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Lipid modification of GRN163, an N3′→P5′ *thio*-phosphoramidate oligonucleotide, enhances the potency of telomerase inhibition

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The vast majority of human cancers express telomerase activity, while most human somatic cells do not have detectable telomerase activity. Since telomerase plays a critical role in cell immortality, it is an attractive target for a selective cancer therapy. Oligonucleotides complementary to the RNA template region of human telomerase (hTR) have been shown to be effective inhibitors of telomerase and, subsequently, cancer cell growth *in vitro*. We show here that a lipid-modified N3′→P5′ *thio*-phosphoramidate oligonucleotide (GRN163L) inhibits telomerase more potently than its parental nonconjugated *thio*-phosphoramidate sequence (GRN163). Cells were treated with both the first- (GRN163) and second-generation (GRN163L) oligonucleotides, including a mismatch control, with or without a transfection enhancer reagent. GRN163L inhibited telomerase activity effectively in a dose-dependent manner, even without the use of a transfection reagent. The IC_{50} values for GRN163 in various cell lines were on average sevenfold higher than for GRN163L. GRN163L inhibition of telomerase activity resulted in a more rapid loss of telomeres and cell growth than GRN163. This report is the first to show that lipid modification enhanced the potency of the novel GRN163 telomerase inhibitor. These results suggest that the lipid-conjugated *thio*-phosphoramidates could be important for improved pharmacodynamics of telomerase inhibitors in cancer therapy.
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Human telomerase is a ribonucleoprotein complex consisting of a cellular reverse transcriptase catalytic subunit (hTERT) that uses the telomerase RNA component (hTR) of the complex as a template for adding TTAGGG repeats to the ends of the chromosomes (reviewed in Cong *et al*., 2002). The lack of telomerase activity in most somatic cells renders the ends of chromosomes, or telomeres, to shorten with each cell division due to the end replication problem. Maintenance of telomeres is essential for preventing critically short telomeres from being fused together or recognized as damaged DNA needing repair (de Lange, 2002). Telomerase activity may preferentially elongate the critically short telomeres, stabilize telomere lengths, and permit continued cancer or immortal cell division. The average telomere lengths in cancer cells are usually well below those of normal cells. These differences, coupled with the much more rapid rate of cell division in some cancer cells, make the inhibition of telomerase an attractive therapeutic target, as current therapies for cancer are generally toxic to normal cells. Telomerase is activated in over 90% of cancers, but not in the majority of normal tissues, so its inhibition might have limited side effects (White *et al*., 2001).

Studies of telomerase inhibition as a cancer therapeutic target have increased over the past several years with the development of a variety of new approaches (reviewed in White *et al*., 2001). The inhibition of telomerase through a variety of mechanisms has been shown previously to lead to progressive telomere shortening and growth arrest or cell death due to the critically short telomeres (Hahn *et al*., 1999; Herbert *et al*., 1999, 2002; Zhang *et al*., 1999; Asai *et al*., 2003). These mechanisms include using hammerhead ribozymes that digest the hTR template, using small molecules such as G-quadruplex stabilizers to inactivate the substrate, targeting the catalytic hTERT component (reverse transcriptase inhibitors, dominant-negative hTERT, and immunotherapy), and using oligonucleotides directed against the hTR template. The use of oligonucleotides has shown promise for antitelomerase cancer therapy (White *et al*., 2001). However, one major drawback in the broad utilization of oligonucleotides as clinical drugs is their low ability to transverse cell membranes. To solve this problem, transfection reagents

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such as lipophilic carriers or liposomes have been used *in vitro* to allow the oligonucleotides to enter cells. Unfortunately, the use of these lipophilic carriers does not easily translate to the clinic. While telomerase inhibition through oligonucleotide chemistries has been obtained *in vitro*, it remains to be shown that these drugs would have low toxicity and be easily administered *in vivo*.

