Telomerase Template Antagonist GRN163L Disrupts Telomere Maintenance, Tumor Growth, and Metastasis of Breast Cancer

Amelia E. Hochreiter,1,2 Hongling Xiao,1,2 Erin M. Goldblatt,1 Sergei M. Gryaznov,5 Kathy D. Miller,2,3 Sunil Badve,2,4 George W. Sledge,2,3 and Brittney-Shea Herbert1,2

Abstract

Purpose: Maintenance of telomeres by telomerase is critical for the continuing proliferation of most advanced cancer cells. Telomerase activity has been detected in the vast majority of cancer cells but not most normal cells, making the enzyme an attractive target for anticancer therapy. The aim of this study was to address the breast cancer translational potential of the novel telomerase inhibitor, GRN163L.

Experimental Design: In the present study, we investigated the effects of GRN163L treatment on a panel of breast cancer cells representing different tumor subtypes with varying genetic backgrounds, including ER+, ER−, HER2+, BRCA1 mutant breast tumor cells as well as doxorubicin-resistant cancer cells. To investigate the in vivo effects of GRN163L, we employed a breast cancer xenograft and metastasis model that simulates a clinical situation in which a patient arrives with a primary tumor that may be then treated or surgically removed.

Results: GRN163L effectively inhibited telomerase activity in a dose-dependent fashion in all breast cancer cell lines resulting in progressive telomere shortening. A mismatch control oligonucleotide showed no effect on telomerase activity and GRN163L did not significantly affect telomere shortening in normal human mammary epithelial cells or in endothelial cells. Breast cancer cells that exhibited telomerase inhibition also exhibited significant reduction in colony formation and tumorigenicity. Furthermore, GRN163L suppressed tumor growth and lung metastases (P = 0.017) of MDA-MB-231 cells in vivo after 4 weeks of treatment.

Conclusions: These results show in vivo effectiveness of GRN163L in breast cancer and support its promising clinical potential for breast cancer treatment.

Breast cancer remains as the most common malignant disease in Western women. Although early detection and improved therapy of early disease has led to an overall decline in breast cancer mortality, metastatic breast cancer remains largely incurable with a median survival of 2 to 3 years. Recent genomic studies have confirmed that breast cancer is a heterogeneous disease consisting of different subtypes with potentially different survival outcomes or responses to treatment (1–3). It is therefore necessary to develop and test new molecular targets for their potential in breast cancer therapy.

A hallmark of cancer is its limitless proliferative potential predominately achieved by telomere maintenance (4,5). Telomeres are specialized structures at the end of chromosomes that protect the ends from fusion, recombination, or being recognized as uncapped DNA breaks (6–8). Human telomerase is a ribonucleoprotein complex consisting of a cellular reverse transcriptase catalytic subunit (hTERT) that uses the telomerase RNA component (hTR or hTERC) of the complex as a template for adding TTAGGG repeats to the ends of the chromosomes (reviewed in ref. 9). Telomerase activity has been detected in the vast majority of human tumors cells as well as in cells of continually renewable tissues (e.g., stem cells) and germ-line cells, but not in most normal somatic cells. The observed differences of telomerase activity in tumor-derived versus normal cells, coupled with the much more rapid rate of cell division of cancer cells, resulted in the hypothesis that telomerase may represent a suitable target for specific anticancer therapies. Telomerase activity has been detected early in breast cancer progression and correlated with hTERT mRNA levels and prognosis (11–14). Furthermore, the observation...
that the majority of epithelial cancers cells have significantly shorter telomeres compared with other normal cells suggests that those cancer cells represent an attractive target for antitelomerase treatments (15–20).

Following this appealing scientific rationale, several classes of telomerase inhibitors targeting different sites of the telomerase complex or telomeres themselves have been evaluated (reviewed in refs. 21, 22). In particular, agents that target the 11-base template region of hTR, such as peptide nucleic acids, 2′-O-methoxymethyl RNA, and phosphoramidate oligonucleotides, have been well studied in a variety of cancer cell lines (21–24). Furthermore, a new class of oligonucleotides targeting the hTR, N3′→P5′ thio phosphoramidates, have been shown to form stable duplexes with ssRNA, to be resistant to nuclease degradation, and to have both high affinity and specificity for targets (25). These N3′→P5′ thio phosphoramidate oligomers (such as GRN163) are effective in telomerase inhibition, telomere shortening, and inhibition of cancer cell growth (25–30). Agents that target the hTR template region act as classic, competitive enzyme inhibitors of telomerase activity and work at pharmacologic concentrations (31–33).

