (PAGE), and by analytical RP HPLC, (C_{18} column, 1%/min CH3CN gradient in 0.1 M triethylammonium acetate, pH 7.2). All the prepared oligonucleotides were solubilized in sterile normal phosphate buffered saline (pH 7.4) and kept at −20°C.

In order to determine in vitro telomerase inhibitory activity of all oligonucleotides, T24-luc cells (2.5×10^4/2 ml media) were plated into 6-well cell culture dishes and then incubated with 0.1, 1.0, and 10 μM of the oligonucleotides for 24 and 72 h. The TRAP assay was used to assess telomerase inhibition after the cells exposure to the compounds (see below).

Telomerase Activity Assay

Telomerase activity from cell extracts was analyzed using a PCR-based telomeric repeat amplification protocol (TRAP) assay [Shay and Bacchetti, 1997]. Typically, 1×10^5 cells were pelleted and lysed for 30 min in ice-cold NP-40 lysis buffer (10 mM Tris–HCl pH 8.0, 1.0 mM $MgCl_2$, 1 mM EDTA, 1% NP-40, 0.25 mM sodium deoxycholate, 10% glycerol, 150 mM NaCl, 5 mM β-mercaptoethanol). Samples were mixed with TRAP-eze kit reagents (Chemicon) according to the manufacturer's instructions, and the telomerase extension products were amplified using PCR (94°C for 30 s, 52°C for 30 s, 72°C for 30 s; 30 cycles) in the presence of a Cy5-labeled TS primer. A standard batch of H1299 lung cancer cell line was used as a positive control, whereas lysis buffer was used as a negative control for each run. PCR samples were resolved by 10% PAGE in 0.5XTBE at 250 V for 2.5 h and scanned using a STORM 860 PhosphorImager scanner system (MolecularDynamics).

Cell Cycle Analyses

T24-luc cells were treated with 1 μM GRN163L following the cells plating, tyripsinized once a week, re-plated and treated with GRN163L (1 μM) every 3 days in vitro. Flow cytometry was used to evaluate the number of cells in the particular phases of the cell cycle. Control and treated cells (1×10^6) were washed twice with PBS (pH 7.4), harvested and resuspended in PBS to achieve a single cell suspension. For fixation and permeabilization of the cells, 70% ice cold ethanol was added dropwise to the cell suspension under vortexing. Following the removal of ethanol by centrifugation, the cells were stained with propidium iodide/RNAse staining solution (BD Biosciences) for flow analysis and the cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences). The obtained histograms were further analyzed and quantified by a curve-fitting program to determine the percentage of cells in G_0/G_1, S, and G_2/M phases.

RESULTS

Short-Term and Long-Term Treatment of T24-luc Cells With GRN163L

Telomerase activity of T24-luc cells was reduced to undetectable levels 24 h after addition of GRN163L (1 μM), whereas the mismatch control oligonucleotide (1 μM) had no effect on the enzyme activity (Fig. 1).

Previous studies with A549-luc lung cancer cells indicated rapid morphological changes within 24 h when cells were treated before cell attachment with a single dose of GRN163L. These morphologic changes were independent of *hTR* expression, telomerase inhibition and unrelated to telomere length of cancer cells. This effect is dependent on the molecular properties of the lipid moiety, the phosphorothioate backbone, and the presence of triplet-G sequences within the GRN163L structure (Jackson et al., 2007). However, administration of GRN163L (1 μM) before cell attachment did not cause any noticeable morphological changes on T24-luc cells after this short-term (24–72 h) exposure (Fig. 2). Interestingly, continuous treatment of the cells with GRN163L for ~2 weeks caused significant morphological changes; the cells started to cluster-up and become weakly attached to the cell culture plates (Fig. 3). Nevertheless, the cell clustering effects as well as the morphological changes were reversible. Removal of GRN163L from the cell culture or cessation of the treatment resulted in complete recovery of cellular phenotype in ~3–4 days (data not shown). Normal