Improvements for the cellular uptake of oligonucleotides include modifying the phosphate backbone to generate nonionic analogs, such as phosphodiester amidates with nonbridging nitrogen atoms (Agrawal *et al*., 1988; Shea *et al*., 1990). Other types of modified ionic oligonucleotides with a 3′-bridging nitrogen, such as N3′→P5′ phosphoramidate and *thio*-phosphoramidates, have already been shown to be potent telomerase inhibitors (Herbert *et al*., 2002; Akiyama *et al*., 2003; Asai *et al*., 2003; Ozawa *et al*., 2004; Wang *et al*., 2004). These telomerase inhibitory effects of the oligonucleotides, however, were significantly more profound *in vitro* in the presence of a transfection reagent. Several modifications of oligonucleotides, such as lipid or cell-penetrating peptide conjugates, have been suggested to improve oligonucleotide *in vitro* activity (Boutorin *et al*., 1989; Letsinger *et al*., 1989; Shea *et al*., 1990; Farooqui *et al*., 1991; Reed *et al*., 1991; Stein *et al*., 1991; MacKellar *et al*., 1992; Oberhauser and Wagner, 1992; Polushin and Cohen, 1994; Mishra *et al*., 1995). In particular, cholesterol modification of phosphorothioate oligonucleotides has been shown to improve inhibition of HIV replication (Letsinger *et al*., 1989; Farooqui *et al*., 1991; Stein *et al*., 1991). However, it was also reported that these antiviral effects of cholesterol-conjugated phosphorothioate oligonucleotides were largely sequence nonspecific.

We hypothesized that a lipid modification of oligonucleotide phosphoramidates, or *thio*-phosphoramidates, and in particular the telomerase inhibitor GRN163, could lead to an improvement of its bioavailability and, consequently, enhancement of the antitelomerase activity. Therefore, we synthesized an oligonucleotide conjugate with a 5′-terminal lipophilic (palmitoyl) group attached through an amide bond of α-amino glycerol to the *thio*-phosphoramidate GRN163 to form GRN163L compound (GRN163L; Figure 1). This modification noticeably increased lipophilicity of the oligonucleotide, as was judged by reverse-phase (RP) HPLC analysis – retention times were ∼11 and 26 min for the parent GRN163 and the conjugated GRN163L oligonucleotides, respectively, under the same experimental conditions. Moreover, the 5′-lipid conjugation did not significantly change the melting temperature (T_m) of the duplex that the oligonucleotide forms with its complimentary RNA target strand. The duplexes T_m values (in PBS) were 70.0 and 66.0°C for GRN163 and GRN163L, respectively.

The effectiveness of GRN163L in inhibiting telomerase activity *in vitro* is demonstrated by the results presented in Figure 2. Extracts from HME50-5E cells lysed with NP-40 lysis buffer were incubated with 0.001–100 nM GRN163 or GRN163L for 30 min and then processed for telomerase activity assays. The IC_{50} values were ∼1.4 and ∼7.8 nM for GRN163 and GRN163L, respectively. Interestingly, in this cell-free-based TRAP assay, GRN163 was still more potent than its modified NPS oligonucleotide, GRN163L. This difference may be due to minimal interference of the lipid moiety with the hTR and hTERT complex. These results also confirm that both GRN163 and GRN163L are specific telomerase inhibitors as shown previously with GRN163 in a variety of tumor cell types (Akiyama *et al*., 2003; Asai *et al*., 2003; Ozawa *et al*., 2004; Wang *et al*., 2004).

To determine and compare the effectiveness of GRN163L with the first-generation NPS GRN163 in cells, we treated immortal HME50-5E cells with GRN163L and GRN163 at various concentrations with or without the transfection reagent, FuGENE6. Figure 3 shows results representative of multiple experiments comparing the inhibitory activity of GRN163 to GRN163L. Without the use of transfection reagent, only the lipid-modified phosphoramidate GRN163L was able to inhibit telomerase activity effectively compared to GRN163 with an estimated IC_{50} of ∼150 nM and >20 μM, respectively (Figure 3a). The inhibition of telomerase activity by GRN163L without the use of a transfection reagent was dose dependent. While GRN163 also inhibited telomerase activity in a dose-dependent fashion, the inhibition was significantly dependent on the use of a transfection reagent (Figure 3b). No toxicity was seen during these short-term cellular assays (data not shown). The fact that GRN163L was more potent than GRN163 *in vitro* compared to the results in the cell-free PCR-based assay suggests that attachment of a terminal lipophilic group to GRN163 improved its cellular uptake so that more molecules were available inside the cell.

