The telomerase template antagonist GRN163L alters MDA-MB-231 breast cancer cell morphology, inhibits growth, and augments the effects of paclitaxel

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Abstract
Telomeres are repetitive (TTAGGG)n DNA sequences found at the end of chromosomes that protect the ends from recombination, end to end fusions, and recognition as damaged DNA. Telomerase activity can be detected in 85% to 90% of human tumors, which stabilizes telomeres to prevent apoptosis or cellular senescence. Previous reports showed the efficacy of the novel telomerase template antagonist, GRN163L, as a potential anticancer agent. The objective of the present study was to elucidate the molecular effects of GRN163L in MDA-MB-231 breast cancer cells and to determine whether GRN163L could be used in mechanism-based combination therapy for breast cancer. We observed that GRN163L reduced MDA-MB-231 growth rates without a significant effect on breast cancer cell viability within the first 14 days in vitro. In addition, GRN163L altered cell morphology, actin filament organization, and focal adhesion formation in MDA-MB-231 cells. Importantly, the cellular response to GRN163L significantly augmented the effects of the microtubule stabilizer paclitaxel in MDA-MB-231 breast cancer cell growth in vitro and in vivo compared with paclitaxel alone or a mismatch control oligonucleotide plus paclitaxel. Furthermore, in vitro MDA-MB-231 invasive potential was significantly inhibited with GRN163L and paclitaxel. These data support a rationale for potentially combining GRN163L with paclitaxel for the treatment of breast cancer in the clinical setting. [Mol Cancer Ther 2009;8(7):2027–35]

Introduction
Breast cancer is one of the most common malignancies among women. Typically after surgical removal of the tumor mass, breast cancer patients are treated with radiation and/or chemotherapeutic drugs that lack selectivity, and resistance often develops quickly. Additionally, ~20% of women with a history of early breast cancer will develop metastatic disease. Metastatic breast cancer, although treatable, is largely incurable with a low number of patients achieving long-term survival after standard chemotherapy (1). Because metastatic tumors are responsible for ~90% of cancer-related deaths, the development of new treatment regimens that reduce metastasis is highly important in cancer therapy. Anticancer therapies aim to prolong survival, control symptoms, and maintain the quality of life for breast cancer patients (2, 3).

Telomerase activity can be detected in 85% to 90% of human tumors, but not in the majority of normal somatic cells, making telomerase an attractive target for cancer therapy (4). Telomerase inhibition results in the erosion of telomeric DNA, genomic instability, and eventually to apoptosis or cellular senescence (5–7). Previous in vitro studies have shown that the telomerase template antagonist GRN163L effectively inhibits the tumorigenicity of breast cancer cells within 2 weeks. In addition, GRN163L reduces human breast tumor volume and metastases in vivo within 30 days of the treatment with GRN163L (8). This reduction in metastases may be explained by an inhibition of migration, adherence, or growth at distant sites by GRN163L (8, 9). Previous reports have also shown that inhibiting telomerase activity alone or in combination with current therapeutic techniques can inhibit tumor cell growth in vitro without significant adverse effects on normal cells (7, 8, 10–12).

Microtubules are the major components of the cytoskeleton. Microtubules are vital for the maintenance of cell shape, signaling, proliferation, and migration, making them an important target for anticancer drugs (13, 14). Paclitaxel is one of the most extensively used anticancer agents, with clinical efficacy in a wide range of cancers (3, 15). Paclitaxel acts to stabilize microtubules, thus inhibiting cell proliferation at the metaphase/anaphase boundary (16–18). However, the success of paclitaxel in the clinical setting can often be tempered by drug resistance, which severely limits the effectiveness of chemotherapy. Although the exact mechanisms remain unclear, this phenomenon is considered to be mediated by

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Synergism between Telomerase Antagonists and Paclitaxel

The overall goal of the present study was to determine the cellular response to GRN163L in MDA-MB-231 breast cancer cells to understand how this compound may be used to augment breast cancer therapy. We observed that GRN163L induced a rapid change in cellular architecture, leading to the hypothesis that GRN163L can augment the effects of the microtubule stabilizer paclitaxel in reducing the cell growth of breast cancer cells. The combined treatment of nmol/L/sub-nmol/L concentrations of GRN163L and paclitaxel resulted in a significant synergistic combination index (CI) in reducing the cellular proliferation and invasive potential of MDA-MB-231 breast cancer cells. Furthermore, this reduction in MDA-MB-231 breast cancer cell growth by combination treatment suggests a potential use for GRN163L in adjuvant therapy for breast cancer.