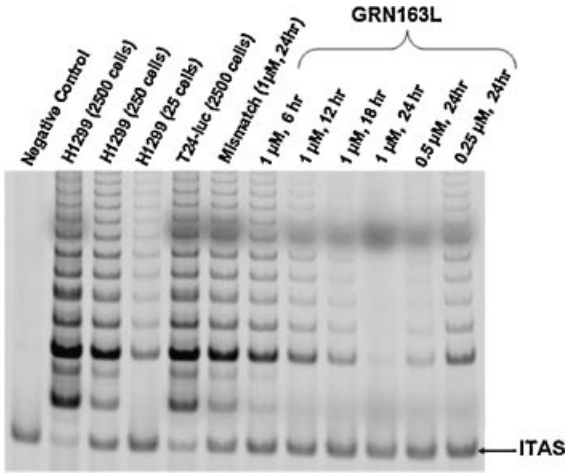


Fig. 1. Time and dose response of T24-luc bladder cancer cells treated with GRN163L. Cells were collected and telomerase activity was measured for 2,500 cell equivalents per lane using TRAP assay. H1299 cells were used as positive controls, and lysis buffer was used as a negative control. **Lane 1**: Negative control (lysis buffer), **lane 2**: H1299 cells-2,500 cells, **lane 3**: H1299 cells-250 cells, **lane 4**: H1299 cells-25 cells, **lane 5**: T24-luc cells (untreated), **lane 6**: T24-luc cells treated with mismatch oligo (1 μM), **lane 7**: T24-luc cells treated with 1 μM GRN163L for 6 h, **lane 8**: T24-luc cells treated with 1 μM GRN163L for 12 h, **lane 9**: T24-luc cells treated with 1 μM GRN163L for 18 h, **lane 10**: T24-luc cells treated with 1 μM GRN163L for 24 h, **lane 11**: T24-luc cells treated with 0.5 μM GRN163L for 24 h, **lane 12**: T24-luc cells treated with 0.25 μM GRN163L for 24 h (ITAS: Internal Telomerase Assay Standard, a DNA standard appearing as a single band at the bottom of the gel, indicating a general efficiency of PCR amplification).

SV40 immortalized non-cancerous human uroepithelial cells (SVHUC) were also treated with GRN163L (1 μM) for 2 weeks, but no significant change in their morphology or any measurable decrease in their proliferation rate was observed (Fig. 4). This strongly suggests a good degree of specificity in action of GRN163L as a potential anti-cancer agent.

We monitored cell cycle profiles of GRN163L and mismatch treated T24-luc cells to determine whether the inhibition of telomerase results into a perturbation in the cell cycle progression. Flow cytometry analysis showed that 86.7% of GRN163L treated cells arrest in G_0/G_1 phase, 8.8% were in M phase and 2.7% were in S phase of the cycle after 2 weeks of in vitro treatment. Following 3 weeks of treatment, 94.9% of GRN163L treated cells were in G_0/G_1 phase, whereas only 2.7% were in M phase and 1.3% were in S phase. These results demonstrated that the T24-luc cells treated with GRN163L for 2–3 weeks were unable to progress from the G_0/G_1 phase to the M phase, as shown in Figure 5.

New Analogues of GRN163L

In order to determine if the morphological changes that occurred after 2 weeks of GRN163L treatment were due to inhibition of telomerase or another structural component of the oligonucleotide, several other analogues of GRN163L were tested on T24-luc cells. These oligonucleotides contained phosphoramidate (NP) internucleoside groups with palmitoyl (C16) or stearoyl (C18) groups. The compounds, designated as 5′-Palm-7NP and 5′-Stearoyl-7NP (Table I), represent a new type of lipid oligonucleotide conjugates without sulfur atoms in their sugar-phosphate backbone. In vitro evaluation of these compounds revealed that none of them inhibited TRAP activity of the cells at 1 μM. Interestingly, when the sulfur atom was re-introduced back into the compounds (5′-Palm-7NPS, a.k.a GRN163L, or 5′-Stearoyl-7NPS), a dramatic increase in cellular anti-telomerase activity was observed (data not shown).