Treatment with specific telomerase inhibitors should initially be nontoxic to the cells. Furthermore, this treatment should theoretically result in the specific loss of telomeres in a time frame determined by initial telomere length, and subsequently should inhibit the cell growth and/or induce apoptosis (White *et al*., 2001). The first-generation compound of NPS telomerase inhibitors, GRN163, was shown previously to inhibit telomerase and cell growth in a telomere size-dependent manner (Herbert *et al*., 2002; Asai *et al*., 2003). We therefore wanted to verify that the second-generation NPS compound GRN163L was a specific and effective telomerase inhibitor, inhibiting growth by a telomere-based mechanism. HME50-5E cells treated with 1 μM GRN163L every third day without transfection reagent, like those treated with GRN163, exhibited telomere shortening (Figure 4a). The mismatch control cells showed no telomere shortening or effect on cell growth over the course of the experiment (Figure 4). The loss of telomeric repeats was more evident in cells treated with GRN163L than in cells treated with GRN163, especially near the end of the experiment (Figure 4a). The average telomere restriction fragment (TRF) lengths at similar time points within the experiment, population doubling level (PDL) 17 for GRN163 and PDL 19 for GRN163L, were 2.6 kbp for GRN163 and 1.8 kbp for GRN163L,

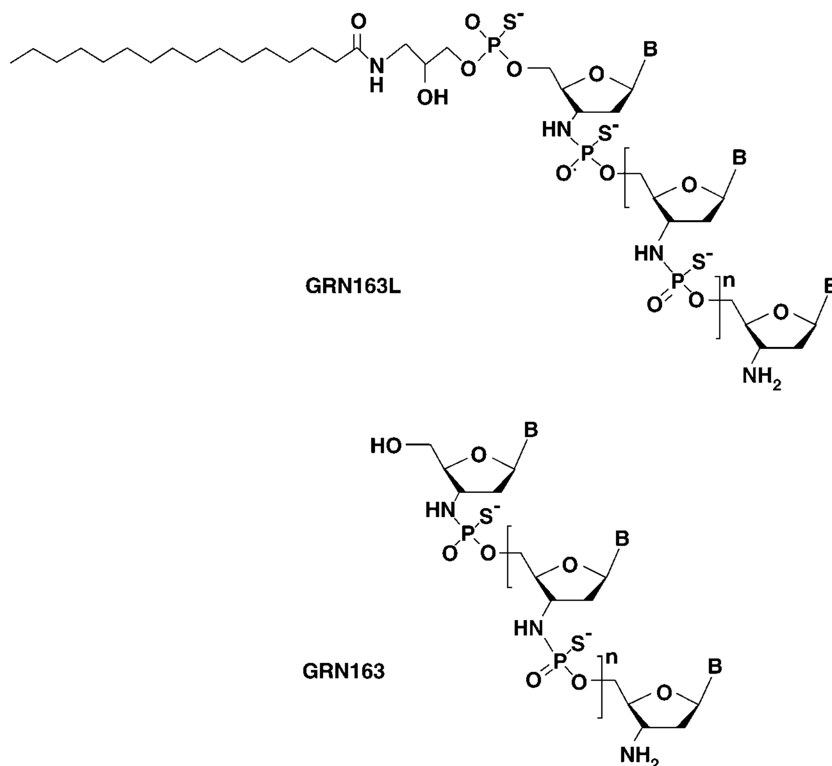

Figure 1 General structure of oligonucleotide N3′→P5′ *thio*-phosphoramidates (GRN163) and the lipid-conjugated N3′→P5′ *thio*-phosphoramidate (GRN163L) used in these studies. The nucleotide sequence is 5′-TAGGGTTAGACAA-3′ for both compounds. N3′→P5′ *thio*-phosphoramidate (NPS) oligonucleotides were prepared on a 1 μmol scale using ABI 394 synthesizers as described previously (Asai *et al*., 2003), except that stepwise sulfurization during synthesis of NPS compounds was accomplished with 0.1 M phenylacetyl disulfide (PADS) in CH_3CN/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. The 3′-aminonucleoside-containing 5′-phosphoramidites and CPG-based solid supports were purchased from Annovis Inc. (now Transgenomic Inc.). The lipid-modified oligonucleotide, GRN163L (5′-Palm-TAGGGTTAGACAA-NH2-3′), was prepared using LCAA CPG support containing *N*-palmitoyl-1-*O*-DMTr-2-*O*-succinyl-amino glycerol linker. Upon completion of the oligonucleotide chain elongation, the lipidated product was released from the support and deprotected with the mixture of concentrated aqueous ammonia/ethanol (1/1, v/v) for ~8 h at 55°C. The 5′-palmitoyl mismatch control oligonucleotide (5′-Palm-TAGG*TG*TA*AG*CAA-NH2-3′ with the mismatch bases in italics) was prepared in a similar manner. Oligonucleotide compounds were analysed and purified, if needed, by ion exchange or RP HPLC. Purity of the 5′-lipidated and nonlipidated oligonucleotides used in this study was higher than 80% by RP HPLC analysis. The compounds were then desalted by either gel filtration on NAP-10 columns (Pharmacia) or by precipitation with 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 (C18 column, 1%/min CH_3CN gradient in 0.1 M triethylammonium acetate, pH 7.2). The RP HPLC retention times for GRN163 and GRN163L typically were 13.2 and 22.3 min, respectively, reflecting higher lipophilicity of the lipid-conjugated molecule

respectively. Comparison of the growth curves (Figure 4b) shows that cells treated with GRN163L initiated a slower growth curve earlier than GRN163-treated cells. In fact, without the use of a transfection reagent, the GRN163L-treated cell population exhibited growth inhibition and apoptosis after only 20 population doublings (PDs, approximately 90 days), whereas GRN163-treated cells were still growing, albeit slowly, at this time (Figure 4b; data not shown). Furthermore, the changes in growth curves for each of the treated cells correlated with the onset of significant telomere shortening (at PD~8 for GRN163L-treated cells and PD~13 for GRN163-treated cells). As stated previously, no immediate toxicity was observed during this long-term treatment. Therefore, at similar concentrations, the lipid-conjugated *thio*-phosphoramidate GRN163L inhibited cell growth more potently than GRN163 even without the use of a transfection reagent.

Other cell lines such as tumorigenic HME50-T breast epithelial cells, MDA-MB-231 breast carcinoma cells, and PC3 human prostate carcinoma cells treated with 1 μM GRN163L or a mismatch control were examined together for effects on telomerase activity (Figure 5). GRN163L was shown to effectively inhibit telomerase activity after 96 h without the use of a transfection reagent for these cell lines (93, 73, and 69% for PC3, HME50-T, and MDA-MB-231 cells, respectively). Mismatch controls did not significantly inhibit telomerase activity (1–29%). Further comparison of a variety of tumor cell types confirmed the improved potency of GRN163L over GRN163 (Table 1). Cervical, glioblastoma, hepatoma, lung, melanoma, myeloma, ovarian, and prostate tumor cell lines were treated for 24 h