The synthesis and properties of a new telomerase template antagonist, GRN163L, were recently reported. This lipid-modified N3′→P5′ thio phosphoramidate oligonucleotide is complementary to the template region of hTR and is a potent inhibitor of telomerase activity in vitro (34). GRN163L is a competitive telomerase enzyme inhibitor in that it does not target hTERT protein per se but blocks the binding of chromosomal telomere substrates to telomerase. Due to its 5′-lipid palmitoyl domain, which increases its lipophilicity, GRN163L was shown to exhibit increased bioavailability, cellular uptake in tumors relative to nonlipidated counterparts, and it is more acid resistant than other telomerase-addressed phosphoramidate oligonucleotides (34). Recent in vivo studies in hepatoma and lung cancer suggest the translational potential for GRN163L as a novel anticancer therapeutic in these cancers (35, 36). Currently, GRN163L is in phase I/II clinical trials for chronic lymphocytic leukemia.

In the present study, we investigated the effects of GRN163L as a telomerase inhibitor on telomere shortening, anchorage-dependent and anchorage-independent growth, and plating efficiency of various breast cancer cell lines representing different lineages and/or genetic backgrounds. Furthermore, in the first evaluation of GRN163L using an in vivo breast xenograft tumor and metastasis model, we show that GRN163L is effective in reducing breast tumor growth and the amount of breast cancer metastases to the lung.

### Materials and Methods

**Oligonucleotides.** The oligonucleotide GRN163L (5′-Palm-TAGGG-TTACGAA-NH2-3′) has a sequence complementary to the hTR template region. This lipid-modified N3′→P5′ thio phosphoramidate oligonucleotide and the 5′-palmitoyl mismatch control oligonucleotide (5′-Palm-TAGGG-TTACGAA-NH2-3′ with the mismatch bases italicized) were prepared and analyzed as described previously (34).

**Cell culture.** A tumorigenic human mammary epithelial (HME) cell line (HME50-T) was established by infecting premortal HME cells from a patient with Li-Fraumeni syndrome, which have a germ-line mutation at codon 133 in one of the two alleles of the p53 gene [Met-to-Thr (M133T)] that affects wild-type p53 protein conformation, with hTERT and H-RasV12, and then cloning those that grew in soft agar and nude mice xenografts (34). These cells and other breast cancer cells (MCF-7, HCC1937, SKBR3, and MDA-MB-231 breast carcinoma cells) were grown in DMEM (Invitrogen, Carlsbad, CA) containing 10% cosmic calf serum (HyClone, Logan, UT) and 50 μg/mL gentamicin (Invitrogen). Nontumorigenic 21NT breast epithelial and normal HME cells were grown in MEGM (Cambrex, East Rutherford, NJ). Human endothelial progenitor cells were maintained as described (37). Population doublings were calculated as the log [(the number of cells collected) / (number of cell initially plated)] / log 2 for each passage.

**Treatment with GRN163L.** To determine the efficacy and dose response for GRN163L in breast cancer cells, a 1:2 serial dilution series of GRN163L or its mismatch control (5.0, 2.5, 1.25, 0.625, and 0.325 μmol/L) was prepared and given to cells plated on cell culture plates. After 24 hours, 1.0 × 105 cells were collected for telomeric repeat amplification protocol (TRAP) assay as described below. In addition, cells were treated with GRN163L at an effective concentration (1.25 μmol/L, decided on by the dose-response data of the cells) for 24, 48, and 72 hours and then collected for TRAP.