We also prepared a nucleoside base modified molecule by replacing cytidine with 5-methyl cytidine. This compound—5-methyl cytidine-containing GRN163L (5-MeCyt-GRN163L, Table I) exhibited a slightly higher thermodynamic stability of duplex with RNA (73°C vs. 69.0°C for GRN163L), and also better

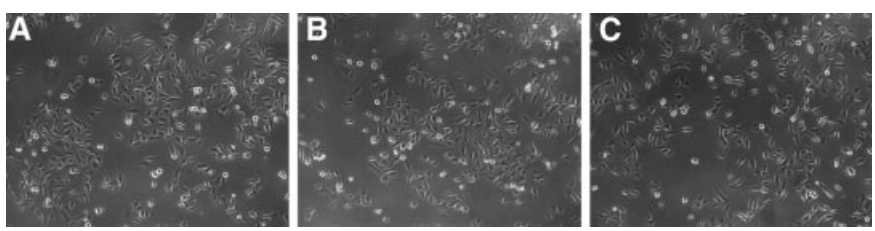


Fig. 2. Short-term treatment (24 h) of T24-luc cells with GRN163L and Mismatch Control Compounds. **A**: T24-luc cells (control), **B**: T24-luc cells treated with mismatch (1 μM), **C**: T24-luc cells treated with GRN163L (1 μM), GRN163L was added to the culture media before cell attachment.

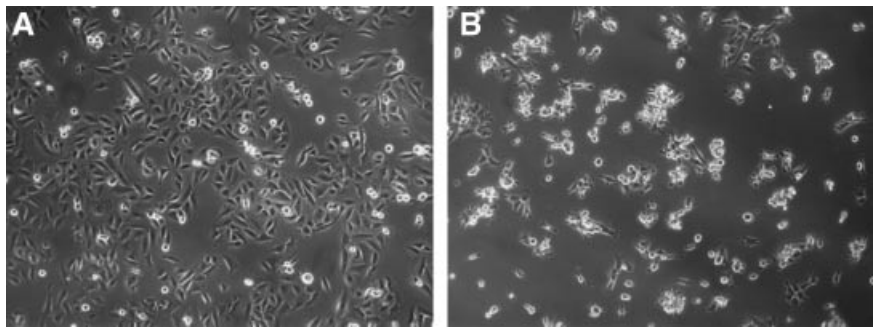


Fig. 3. Morphological changes of T24-luc cells induced by continuous GRN163L treatment: **A**: T24-luc cells (untreated control), **B**: T24-luc cells treated with 1 μM GRN163L in vitro for 2 weeks were clustered up; GRN163L was added to the culture media before cell attachment.

manufacturing characteristics. Telomerase inhibitory activity of MeCyt-GRN163L in T24-luc cells (24 h assays) was similar to that for GRN163L at 1 μM concentrations.

5′-Oleic-163 (Table I) is another lipid oligonucleotide conjugate isosequential to GRN163L, but carrying oleic (C18:1), a monounsaturated fatty acid with one double bond between C9 and C10, instead of palmitoyl (C16:0) group as in GRN163L. 5′ Oleic-163 at 1 μM showed ~30% less anti-telomerase activity as compared with GRN163L in T24-luc cells (24 h assay).

We also tested two non-template region addressed N3′-P5′ phosphoramidate oligonucleotide-palmitic acid conjugates (complementary to 137–151 nucleotides region of *hTR*, which was shown to be crucial for telomerase activity in biochemical assays) with sulfur (5′-Palm-73S) and without internucleoside sulfur atoms (5′-Palm-73) in the sugar-phosphate backbone (Table I). Similar to GRN163L, these compounds are also G-rich (5′-Palm-GTGG-AAGGCGGCAGG), but unlike GRN163L, they do not contain GGG-motif. Those molecules did not inhibit telomerase activity in T24-luc cells at 1 μM after 24 or 72 h exposure.

Other sugar-phosphate backbone modified oligonucleotides were designed and evaluated.