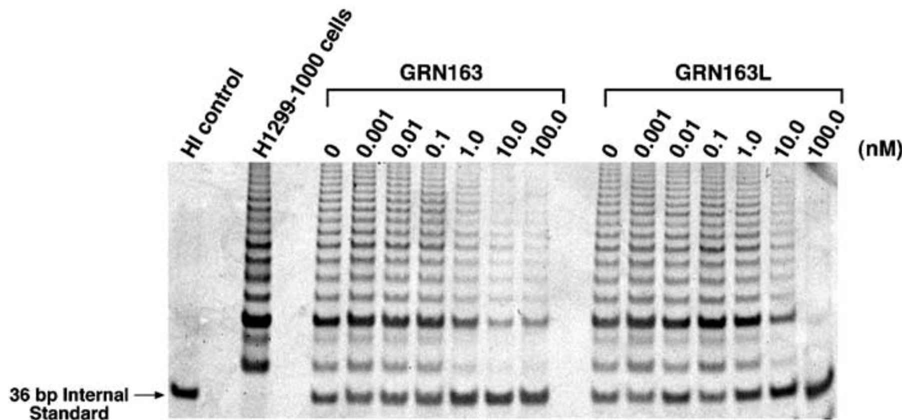

Figure 2 Telomerase inhibition by GRN163 and GRN163L in a cell-free TRAP assay. Oligonucleotides were incubated with TRAP extracts from HME50-5E cells for 30 min. An aliquot of 100 cell equivalents per lane was analysed by a polymerase chain reaction (PCR)-based TRAP assay as described (Asai *et al*., 2003). PCR reaction products were resolved on polyacrylamide gels and visualized by SYBR Green I. A ladder of bands represents the extension of the substrate primer by telomerase. Lysis buffer alone served as a negative control and H1299 lung carcinoma cells served as a positive control. Results are representative of multiple experiments

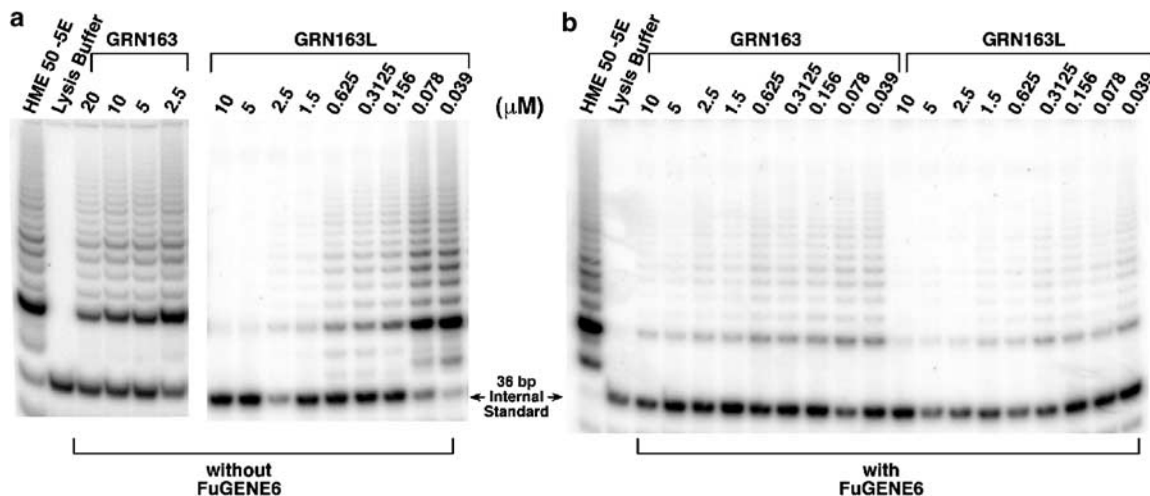

Figure 3 Comparison of the dose-dependent inhibition of telomerase activity between the *thio*-phosphoramidate oligomer GRN163 and the modified *thio*-phosphoramidate oligomer GRN163L delivered into HME50-5E cells with (**b**) or without (**a**) the use of lipid carrier FuGENE6, as measured 24 h post-transfection. After transfection, cells were collected and telomerase activity was measured for 250 cell equivalents per lane using a radiolabeled oligonucleotide primer and a PCR-based TRAP assay. The TRAP assay was performed with the TRAP-eze Telomerase Detection kit (Serologicals/Invitrogen) and established protocols (Kim *et al*., 1994; Piatyszek *et al*., 1995; Wright *et al*., 1995). Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging. The gel was exposed without drying to a phosphor screen and visualized on a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). A ladder of bands represents the extension of the substrate primer by telomerase. Telomerase activity was estimated as the ratio of the telomerase products (the 6 bp telomerase-specific ladder) to the 36 bp internal standard. This can then be plotted on a logarithmic curve to determine linearity of the assay. The TRAP assay has been shown to quantitate accurately the level of telomerase activity in samples (Piatyszek *et al*., 1995; Holt *et al*., 1996; Kim and Wu, 1997). Lysis buffer alone served as a negative control. Results are representative of multiple experiments