For long-term treatment of GRN163L, cells were maintained in triplicate in six-well cell culture plates at an initial seeding of 2.5 × 104 to 5.0 × 104 per well. Cells were fed medium containing 1 to 2.5 μmol/L GRN163L, mismatch control oligonucleotide, or medium alone every 3 to 4 days. Cells were passaged weekly at 80% confluency and replated as described above in the presence of treatment. At each passage, cells were collected for other analyses described below. Throughout the experiment, all cells were examined for morphologic changes.

**Telomeric repeat amplification protocol.** The TRAP assay was done according to the TRAPeze Telomerase Detection kit (Serologicals, Norcross, GA) using a Cy5 fluorescently labeled primer and established protocols (38, 39). Following PCR, the TRAP reaction products were run on a 10% nondenaturing acrylamide gel. The gel was fixed in 0.5 mol/L NaCl, 50% ethanol, and 40 mmol/L sodium acetate (pH 4.2) for 30 minutes and visualized, without drying, on a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Telomerase activity was shown as a 6-bp telomerase-specific ladder above the 36-bp internal standard control band.

**Terminal restriction fragment length assay.** Measurements of telomere lengths were done as described previously (40). Briefly, DNA was isolated and the proteins were digested in 10 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 100 mmol/L EDTA (pH 8.0), 1% Triton X-100, and 2 mg/mL protease K for 4 hours at 55°C followed by inactivation of protease K for 30 minutes at 70°C and dialysis in 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA (pH 8.0) at 4°C overnight. Genomic DNA was digested to completion with multiple restriction enzyme mix (–IU/g each of AluI, DdeI, HaeIII, HinfI, MspI, and BsuRI, Roche Boehringer Mannheim, Indianapolis, IN). The digested DNA was separated on a 0.7% agarose gel in 0.5× Tris-borate EDTA [0.5 mol/L Tris-borate (pH 8.3), 10 mmol/L EDTA] or 1× TAE [0.04 mol/L Tris-acetate, 0.002 mol/L EDTA (pH 7.6)]. The gel was denatured for 20 minutes in 0.5 mol/L NaOH/1.5 mol/L NaCl, rinsed with distilled H2O for 10 minutes, dried on Whatman No. 3MM paper under vacuum for 1 hour at 55°C, and neutralized for 15 minutes in 1.5 mol/L NaCl, 0.5 mol/L Tris-HCl (pH 8.0). The gel was probed with a radiolabeled telomeric probe for 16 hours at 42°C in 5× SSC buffer, 5× Denhardt’s solution, 10 mmol/L NaHPO4, and 1 mmol/L Na2HPO4. The gel was then washed once for 20 minutes in 2× SSC, twice for 15 minutes each in 0.1× SSC at room temperature, and exposed to a phosphor screen (PhosphorImager).

**Replicative capacity as measured by colony formation assay.** GRN163L and control pretreated and untreated breast cancer cells were seeded at 103 on 10 cm2 plates and grown for 11 to 18 days in a humidified chamber at 37°C and 5% CO2. Oligonucleotide treatment was conducted twice after plating. Medium was then aspirated and the cells were washed once with HBSS before fixing with 70% ethanol.
Plates were stained with 20% Giemsa in double-distilled water for 1 hour and gently rinsed with water to remove excess stain, and images were captured using a CCD camera (UVP GDS-8000, Imaging System, Upland, CA). Colonies that consisted of ≥50 cells were counted.

**Anchorage-independent growth/soft agar studies.** Twelve-well cell culture plates were prepared with a bottom layer of 0.5% sterile agar in culture medium. Cell suspensions of 500 and 1,000 cells for controls and GRN163L-treated cells at the indicated passages were prepared in triplicate and mixed with 0.375% agar in culture medium. This top layer suspension was mixed quickly and then poured onto the bottom layer. The plates were incubated in a humidified chamber at 37°C and 5% CO2 for 14 to 21 days until colony size was ≥50 cells. The colonies were counted using an inverted microscope and stained with 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