Materials and Methods

Cell Culture and Reagents
MDA-MB-231 breast cancer cells, MDA-087 human mammary stromal cells, and BJ foreskin fibroblasts were cultured in DMEM media containing 10% cosmic calf serum (HyClone). Nontumorigenic MCF10A breast epithelial cells were cultured in supplemented MEGM (Cascade Biologicals). The lipid-modified telomerase template antagonist GRN163L and its mismatch control oligonucleotide were prepared as previously described (6). Paclitaxel was purchased from Sigma and dissolved as a 10 mmol/L stock in DMSO.

Telomerase Activity Assay
Telomerase activity was measured using the PCR-based Telomeric Repeat Amplification Protocol as previously described (6, 20).

Cell Growth and Viability Assays
To determine the effect of GRN163L on cell growth and viability, 5 × 10⁴ cells were plated in triplicate with 2.5 μmol/L GRN163L, mismatch control oligonucleotide, or media only (untreated) on 12-well dishes. Cells were collected weekly and counted via a Beckman Coulter Counter. The trypan blue exclusion assay was used to determine the combined effect of multiple drug treatment via the Chou and Talalay method (21). A CI value of <1.0 denotes synergistic interaction (more than additive), a CI of 1.0 shows summation (additive), and a CI of >1.0 indicates antagonistic interaction (less than additive).

Colony Formation Assays
MDA-MB-231 cells were treated with 40 to 80 nmol/L GRN163L/mismatch control oligonucleotides, media only, or combination of 40 nmol/L GRN163L plus 4 nmol/L paclitaxel for 5 d before collection. Cells (2 × 10⁵) from each treatment group were plated in triplicate onto 6-well dishes and colonies were allowed to grow for 10 d without changing the media or adding any drugs before staining with 20% Giemsa. Colonies with >50 cells were counted independently by two individuals and averaged. Plating efficiency was calculated by dividing the average number of colonies by the number of cells initially plated.

Xenograft mice studies
Athymic nude mice (nu/nu; Harlan Sprague-Dawley, Inc.) were maintained in pathogen-free conditions within the Laboratory Animal Resources Center at the Indiana University School of Medicine according to approved institutional protocols. MDA-MB-231 breast cancer cells (1 × 10⁶) were injected into the left and right mammary fat pads of 6- to 8-wk-old mice. Following a recovery period of 2 d, mice began receiving treatment of 30 mg/kg GRN163L (n = 15) or a PBS solvent control (n = 14) thrice weekly i.p., or 15 mg/kg paclitaxel (n = 15) once weekly via i.p., alone or in combination with 30 mg/kg GRN163L (n = 14). Average weights of animals were similar among treatment groups. Tumor volume was calculated as (length × width²)/2 (in millimeters). Mixed-effects statistical model was fitted to

Western Immunoblotting
Cells were harvested after treatment and lysed in buffer containing 1M Tris (pH 7.0), 2% SDS, and 5% sucrose. Protein concentration was quantified using the Pierce BCA Protein Assay according to manufacturer’s instructions. Fifty-microgram protein lysate was subjected to SDS-PAGE followed by transfer to polyvinylidene difluoride membrane. Blocking and antibody incubation were done in 5% milk in PBS containing 0.05% Tween 20. Membranes were exposed to X-ray film using the ECL Western Blotting Substrate (Pierce). Antibodies used in this study included the following: anti-β-tubulin and anti-γ-tubulin (Sigma), anti-Pyk2 and anti-FAK (Upstate Cell Signaling Solutions), and glyceraldehyde-3-phosphate dehydrogenase (Chemicon International). Densitometry levels for each blot were determined using glyceraldehyde-3-phosphate dehydrogenase as a loading control; relative intensity levels for each sample were plotted and normalized to untreated samples.