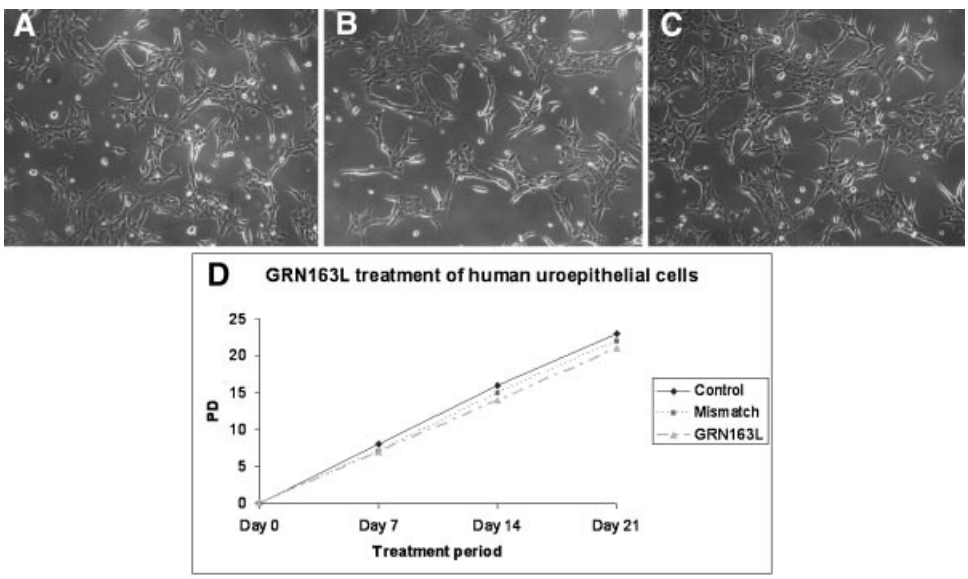
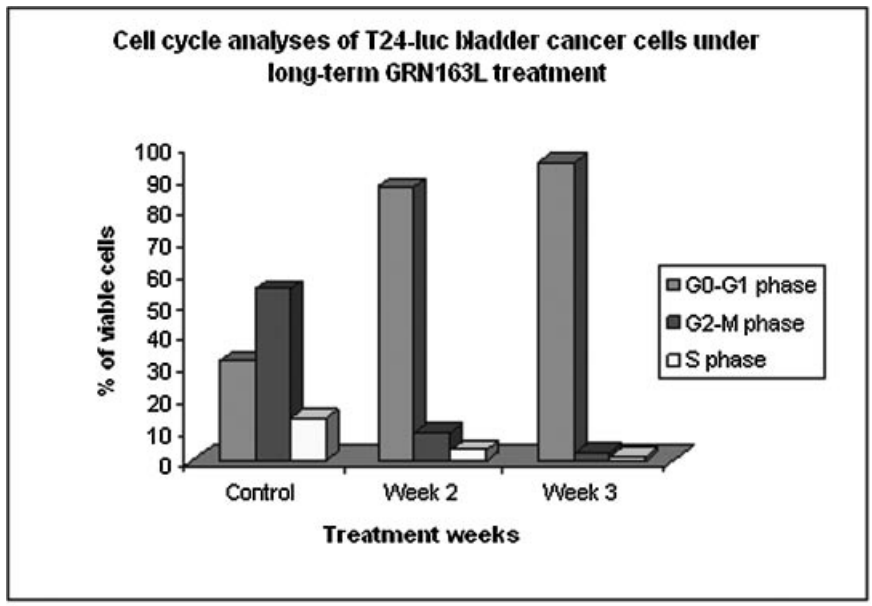


Fig. 4. Non-cancerous uroepithelial cells (SVHUC) under long-term treatment with GRN163L and Mismatch Control Oligos: **A**: SVHUC cells (untreated control), **B**: SVHUC cells treated with mismatch control compound (1 μM), **C**: SVHUC cells treated with GRN163L (1 μM); GRN163L was added to the culture media before cell attachment, **D**: population doublings (PD) of human uroepithelial cells cultured in medium with mismatch (1 μM) and GRN163L (1 μM) for 3 weeks.



	G0/G1 phase	G2-M phase	Sphase
Controls (untreated)	31.5 %	54.4 %	13.5 %
GRN163L treatment for 2 weeks	86.7 %	8.8 %	2.7 %
GRN163L treatment for 3 weeks	94.9 %	2.7 %	1.3 %

Fig. 5. Cell cycle analyses of T24-luc cells under GRN163L (1 μM) treatment for 2 and 3 weeks.

Thus, we tested GRN163L-2′-OH-A, (Table I). This is a new analogue of GRN163L, where all 2′-deoxy adenosine nucleosides were replaced by 2′-ribo-adenosines (5′-Palm-T*r*AGGGTT*r*A-G*r*AC*r*A*r*A). This compound almost completely inhibits telomerase activity in T24-luc cells at 1 μM. However, when all thirteen 2′-deoxy 3′-aminonucleosides were replaced with their 2′-ribo-3′-amino counterparts (*ribo*-GRN163L), then telomerase inhibitory activity was minimal at 1 μM, as compared with the compound containing all deoxyribonuclosides in the backbone (GRN163L).