**Xenograft mice studies.** Athymic nude mice (nu/nu; Harlan Sprague-Dawley, Inc., Indianapolis, IN) were maintained in pathogen-free conditions within the Laboratory Animal Resources Center at the Indiana University School of Medicine according to an approved protocol by institutional Laboratory Animal Resources Center and Institutional Animal Care and Use Committee. MDA-MB-231 cells (1 × 106) were injected into the mammary fat pads of 4- to 6-week-old mice. These cells have been shown previously to develop primary tumors and metastasize efficiently to the lungs when the primary tumors have been removed (41). For metastases studies, tumors were allowed to grow to ~100 mm3 and then resected. Mice were assigned to treatment group based on average tumor size/weight. Following a recovery period of 2 days, mice began receiving treatment of 30 mg/kg GRN163L (n = 14) or a PBS solvent control (n = 13) every 3 days i.p. After 4 weeks, mice were euthanized and the lungs were resected, fixed in formalin solution, sectioned, and stained with H&E for analysis. The number and size of metastasis in five fields per sample was calculated and a score of 4+ was given to a sample with highest metastasis index and relative metastasis in other samples were calculated (i.e., 1+, 2+, and 3+) by a pathologist blinded to treatment arm (41). In a separate study to evaluate effects on primary tumor growth, mice began treatment with 30 mg/kg GRN163L (n = 10) or a PBS solvent control (n = 10) every 3 days i.p. when the established primary tumors in the mammary fat pad reached an average size of ~50 mm3. Average weight of animals and size of tumors were similar among different groups. Tumor volume was calculated as (length × width2) / 2 (mm).

**Statistical analysis.** Statistical analysis was accomplished using Microsoft Excel and GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA). A two-tailed Student’s t test with unequal variance was used to calculate the P values of lung metastases indices. P values for other assays were calculated using a Student’s t test or ANOVA. In all assays, P < 0.05 were considered statistically significant.

**Table 1.** Classification of breast cancer cell lines

<table>
<thead>
<tr>
<th>Name</th>
<th>Mean TRF (kb)</th>
<th>Tumor subtype/genetic background*</th>
</tr>
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<tbody>
<tr>
<td>MCF-7 adenocarcinoma</td>
<td>3.0</td>
<td>ER+, p53+, luminal</td>
</tr>
<tr>
<td>MDA-MB-231 adenocarcinoma</td>
<td>4.5</td>
<td>ER–, p53–, basal-like</td>
</tr>
<tr>
<td>SKBR3 adenocarcinoma</td>
<td>6.2·7.5</td>
<td>HER2+, ER–, p53–</td>
</tr>
<tr>
<td>HCC1937 primary ductal carcinoma</td>
<td>3.5</td>
<td>BRCA1mut, ER–, p53–, basal-like</td>
</tr>
<tr>
<td>21NT nonmutomogenic</td>
<td>2.7</td>
<td>HER2+, p53–</td>
</tr>
<tr>
<td>HME50-T (transformed HME)</td>
<td>10.5 and 2.5</td>
<td>p53mut, exohTERT+, H-RasV12+</td>
</tr>
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*P53– indicates p53 is nonfunctional or not expressed; HER-2+ indicates amplification of HER2 gene; BRCA1mut indicates an inherited mutation in BRCA1 (5382insC); exohTERT+ indicates exogenous overexpression of TERT.
types of breast cancer cells tested as evidenced by a lower average molecular weight of the telomere restriction fragments (TRF) compared with the untreated or mismatch controls (Fig. 2). Importantly, treatment with a mismatch oligonucleotide did not result in shortening of average TRF lengths over the entire treatment time (up to 40-90 population doublings or 6-12 passages). Interestingly, GRN163L-treated HME50-T cells lost the shorter TRF population at PD87, as their longer bulk telomeres continued to progressively shorten (Fig. 2F). To determine whether an alternative mechanism for maintaining telomeres was activated, we investigated these cells using fluorescence in situ hybridization of telomeres and PML/TRF1 colocalization (a characteristic of cells undergoing alternative mechanism for maintaining telomeres). We did not find any evidence that these cells underwent the alternative mechanism for maintaining telomere pathway (data not shown). The disruption of telomere maintenance shows the sequence-specific effects of GRN163L as a telomerase inhibitor.