Colony Growth and Viability Assays to Determine the CI
Cells (5 × 10⁴) were plated in triplicate with 1:2 serial dilutions of GRN163L (0.005–1.25 μmol/L) or paclitaxel (0.5–12.5 nmol/L) in culture media as monotherapy, or combined in which the two agents were at a 1:10 ratio of each other (paclitaxel/GRN163L). Cells were collected after 5 d to obtain the number of viable cells as measured by the trypan blue exclusion assay. CalcuSyn software (Biosoft) was used to determine the combined effect of multiple drug treatment via the Chou and Talalay method (21). A CI value of <1.0 denotes synergistic interaction (more than additive), a CI of 1.0 shows summation (additive), and a CI of >1.0 indicates antagonistic interaction (less than additive).

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the data. Fixed effects include group, a linear and a quadratic component of day, and the interaction between group and the linear/quadratic component. Random effects include mouse, tumor within mouse, slope of day within mouse, and slope of day within tumor. The analysis was conducted by SAS 9.1.

Cell Invasion Assay

Matrigel invasion assays were done using the Cell Invasion Assay kit from Chemicon International per manufacturer’s protocol. MDA-MB-231 cells were treated with 0.156 to 0.3125 μmol/L GRN163L or mismatch oligonucleotides, or 16 to 31 nmol/L paclitaxel for 5 d. Parallel studies were done in combination with paclitaxel (16–31 nmol/L), in which the two agents were at a 1:10 ratio of each other. After 5 d of treatment, cells were collected and the invasive potential was measured. Normal human BJ fibroblasts served as negative controls for the assay. Statistical analysis was done using a Student’s t test (Microsoft Excel).

Results

Treatment With GRN163L Alters the Morphology of Breast Cancer Cells

To test for the cellular responses to GRN163L, MDA-MB-231 breast cancer cells were treated with 19.5 nmol/L to 2.5 μmol/L GRN163L or 10 μmol/L mismatch control oligonucleotides, and morphology was monitored daily. When GRN163L was given to the cells at the same time as plating, the morphology of MDA-MB-231 cells was altered, resulting in a rounding-up effect on adherent cells as well the extension of filipodia-like structures (Fig. 1). This morphologic effect by GRN163L was not observed with doses lower than 0.3125 μmol/L. In contrast, normal mammary epithelial cells did not show a morphologic response to treatment with GRN163L, suggesting that this response was cancer cell specific. Interestingly, MDA087 immortalized breast fibroblasts, which use the alternative lengthening of telomeres pathway and do not contain telomerase activity, also showed a

Figure 1. GRN163L induces morphologic changes in MDA-MB-231 breast cancer cells, MDA-MB-231 breast cancer cells, MDA087 telomerase-negative, immortalized breast fibroblasts, and nontumorigenic MCF10A cells were treated with 2.5 μmol/L GRN163L or mismatch (MM) control oligonucleotide for 72 h. Arrows, rounded cells; arrowheads, filipodia-like structures (×200).
A morphologic response to treatment with GRN163L similar to that observed in MDA-MB-231 cells. This finding indicates that the effect of this telomerase template antagonist may be independent of telomerase inhibition. The mismatch control oligonucleotide did not have an effect on cell morphology. This differential response may be due to the triplet guanine sequence unique to GRN163L, which is not contained in the mismatch control oligonucleotide. Additionally, we observed a cell type–specific change in morphology in response to GRN163L (Supplementary Table S1; Supplementary Fig. S1). For example, luminal breast cancer cell lines displayed complete loss of adherence to culture substrates yet displayed cohesion, or adhered to each other in cell aggregates. The effects of GRN163L on cell adhesion and architecture was reversible with the addition of extracellular matrix proteins to cell culture dishes.