We next studied the in vitro effects of GRN163L-A/link molecule (Table I), which is another analogue of GRN163L, where all

TABLE I. Summary of In Vitro Experiments Testing Various Modifications of Oligomer Chemistry on T24-luc Telomerase Activity (24 h)

Oligonucleotide	Sequence	Lipidation	Backbone chemistry	GGG-motif	TRAP inhibition
Mismatch	5′-Palm-TAG GTG TAA GCA A-3′	Palmitoyl	NPS	No	No
GRN163L	5′-Palm-TAGGGTTAGACAA-3′	Palmitoyl	NPS	Yes	Yes
5′Palm NP	5′-Palm-TAGGGT TAG ACAA-3′	Palmitoyl	NP	Yes	No
5′S 7NPS	5′-Stearoyl-TAGGGT TAG ACAA-3′	Stearoyl	NPS	Yes	Yes
5′S 7NP	5′-Stearoyl-TAGGGT TAG ACAA-3′	Stearoyl	NP	Yes	No
5′-MeCyt-163L	5′-Methyl Cyt-163L	Palmitoyl	NPS	Yes	Yes
5′Palm73	5′-Palm-GTGGAAGGCGGCAGG-3′	Palmitoyl	NP	No	No
5′Palm73S	5′-Palm-GTGGAAGGCGGCAGG-3′	Palmitoyl	NPS	No	No
2′-OH deoxyA	5′-Palm-T*r*AGGGTT*r*AG*r*AC*r*A*r*A-3′	Palmitoyl	NPS	Yes	Yes
ribo-163L	5′-Palm-*r*TAGGGTTAGACAA-3′	Palmitoyl	NPS	Yes	No
163LA/link	5′-Palm-TLGGGTTLGLCLL-3′	Palmitoyl	NPS	Yes	No

Column 1: oligonucleotide designation; Column 2: sequence of the oligonucleotide; Column 3: lipid modifications; Column 4: backbone chemistry: NP (phosphoramidate) and NPS (*thio*-phosphoramidate); Column 5: G3 repeat; Column 6: inhibition of telomerase by TRAP at1 μM of oligonucleotide: Yes = complete inhibition, No = no detectable inhibition.

adenosine nucleosides were replaced by a non-nucleosidic abasic C_3 linker [5′–O–$CH_2CH_2CH_2$–O– P(O)(S–)–]. This compound is an NPS oligonucleotide analogue with 5′-Palm-TL-GGGTTLGLCLL sequence, containing a G3 motif and sulfur atoms (as does GRN163L), but incapable of forming a stable duplex with *hTR* under physiological salt and temperature conditions (duplex T_m ~30.5°C vs. 69.0°C for GRN163L). This compound does not form duplex with *hTR*, and as expected, no telomerase inhibition was observed by TRAP with T24-luc cells at 1 μM.

Structural analogues of GRN163L carrying two lipid groups were also designed and tested in T24-luc cells. The analogues carrying palmitoyl group attached to the 5′-terminus in a "linear manner," were named as "I-type" compounds, for instance 5′-Palm-L-TAGGGTTA-GACAA$_{NH2}$-3′ (GRN163L; Fig. 6A). At the same time oligonucleotides with a lipid group attached to the N4 position of cytosine were named "T-type" molecules, for instance 5′-TAG-GGTTAGAC*AA$_{NH2}$-3′, (Fig. 6B). The "T-type" oligonucleotides carrying only one C_{18} group attached to the cytosine was somewhat less potent at 1 μM than GRN163L. Addition of the second 5′-Palm lipid group to the oligonucleotides (5′-Palm-L-TAGGGTTAGAC*AA$_{NH2}$-3′) apparently did not enhance the cellular uptake of this "T-type" oligo, and did not inhibit telomerase activity, but showed non-specific toxic effects in T24 cells within 2–3 days of treatment (data not shown).