Treatment with GRN163L does not significantly affect normal mammary epithelial or human endothelial cell telomere length. To offer insight into whether GRN163L induces significant telomere shortening in normal cells, we treated HME cells and human CFB8 endothelial progenitor cells with either GRN163L (1 μmol/L), its mismatch control (1 μmol/L), or no treatment for six passages. The dose (1 μmol/L) was chosen as a comparison to breast cancer cells because it was used in the majority of the breast cancer cell line experiments. HME cells with no detectable levels of telomerase activity exhibit telomere shortening at a rate of ~50 to 100 bp per division during their life span as part of the replicative aging process in vitro (5, 42). GRN163L did not alter the rate of telomere shortening in HME cells compared with controls after six passages of treatment (Fig. 3A). At the beginning of the experiment, average TRF lengths were 5.0 and 4.9 kb for untreated/control and GRN163L-treated cells, respectively. After six passages, average TRF lengths were 3.7 and 3.8 kb for untreated/control and GRN163L-treated cells, respectively. Therefore, treatment of normal HME cells with GRN163L did not result in increased telomere shortening compared with untreated samples. Although the presence of the oligonucleotides, either mismatch or GRN163L, in the medium did not result in cytotoxicity during the 6 weeks of continuous treatment, a nonsignificant cytotoxic effect on HME cell growth was observed as these cells reached replicative senescence. A similar observation was observed in normal BJ fibroblasts as they approached replicative senescence, which may be due to cellular responses to the oligonucleotides at this stage (30). Furthermore, therapeutically relevant doses of GRN163L did not result in the presence of critically shortened telomeres in CFB8 human endothelial cell population compared with untreated or

Fig. 1. Dose-dependent and long-term inhibition of telomerase activity of the lipid-modified thiophosphoramidate oligonucleotide GRN163L delivered into a panel of breast cancer cell lines (see Table 1 for descriptions). A, breast cancer cell lines were treated with 5.0 (lane 3), 2.5 (lane 4), 1.25 (lane 5), 0.625 (lane 6), and 0.3125 (lane 7) μmol/L GRN163L or its mismatch control for 24 hours. Untreated (UT, lane 2), mismatch treated, and GRN163L-treated cells were collected and telomerase activity was measured for 500 to 1,000 cell equivalents per lane using a Cy5-labeled oligonucleotide primer and a PCR-based TRAP assay as described in Materials and Methods. Labeled PCR reaction products were resolved on polyacrylamide gels and visualized on a PhosphorImager using ImageQuant software. A ladder of bands above a 36-bp internal standard control band represents the extension of the substrate primer by telomerase. LB, lysis buffer. B, effects of long-term treatment of GRN163L on telomerase activity. Cells were treated twice weekly with 1 μmol/L GRN163L (MDA-MB-231 cells with 2.5 μmol/L) and collected at each passage for the TRAP assay as described above. Lane number, passage number. Lysis buffer alone served as a negative control. Representative mismatch controls (MM) were obtained using MCF-7 cells. Representative of multiple experiments.
mismatch controls (Fig. 3B). CFB8 endothelial progenitor cells have endogenous telomerase activity (37), which maintains telomere lengths in the population. These cells also possess long telomeres as shown by their large TRFs. At the same dose and time frame used in breast cancer cell line studies (1 μmol/L), long-term treatment with GRN163L did not result in critically short telomeres or cell growth inhibition in these endothelial cells compared with controls and the breast cancer cells treated at the same concentration and time (Fig. 1). These results offer preliminary suggestions to the ability of GRN163L to alter telomere maintenance and cell growth in cancer but not in normal cells. Further investigation is warranted into the effects of GRN163L on other types of normal cells, such as T cells.