with 0.1–10 μM of either GRN163 or GRN163L and then collected for the conventional TRAP assay. Table 1 lists the IC_{50} values from multiple experiments for telomerase activity with GRN163 and GRN163L. The fold change in effective telomerase inhibition with GRN163L ranged from 1.4- to 39-fold (Table 1). GRN163L was universally more potent at inhibiting telomerase activity than GRN163, without the use of a transfection reagent. However, this doesnot undermine the effectiveness of GRN163 in inhibiting telomerase activity as was previously shown in a variety of tumor types *in vitro* and *in vivo* (Akiyama *et al*., 2003; Asai *et al*., 2003; Ozawa *et al*., 2004; Wang *et al*., 2004). Taken together, these results further support the improved potency of GRN163L over GRN163 in a wide range of tumor cell types.

To determine whether GRN163L can be delivered into a xenograft tumor and inhibit telomerase activity *in*

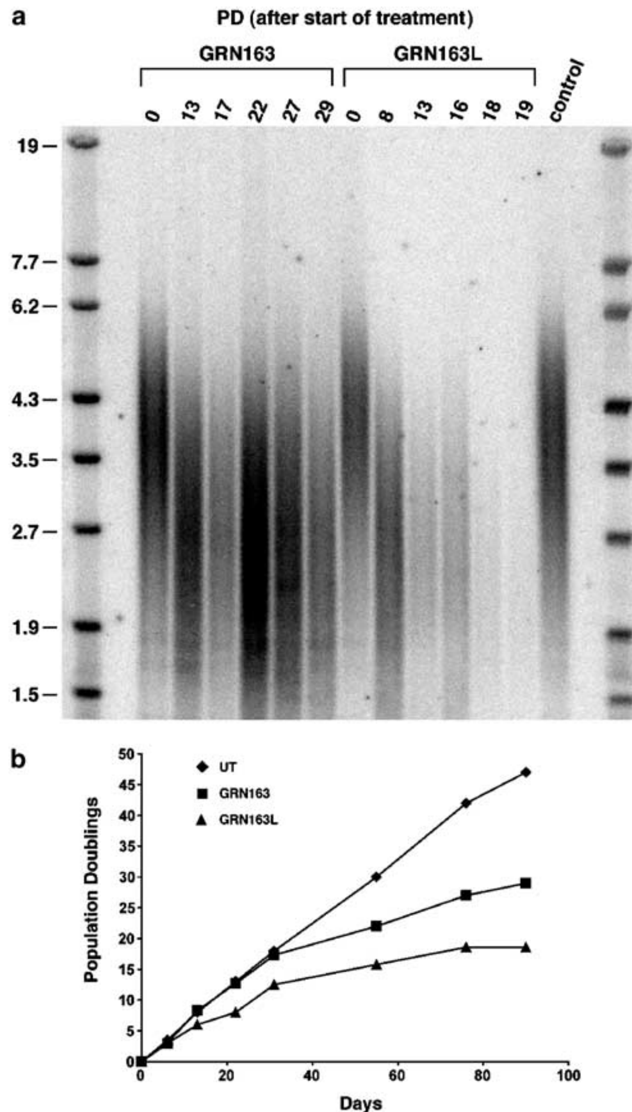

Figure 4 Effect of long-term treatment with GRN163L on telomere length and cell growth. (**a**) Measurement of TRF length in HME50-5E cells subjected to long-term treatment with *thio*-phosphoramidate oligomers GRN163 or GRN163L, or a mismatch control (at PD 33) without the use of transfection reagent. Measurements of telomere lengths were performed as described previously (Ouellette *et al*., 1999). PDs were calculated after initiation of treatment. PDs were calculated as log((the number of cells collected)/(number of cells initially plated))/log 2. Equivalent amounts of DNA were loaded in each lane. Results are representative of multiple experiments. (**b**) Effects of GRN163 and GRN163L *thio*-phosphoramidate oligonucleotides (without the use of lipid carrier) on HME50-5E cell growth, compared to untreated (UT) cells, during long-term treatment experiments. The concentration of the oligonucleotides was 1 μM and were provided every 3 days

vivo, we administered 50 mg/kg GRN163L, GRN163, or PBS into the tail vein of three separate nude mice, each with DU145 prostate cancer xenograft tumors located on the right and left hind legs. Half of the oligonucleotide dose given to the mice was GRN163L conjugated with FITC in order to visualize uptake into the tumor. At 24 h after administering the oligonucleotides, the