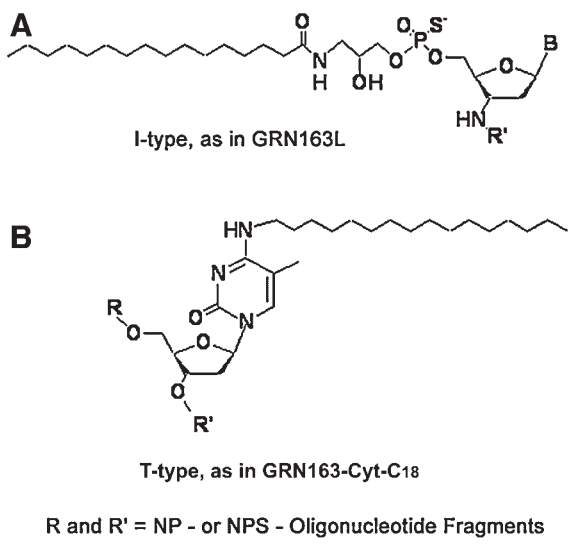


Fig. 6. Structural analogues of GRN163L. **A**: GRN163L (I-type oligo) = 5′-(Palm-L-)-TAGGGTTAGACAA$_{NH2}$-3′ (one palm-group is attached to the 5′-terminus). **B**: GRN163-Cyt-C_{18} (T-type oligo) = 5′-TAGGGTTAGAC*AA$_{NH2}$ 3′ (one palm-group is attached to the cytosine base).

Two new analogues of GRN163L with aminoethyl linker between the lipid and oligonucleotide parts of the conjugates with phosphodiester (PO) and phosphorothioate (PS) groups were prepared (Fig. 7), and their telomerase inhibitory activity was compared with aminoglycerol containing linker GRN163L. The activity of these two analogues was approximately three times lower than that for GRN163L in T24-luc cells.

DISCUSSION

This study was undertaken to investigate the in vitro effects of the telomerase inhibitor GRN163L in T24-luc bladder cancer cells as a potential therapeutic modality against bladder cancer. Additionally, SV40 immortalized non-cancerous uroepithelial cells were also used in this study to evaluate selectivity of this compound against cancerous relative to a related non-cancerous uroepithelial cell line. Several new GRN163L analogues were also systematically designed and tested in T24-luc cells for their ability to inhibit telomerase as a part of our broader structure-activity relationship (SAR) evaluation of oligonucleotide-based telomerase inhibitors (all listed in Table I).

It has been shown that oligonucleotide *thio*-phosphoramides (NPS) were more potent telomerase inhibitors than their phosphoramidates (NP) counterparts [Pongracz and Gryaznov, 1999; Herbert et al., 2002]. NPS oligonucleotides exert their anti-telomerase activity via forming thermodynamically stable duplexes with the template region of the RNA subunit of telomerase *hTR*. The greater potency of NPS versus NP compound was attributed to the stabilizing interactions between the oligonucleotide's backbone (with soft nucleophile sulfur atoms) and amino acid residues of hTERT (soft electrophiles), that are adjacent to the template region of *hTR* [Asai et al., 2003]. A noticeably better cellular up-take of NPS relative to NP compounds was also suggested as a significant factor for superior in vitro activity. In our study, we observed that the telomerase inhibitory activity of oligonucleotides was reduced when every other sulfur atom in the backbone was replaced with oxygen. This effect was not influenced by the chemical nature of the lipid

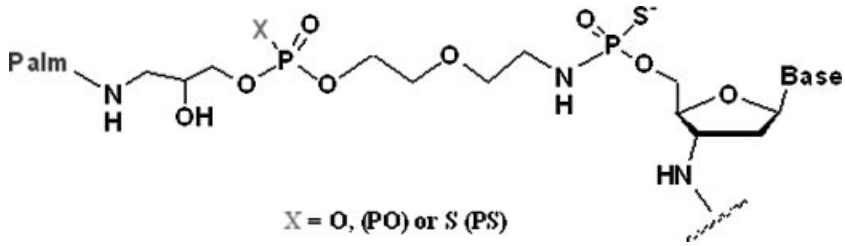


Fig. 7. A general chemical structure of oligonucleotide conjugate with aminoethylen glycol (AEG) linker between lipid and oligonucleotide groups.

attached to the 5′-terminus of the oligonucleotides—compounds with C_{16} and C_{18} groups had a similar biological activity. Moreover, all-NP compounds showed no measurable anti-telomerase activity in T24-luc cells at 1 μM. Additionally, when the non-template addressed oligonucleotides were tested in T24-luc cells, neither NPS nor NP analogues significantly inhibited telomerase activity. These findings stress an importance of targeting the *hTR* template region for potent anti-telomerase effects. Also, the presence of some sulfur atoms in the oligonucleotide backbone (~50–100%) apparently increases oligonucleotides cellular up-take and/or intracellular stability and consequently enhances activity of the tested compounds in T24 cells.