**Telomerase template antagonist GRN163L inhibits replicative capacity and anchorage-independent growth of breast cancer cells in vitro.** To further evaluate the effects of sustained telomerase inhibition and telomere shortening in different subtypes of breast cancer cell lines treated with GRN163L (or its mismatch control), we did various cell growth assays. It was reported previously that telomerase inhibition resulted in complete growth arrest or death of nontumorigenic yet immortal mammary epithelial HME50-5E cells (25, 34). Interestingly, population doubling levels or anchorage dependence did not alter greatly over time in breast cancer cells grown on plastic and subcultured weekly (Supplementary Fig. S1A), whereas telomerase activity was decreased (Fig. 1B), telomeres shortened, and cell morphology was compromised (Supplementary Fig. S1B). Even increasing the dose of GRN163L from 1 μmol/L to 1.75 to 2.5 μmol/L to ensure 100% inhibition of telomerase activity did not result in complete growth inhibition of the cells. However, the immortalized, nontumorigenic 21NT breast epithelial cells showed complete cell growth inhibition and positive senescence as shown by the β-galactosidase staining assay (Supplementary Fig. S1A and S1C). These results were similar to previous studies with template antagonists using...
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Fig. 3. Treatment with GRN163L does not significantly affect normal HME or endothelial cell growth and telomere length. Analyses of TRF lengths in normal HME cells and human endothelial cells (CFB8) treated long-term with thiophosphoramidate oligomers GRN163L, a mismatch control, or medium only (untreated). HME and CFB8 cells were treated at 1 μmol/L for six passages before collecting for TRF analyses. Throughout the treatment period, cellular morphology was monitored and images shown are of passage 6 of treated CFB8 endothelial cells. Representative of multiple experiments.

Discussion

In the present study, we analyzed the in vivo effects of the telomerase template antagonist GRN163L on human breast tumor growth. Our results show the antitumor properties of the short oligonucleotide thiophosphoramidate GRN163L in an in vivo xenograft breast cancer metastasis model that mimics breast cancer progression in the clinic (primary tumor resection followed by lung metastases). In addition, we investigated the in vitro effects of GRN163L on telomerase activity, telomere maintenance, and cell growth in a variety of breast cancer cell lines representing different subtypes. In general, GRN163L was effective in inhibiting telomerase for all subtypes of breast cancer cell lines, which further supports GRN163L as a universal antitelomerase agent. Long-term inhibition of telomerase with GRN163L in vitro resulted in effective telomere shortening and reduced replicative/tumorigenic capacity in the breast cancer cell lines. However, the observed differential responses to GRN163L...
may reflect the varied genetic backgrounds of these breast cancer cell lines.

Until recently, telomerase activity was detected only in the vast majority of immortal cell lines (including human tumor cell lines), cells of constantly renewable tissues (stem/progenitor cells), and germ-line cells. However, recent evidence from foreskin fibroblasts has suggested other potential roles for telomerase different from its telomere maintenance function (43, 44). It is therefore necessary to understand the mechanisms of telomerase inhibitors and resulting telomere dysfunction to critically evaluate their effects on normal and cancerous cells. In this study, we did not observe significant changes in cell growth or critically short telomeres in normal mammary epithelial and endothelial cells treated with GRN163L at therapeutically relevant doses. It will be critical to further evaluate the effect of telomerase inhibitors on other normal cells to understand the clinical potential of these agents in cancer treatment.

We noted that GRN163L markedly reduced plating efficiency and anchorage-independent tumor cell growth, which takes place well before the bulk of telomeres of the population became critically short. Furthermore, administration of GRN163L to mice that underwent surgical removal of a xenograft tumor from the mammary fat pad resulted in a significant reduction in lung metastases within 4 weeks. These observations may be due to (a) generalized telomere shortening making cells more susceptible to anchorage-independent growth inhibition; (b) an acute DNA damage response to GRN163L; or (c) a novel, unknown telomere-independent function for telomerase that is critical for tumorigenic and metastatic potential. These rapid effects of GRN163L in breast cancer cell lines agree with other reports...