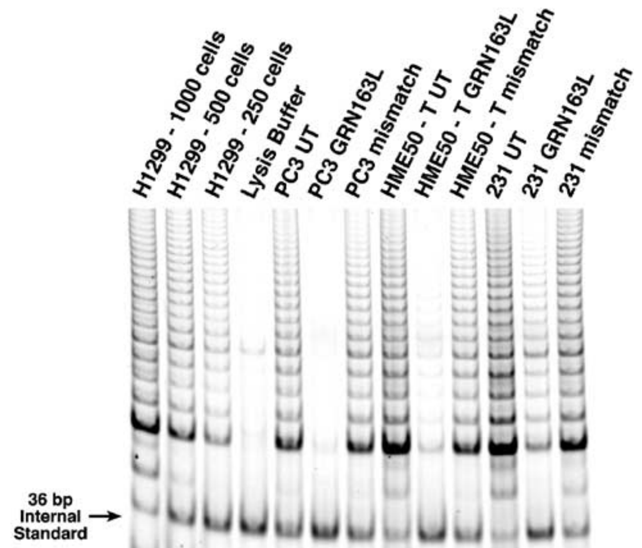

Figure 5 GRN163L is more potent in inhibiting telomerase activity in different tumor cell lines. Comparison of telomerase activity in PC3, HME50-T, or MDA-MB-231 cells with 1 μM GRN163L, mismatch control, or no treatment (UT). These cells were continuously treated in culture, without the use of a transfection reagent, for 50 days with routine passaging and collected every 96 h for TRAP. After treatment, cells were collected and telomerase activity was measured for 250 cell equivalents per lane using a radiolabeled oligonucleotide primer and a PCR-based TRAP assay. For the assay, 250 cell equivalents from H1299 cells served as positive control. Lysis buffer alone served as a negative control. The tumorigenic LFS-HME cell line (HME50-T) was established by infecting the preimmortal LFS-HME cells (Shay *et al*., 1995) with hTERT (Yi *et al*., 2000) and H-RasV12 (retroviral supernatant provided by Dr Michael White, University of Texas Southwestern Medical Center; Morales *et al*., 1999) and then collecting clones that grew in soft agar and nude mice xenografts

Table 1 Comparison of telomerase activity IC_{50} values between GRN163L and GRN163 in tumor cell lines[a]

Cell type	*Cell line*	*GRN163L: IC_{50} (μM)*	*GRN163: IC_{50} (μM)*	*Fold Δ*
Cervical	HT-3	0.29	1.39	4.8
Glioblastoma	U251	0.17	1.75	10
	U87	0.18	0.8	4.4
Hepatoma	Hep3B	1.35	3.02	2.2
	HepG2	0.48	2.72	5.7
Lung	NCI-H522	0.23	0.75	3.3
Melanoma	M14	0.35	0.69	2.0
	SK-MEL-2	0.19	1.13	5.9
	SK-MEL-5	0.38	0.54	1.4
	SK-MEL-28	0.94	3.29	3.5
Myeloma	RPMI 8226	0.38	2.69	7.1
Ovarian	Ovcar5	0.92	3.03	3.3
Prostate	DU145	0.15	5.8	39

[a]U251 glioblastoma, U81 glioblastoma, and NCI-H522 lung carcinoma cells were obtained from UCSF, Duke, and NCI repositories, respectively. All other cells listed were obtained from the American Type Tissue Collection (ATTC). Cells were treated for 24 h with 0.1–10 μM of either GRN163 or GRN163L and then collected and lysed for the conventional TRAP assay. The TRAP assay was performed with the TRAP-eze Telomerase Detection kit (Serologicals/Invitrogen) and established protocols (Kim *et al*., 1994; Piatyszek *et al*., 1995; Wright *et al*., 1995). The relative percentages of telomerase activity were calculated and averaged from at least three experiments and then plotted on a graph to calculate the IC_{50} for each cell line