**GRN163L Significantly Inhibits Cell Proliferation in MDA-MB-231 Cells**

To confirm GRN163L as an effective telomerase inhibitor during the course of these studies, MDA-MB-231 breast cancer cells were treated with 2.5 μmol/L GRN163L or mismatch control oligonucleotide (MM) for 6 days. As shown in Fig. 2A, GRN163L effectively inhibited telomerase activity in MDA-MB-231 cells through 6 days after a single treatment. The mismatch control oligonucleotide had no significant effect on telomerase activity. As shown in Fig. 2B, treatment with GRN163L at the same time as platting dramatically inhibited cell growth. Treatment with mismatch control oligonucleotide had no effect on cell growth, indicating that the effects on cell growth by GRN163L are not related to the use of lipidated oligonucleotides. The total population doublings per week were calculated to determine the rate of growth inhibition. As shown in Fig. 2C, the average time for one population doubling increased from 1.5 days in untreated cells, to 4.25 days in cells treated with GRN163L (P < 0.001). As expected, the mismatch control oligonucleotide had no effect on population doubling rate compared with untreated cells (P = 0.22). Treatment with GRN163L did not have a significant effect on normal human mammary epithelial cells or BJ fibroblasts, as previously reported (data not shown; refs. 7, 8).

One possible explanation for the morphologic changes, reduction in cell growth, and longer population doubling time in GRN163L–treated MDA-MB-231 cells is the induction of cell death, particularly apoptosis. Treatment with GRN163L did not induce apoptotic cell death in MDA-MB-231 cells within 72 hours (Supplementary Fig. S2). Alternatively to cell death, the marked decrease in MDA-MB-231 cell growth may be due to a reduction in cell viability. MDA-MB-231 cells were treated with 2.5 μmol/L GRN163L or mismatch oligonucleotides, and collected and counted via the trypan blue exclusion assay daily for 14 days. Although there was a decrease in cell number, the remaining cells did not show significantly decreased viability (Supplementary Fig. S2). These results taken together indicate that the growth inhibition seen in MDA-MB-231 breast cancer cells for the first 2 weeks of treatment with GRN163L is independent of a reduction in viability or an induction of apoptosis.

**GRN163L Disrupts Focal Adhesion Formation and Actin Filament Organization**

The altered morphology and adhesion capacity observed in response to treatment with GRN163L suggests a change in the cell membrane. To test the effect of GRN163L on the cell structure, actin filaments and focal adhesions were examined to determine whether this telomerase template...
antagonist can disrupt structural organization of breast cancer cells. As shown in Fig. 3, untreated cells and mismatch control–treated cells displayed well-organized actin filaments (phalloidin staining, in red) in long, parallel projections equally distributed throughout the cell (22). However, in GRN163L–treated cells, the actin fiber organization was observed primarily along the edges of the cell rather than in filaments throughout the cell. This dense accumulation around the perimeter of the cell indicates a reduction in stress fiber contractility (22). Furthermore, under normal physiologic conditions, cells form focal adhesions in multiple places along the cell membrane, primarily located at the ends of actin filaments (22). As shown in Fig. 3, untreated and mismatch control treated cells showed this pattern of focal adhesion formation (vinculin staining, in green). However, GRN163L–treated cells displayed punctate staining concentrated around the nuclei of the cells with little staining throughout the remaining cell membrane. This disruption of regular adhesion proteins/elements could help to explain the rounding-up/detachment or disruption of cell spreading effects observed in breast cancer cells in response to GRN163L.

**GRN163L Alters the Expression of Proteins Involved in Cellular Structure and Function**

The morphologic and structural changes induced by GRN163L indicate that the expression of proteins involved in cellular attachment and intracellular organization may be altered. The protein levels of β- and γ-tubulin were measured to determine changes in two of the main components of microtubules. Additionally, expression of FAK and Pyk2 were measured, as these proteins play a major role in microtubule organization, cell spreading, growth, and proliferation (23–27). As shown in Fig. 4, the expression of Pyk2 increased in a cyclical fashion after 12- and 72-hour treatment.
with GRN163L, whereas the other protein levels did not change. Although the exact role of Pyk2 in cancer remains unclear, it has been shown that Pyk2 is involved in maintaining cell structure and inducing proliferation (28, 29). Speculatively, the up-regulation of Pyk2 may be induced to aid the cell in reshaping its actin filaments to allow the cell to continue replicating in a normal fashion.