Fig. 4. Effects of GRN163L on replicative capacity and anchorage-independent growth. A. colony formation assay of control and GRN163L-treated breast cancer cells. Cells were pretreated with GRN163L and controls for two to three passages before plating at low density. After 11 to 18 days, the plates were stained with Giemsa and imaged. B. analyses of soft agar growth in control and GRN163L-treated breast cancer cells at the indicated passage. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Actual Ps = 0.0008 and 0.0005 (MCF-7 P9 and P12, respectively); 0.046, 0.002, and 0.0007 (MDA-MB-231 P3, P6, and P9, respectively); and 0.010, 0.008, and 0.011 (HME50-T P3, P6, and P9, respectively).
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Fig. 5. *In vivo* effects of lung metastases and primary tumor growth of MDA-MB-231 xenografts. See Materials and Methods for experimental details. A, efficacy of GRN163L in orthotopic mammary fat pad model. When tumors reached an average size of ~50 mm³, mice were given either 30 mg/kg GRN163L or PBS solvent control thrice weekly i.p. for 4 weeks (total of 12 treatments). Primary tumor growth was measured weekly and the average ± SE tumor volume (in mm³) per group (n = 10 per group) was plotted over time. B, lung metastasis index in PBS control and GRN163L-treated mice. Mice were given either 30 mg/kg GRN163L or PBS solvent control thrice weekly via i.p. for 4 weeks (total of 12 treatments) 2 days after resection of a primary tumor in the mammary fat pad. The number and size of metastasis in two to five fields per sample were calculated and a score of 4+ was given to a sample with highest metastasis and relative metastasis in other samples were calculated (i.e., 1+, 2+, and 3+) by pathologist blinded to treatment arm. The average ± SD scores were calculated and plotted with the indicated number of mice per treatment group. *, P < 0.05; P = 0.017. C, H&E staining of lung tissue in PBS controls (left) and GRN163L-treated mice (×200). Arrows, evidence of macrometastases on the lungs of the PBS control-treated mice.

with similar findings and conclusions (24, 45). Generalized telomere shortening after telomerase inhibition may also render cells more susceptible to chemotherapy or irradiation as reported by Chen et al. (24). We have also observed that pretreatment with GRN163L augments cell growth inhibition by doxorubicin in MDA-MB-231 breast cancer cells (data not shown).

These observations leave us with the question: What is the role of telomerase or dysfunctional telomeres in cell survival or tumorigenic potential? The maintenance of functional telomeres at chromosome ends is essential for cell survival and preventing critically short telomeres from being fused together, inducing genomic instability, or recognized as damaged DNA needing repair (7, 8). Furthermore, it has been shown that the shortest telomere is critical for cell growth, viability, and genomic stability (46–48). Telomerase inhibition in cancer cells has been proposed to induce either cellular apoptosis or senescence due to telomere shortening and presence of DNA damage signals (49). The factors determining the cellular commitment to undergo apoptosis or senescence are not completely understood; however, Lechel et al. suggest that the cellular level of telomere dysfunction influenced by the genetic background (e.g., p53 status) may be responsible for this decision (50). On the other hand, Djojosubroto et al. did not reach the same conclusion when using GRN163 and GRN163L as the cells used in that study both contained p53 mutations and dysfunctional telomeres yet still displayed rapid inhibition of tumor growth after treatment (36). Therefore, the genetic alterations influencing the cellular responses to GRN163L remain to be identified.

Our results provide the first set of *in vivo* data for the therapeutic effectiveness of GRN163L in human breast cancer xenograft and lung metastases models. The *in vivo* metastasis model used in this study is particularly powerful and mimics the adjuvant breast cancer setting, with primary tumor resection followed by subsequent treatment to eliminate or delay emergence of metastases. This oligonucleotide, being well tolerated *in vivo*, shows noticeable efficacy in limiting tumor growth overall and, importantly, reduction of metastases formation. These results are also corroborated with two other recent reports using GRN163L in lung and hepatoma *in vivo* models (35, 36). The preclinical data suggest that telomerase inhibition is likely to have the greatest clinical effect in combination with other therapeutic agents or in patients with only microscopic disease (i.e., in the adjuvant setting).

In summary, we have shown that GRN163L can rapidly modulate cell growth, plating efficiency, and tumorigenicity *in vitro* and *in vivo* in a variety of different breast cancer cell lines. The inhibition of metastases and reduced colony formation by GRN163L suggest a mechanism that involves inhibition of tumor-initiating cells to repopulate independent of average telomere length, which may prove useful for multiple tumor types. Therefore, these data further support continued *in vivo* studies using GRN163L, such as designing rational combination treatments with chemotherapy or irradiation, as well as the development of GRN163L as a novel agent for breast cancer therapy.

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References

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