We found that an exact chemical structure of nucleoside sugar rings plays an important role defining anti-telomerase activity in vitro. Thus, by introducing into GRN163L sugar-phosphate backbone *ribo*-nucleoside sugar ring (either partial or complete substitutions of 2′-hydrogen by 2′-hydroxyl group), we found that the oligonucleotides containing all 2′-deoxyribonuclosides showed better anti-telomerase activity, as compared to their counterparts with all ribonucleosides in the backbone. This difference may be due to the structural and functional effects of 2′-ribonucleosides, which may be attributed to a markedly lower cellular uptake of ribo-compounds, and/or to formation of complex and stable secondary structures (such as G-quadruplexes) interfering with the oligonucleotides ability to bind with *hTR*.

The lipid-modified N3′ → P5′ *thio*-phosphoramidate oligonucleotide GRN163L with 5′-aminoglycerol linker (between the lipid and the oligonucleotide parts of the molecule) has been shown to inhibit telomerase more potently than its parental non-conjugated *thio*-phosphoramidate GRN163 [Herbert et al., 2005]. In this study, we tested if the attachment site of the lipid group, or an addition of the second lipid group, enhanced the cellular uptake and thus anti-telomerase efficacy of the oligonucleotides. Interestingly, addition of the second lipid group to the 5-Me-cytosine nucleoside base (at N4-position) of GRN163L did not enhance the cellular uptake of the oligonucleotide or its activity, but resulted in toxic effects in the cells within 48–72 h. Additionally, replacement of the aminoglycerol group (–NH–CH_2CH(OH)CH_2–O–, abbreviated as "C_3") connecting oligonucleotide and lipid parts by a longer and more flexible linker (–NH–CH_2CH_2–O–CH_2CH_2–O–, abbreviated as "C_5"), resulted in a measurable reduction in telomerase inhibitory of the conjugates. This may be due to the length of new (C_5) linker, which is longer and less hydrophilic, that potentially interferes with cellular uptake of the oligonucleotide conjugates.

We also observed that the anti-telomerase activity of 5-MeCyt-GRN163L is similar to GRN163L in T24-Luc cells despite the increase in thermal stability of duplex with RNA under close to physiological salt conditions—in PBS T_m of 74 and 69°C for 5-MeCyt-GRN163L and GRN163L, respectively. However, the ease of chemical manufacturing and increase in stability of 5-MeCyt- relative to cytidine-containing oligonucleotides marks 5-MeCyt-GRN163L as a molecule warranting further detailed in vitro and in vivo evaluation.

In order to examine the effects of long-term inhibition of telomerase by GRN163L on cell proliferation and cell cycle, we performed flow cytometry analysis. The proportion of T24-luc cells in G_0/G_1 phase was 86.7% after 2 weeks and 94.9% after 3 weeks of in vitro exposure to GRN163L (1 μM). At the same time only 2.7% of the 2 weeks treated cells and 1.3% of the 3 weeks treated cells were able to enter S-phase. These results indicate that this long-term treatment with GRN163L and continuous inhibition of telomerase activity [likely

resulting in telomeres shortening as was observed before, Gellert et al., 2006], suppressed the viability of T24-luc cells by inducing an efficient block in the G_0/G_1 phase of the cell cycle preventing the cells from entering the S-phase as shown in Figure 5. Based on these findings we conclude, that GRN163L-induced cell cycle arrest in the G_0/G_1 phase or at the G1/S transition preventing T24-luc cells from entering mitosis has an important biological and potentially clinical significance. Current in vitro data strongly suggest that GRN163L may be a new promising treatment option (after or in addition to surgery) for preventing bladder tumor recurrence and decreasing the risk of tumor progression, in combination with existing treatment modalities.

We believe that pre-clinical animal models with human bladder cancer cells have a good potential for development of oligonucleotide-based telomerase addressed therapies. This is due to the accessibility of treatment site by oligonucleotides (either secreted via bladder following IP/IV administrations, or directly infused into bladder via a catheter), and ease of follow-up diagnostics through cystoscopy and urine cytology [So et al., 2005]. Therefore, further in vivo experiments are warranted to evaluate any potential therapeutic benefits of intravesical GRN163L instillation in animal models with human bladder cancer.

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