In a separate attempt to understand the mechanisms underlying these alterations in focal adhesion and actin organization, global proteomics and Ingenuity Pathway Analyses were done on MDA-MB-231 breast cancer cells treated with GRN163L compared with mismatch control and untreated cells (Supplementary Table S2). Although the analyses suggested that there were not alterations in a singular pathway that explain the phenotypic change induced by GRN163L, there were changes in pathways involved in structural functions as reported by others (30). These results support the hypothesis that the phenotypic change can be explained by alterations in a structural function of the cells.

**GRN163L Synergistically Enhances the Effects of Paclitaxel on Inhibiting Cell Proliferation in MDA-MB-231 Breast Cancer Cells**

The disruption of actin structures in MDA-MB-231 cells by GRN163L suggests that GRN163L may work synergistically with paclitaxel to inhibit breast cancer cell growth. To test the effect of combination therapy on cell growth, cells were plated with 0.005 to 1.25 μmol/L GRN163L or mismatch oligonucleotides for the depicted times and Western immunoblotting for the indicated adhesion and morphology proteins was done (A). Densitometry levels were determined by comparing intensity of FAK, Pyk2, γ-tubulin, and β-tubulin to glyceraldehyde-3-phosphate dehydrogenase and relative intensity levels for each sample were plotted and normalized to untreated samples (B). Untreated and mismatch control-treated cells were analyzed after 72 h. Results are representative of at least two independent experiments (±SE).

As shown in Fig. 5B, GRN163L acted synergistically (CI, <1.0) with paclitaxel to inhibit growth of MDA-MB-231 cells. To address the effects of low dose combination treatment on plating efficiency/survival capacity, MDA-MB-231 cells were treated with 40 nmol/L GRN163L in combination with 4 nmol/L paclitaxel for 5 days before collection and replating at low density. After 10 days, colonies were counted and compared with controls (media only- and mismatch oligonucleotide-treated cells). GRN163L in combination with paclitaxel at low nmol/L doses significantly reduced the plating efficiency of MDA-MB-231 breast cancer cells compared with either drug alone and mismatch controls (Fig. 5C).

**The Combination of GRN163L and Paclitaxel Significantly Inhibit Tumor Growth In vivo and Cell Invasion In vitro**

To test that the effects of combining GRN163L and paclitaxel can be seen in vivo as well as in vitro, the combination treatment was examined on the growth of orthotopic MDA-MB-231 tumors. As shown in Fig. 6A, the combination of GRN163L plus paclitaxel significantly decreased tumor volume progression over PBS control–treated animals.
(difference in quadratic effect of day: $P < 0.0001$) and displayed reduced tumor growth compared with either drug alone (difference in quadratic effect of day: $P < 0.0005$ for paclitaxel and $P < 0.005$ for GRN163L).

In addition to testing the effects of combination therapy on primary tumor growth, in vitro invasion assays were done to determine the metastatic potential of MDA-MB-231 cells after treatment with GRN163L (or MM) and paclitaxel. As shown in Fig. 6B, GRN163L and paclitaxel alone inhibited cell invasion compared with untreated control cells ($P = 0.046$ and 0.006, respectively), whereas the mismatch oligonucleotide had no effect ($P = 0.47$). The combination of GRN163L and paclitaxel significantly decreased invasion compared with either drug alone ($P = 0.002$ versus GRN163L alone; $P = 0.017$ versus paclitaxel alone). The combination of mismatch oligonucleotide and paclitaxel had no significant effect on the inhibition of invasion induced by paclitaxel alone ($P = 0.23$). Invasion was completely inhibited with 31 nmol/L paclitaxel, and the addition of GRN163L or mismatch oligonucleotides had no further effect (data not shown).