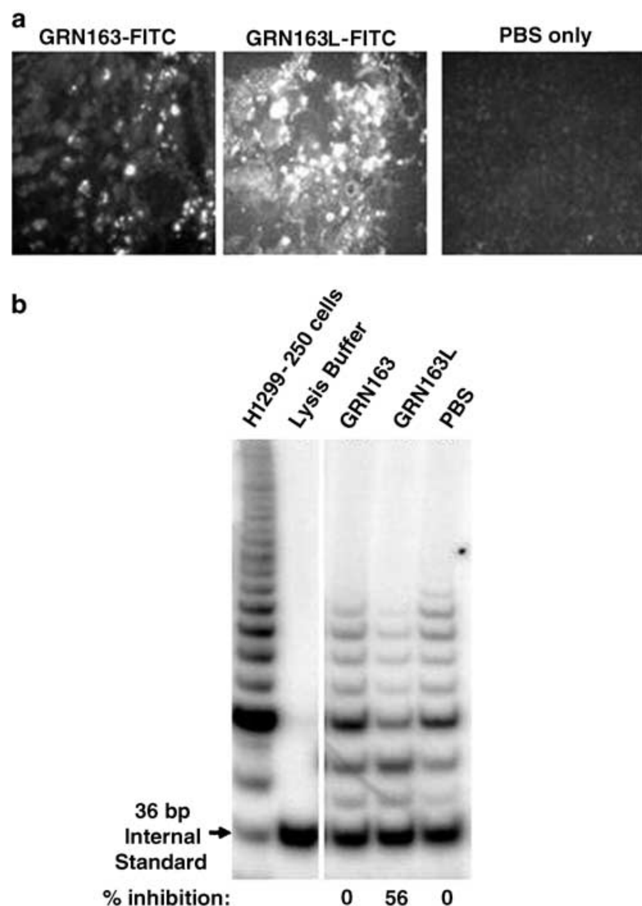

Figure 6 Fluorescent imaging (**a**) and analysis of telomerase activity (**b**) of established DU145 xenograft tumors, as measured 24 h post-treatment. When tumors reached 50 mm^3, three separate nude mice were given 50 mg/kg of the modified *thio*-phosphoramidate oligomer GRN163L, the unmodified *thio*-phosphoramidate oligomer GRN163, or PBS delivered via the tail vein. After treatment, the mouse was imaged live using a fluorescent CCD camera, and then various tissues were collected and processed for fluorescent microscopy and telomerase activity. Shown are tissue sections of the processed FITC-containing xenograft tumors (**a**). Telomerase activity was measured using 0.75 μg protein per lane. Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging (**b**). For the assay, 250 cell equivalents from H1299 cells served as positive control. Lysis buffer alone served as a negative control

mice were visualized for FITC fluorescence within the xenograft tumor *in vivo*, which showed that the xenograft tumor expressed the labeled oligomer (Figure 6). Then, the left flanks were taken for tissue processing while the right flanks were taken for telomerase activity assays. As Figure 6a shows, the oligonucleotides could be taken up by the xenograft tumors. GRN163L showed more prominent uptake than GRN163 into the xenograft tumor and GRN163L was taken up into other tissues of the mouse, such as the liver (data not shown). Analysis of right flanks of the treated mice showed that GRN163L inhibited telomerase activity by $\sim$56% after 24 h compared to the GRN163- or PBS control-treated mice (Figure 6b). These results are consistent with the increased potency of GRN163L compared to GRN163.

In summary, the 5′-lipid modification of GRN163, an N3′$\rightarrow$P5′ *thio*-phosphoramidate oligonucleotide, with palmitoylated amino glycerol group enhanced the potency of telomerase inhibition *in vitro* and *in vivo* without the use of a lipid carrier. This is the first report of an effective telomerase inhibitor that can be delivered into cells without the need of a transfection reagent to increase its cellular uptake. Long-term inhibition of telomerase with GRN163L *in vitro* resulted in effective telomere shortening and subsequently in growth inhibition. Oligonucleotide phosphoramidates, particularly *thio*-phosphoramidates, modified with various lipid groups thus show significant promise as efficient antitelomerase reagents.

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