**Discussion**

GRN163L is an effective telomerase inhibitor; however, the mechanisms underlying all cellular responses are not completely understood. In addition to its effects as a telomerase inhibitor, GRN163L likely disrupts focal adhesion formation and actin filament organization, thus impairing cancer cell adhesion and morphology. This response significantly delays the growth of MDA-MB-231 cells without a reduction in cell viability or the induction of apoptosis in the short term. Importantly, this effect of GRN163L-sensitized breast cancer cells to paclitaxel, an important chemotherapeutic agent used in the treatment of breast cancer. Although the effect of combination therapy on the rate or incidence of metastasis was not tested in vivo, our in vitro invasion assay results suggest that nanomolar amounts of GRN163L plus paclitaxel significantly reduces the invasive potential of MDA-MB-231 cells.

Under normal growth conditions, cells require attachment to a solid surface before they can grow. The formation of focal adhesions helps in the organization of the cytoskeleton and activates intracellular signaling pathways, including signals for proliferation, survival, and migration (23–25). Migration occurs through a continuing cycle of disrupting focal adhesions, extension of membrane protrusions at the advancing edge and contraction of the trailing ends of the cell, and the formation of new focal adhesions (31). The response to GRN163L may disrupt this process through its triplet-G motif (9), thus inhibiting the migratory potential of cancer cells and reducing metastatic disease. In addition to the effect on cell growth, we observed that GRN163L has an effect on cell architecture, resulting in altered actin filament organization and focal adhesion formation. A reduction in migration after treatment with GRN163L has been shown in lung cancer cells (9), although the mechanisms underlying this effect remain to be elucidated. The results shown in Figs. 5 and 6 indicate that the combination of GRN163L and paclitaxel, a microtubule stabilizer and mitotic inhibitor, allows for an effective growth inhibition at low nanomolar doses of both drugs.
Telomerase inhibition has often shown a long lag phase before critical telomere shortening and cell growth inhibition, which may effect the fitness of normal cells that use telomerase for survival (32–35). The research presented here, however, shows a rapid response to GRN163L in cancer cells, with significant effects on MDA-MB-231 breast cancer cell growth and morphology within 5 days. Although the response to GRN163L did not induce rapid senescence, apoptotic cell death, or decreased cell viability over 2 weeks, this does not rule out the fact that loss of a sentinel, critically short telomere(s) may affect cell survival and trigger alternate forms of growth arrest or cell death, such as autophagy, as shown by the recent report on telomere-targeting, G-rich oligonucleotides (36). It is also possible that early-stage telomere dysfunction induced by GRN163L induces mitotic catastrophe, which would result in failure of cell cycle arrest before or at mitosis due to abnormal spindle formation (37, 38). One concern with the use of telomerase inhibitors in the clinical setting is the effect on stem and progenitor cells found in highly replicative tissues. The rapid response to GRN163L and paclitaxel shown here indicates that it is reasonable to expect that quiescent stem cells will not be significantly affected by short courses of telomerase inhibition therapy (39–41). The effect of GRN163L on cell adhesion and morphology is reversible (data not shown; ref. 9), with the return of the pretreatment telomerase activity levels, telomere elongation and maintenance, and normal cell architecture. Taking advantage of this rapid response by treating cells with GRN163L and paclitaxel indicates that shorter exposure times may be possible to reduce secondary effects of treatment with other therapeutics (IR, chemotherapy, or molecular-targeted therapy).

Taken together with previously published reports demonstrating that GRN163L inhibits primary breast cancer tumor growth in vivo (8, 12), these results provide a rationale for the continued investigation of this drug in breast cancer preclinical/clinical trials. Therefore, treatment with telomerase template antagonists could have beneficial outcomes not only on primary tumor growth, due to the effects of telomere shortening, but also in therapeutic regimens for sensitization to therapy and reduction in metastasis.

**Disclosure of Potential Conflicts of Interest**

S.M. Gryaznov: employee, Geron Corporation; inventor, GRN163L. B-S. Herbert, research support, Geron Corporation.
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