# INVESTIGATIONS OF THE TELOMERASE TEMPLATE ANTAGONIST GRN163L AND IMPLICATIONS FOR AUGMENTING BREAST CANCER THERAPY

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ABSTRACT

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INVESTIGATIONS OF THE TELOMERASE TEMPLATE ANTAGONIST GRN163L AND IMPLICATIONS FOR AUGMENTING BREAST CANCER THERAPY

Breast cancer is the second most common cancer among women in the US after skin cancer. While early detection and improved therapy has led to an overall decline in breast cancer mortality, metastatic disease remains largely incurable, indicating a need for improved therapeutic options for patients. 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. The enzyme telomerase acts to stabilize short telomeres, preventing apoptosis or senescence due to genomic instability. Telomerase is active in 85-90% of cancers, and inactive in most normal cells, making telomerase an attractive target for cancer therapy. Use of the telomerase-specific, lipidated oligonucleotide GRN163L can antagonize telomerase activity and telomere maintenance in cancer cells by preventing telomerase from binding to telomeres. GRN163L has been shown by our laboratory to inhibit breast cancer cell growth and metastasis in animal models. However, the mechanisms of cancer cell growth and metastatic inhibition via GRN163L are not completely understood. The overall goal of this research project was to further elucidate the role of telomerase in breast cancer cell survival by: 1) determining the effects of combining telomere dysfunction induced by GRN163L with a DNA damage inducer (irradiation); 2) elucidating the mechanisms underlying the cellular response to GRN163L and the effect of combination therapy with

the mitotic inhibitor paclitaxel; and 3) testing the hypothesis that a telomerase inhibitor can augment the effects of trastuzumab in breast cancer cells with HER2 amplification. Results support the central hypothesis that the telomere dysfunction, structural and proliferative changes in breast cancer cells induced by GRN163L can synergize with irradiation, paclitaxel, and trastuzumab to inhibit the tumorigenicity of breast cancer cells both *in vitro* and *in vivo*. Furthermore, GRN163L can restore sensitivity of therapeutically resistant breast cancer cells to trastuzumab. These results provide insight into the role of telomerase in cancer cell growth. Additionally, implications of this research support GRN163L as an important part of therapeutic regimens for primary tumors, recurrence, and metastatic disease.

Brittney-Shea Herbert, Ph.D., Chair

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# ABBREVIATIONS

| | |
|---|---|
| ALT | Alternative lengthening of telomeres |
| AS-ODNs | Antisense oligonucleotides |
| AU | Arbitrary units |
| AZT | Azidothymidine |
| BCA | Bicinchoninic acid |
| BFB | Breakage fusion breakage |
| BIBR1532 | 2-[(E)-3-naphthalen-2-yl-but-2-enoylamino]-benzoic acid |
| bp | Base pairs |
| BRCA1 | Breast cancer 1, early onset |
| BrdU | 5-bromo-2-deoxyuridine |
| BSA | Bovine serum albumin |
| CI | Combination index |
| CLL | Chronic lymphocytic leukemia |
| CSC | Cancer stem cell |
| CTC | Circulating tumor cell |
| DAPI | 4'-6-Diamidino-2-phenylindole |
| DCIS | Ductal carcinoma in situ |
| DKC | Dyskeratosis congenital |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DN-hTERT | Dominant negative hTERT |
| $EC_{50}$ | Effective concentration inhibiting 50% of target |

| | |
|---|---|
| ECL | Enhanced Chemiluminescence |
| ECM | Extracellular matrix |
| EGFR | Epidermal growth factor receptor |
| ELISA | Enzyme linked immunosorbent assay |
| ER | Estrogen receptor |
| FAK | Focal adhesion kinase |
| FISH | Fluorescence in situ hybridization |
| HER2 | Human epidermal growth factor receptor 2 |
| HI | Heat inactivated |
| HMEC | Human mammary epithelial cell |
| HR | Homologous recombination |
| hTERT | catalytic subunit of human telomerase |
| hTR | RNA template component of human telomerase |
| IC | Internal control |
| IGF-I | Insulin-like growth factor I |
| IP | Intraperitoneal |
| IPF | Idiopathic pulmonary fibrosis |
| IR | Irradiation |
| Kb | Kilobase |
| LB | Lysis Buffer |
| LC3 | Microtubule-associated protein-1 light chain-3 |
| MAPK | Mitogen-activated protein kinase |
| miRNA | Micro ribonucleic acid |

| | |
|---|---|
| mM | Millimolar |
| MM | Mismatch control oligonucleotide |
| MTD | Maximum tolerated dose |
| mTR | Mouse RNA component of telomerase |
| mTR-/- | Mouse model with mTR knocked out |
| nM | Nanomolar |
| NSCLC | Non-small cell lung cancer |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PD | Population doubling |
| PE | Plating efficiency |
| PI3K | Phosphoinositide 3-kinase |
| POT1 | Protection of telomeres-1 |
| PR | Progesterone receptor |
| PVDF | polyvinylidene fluoride |
| Rb | Retinoblastoma |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| RTA | Relative telomerase activity |
| SD | Standard deviation |
| SDS | Sodium Dodecyl Sulfate |
| SE | Standard error |

| | |
|---|---|
| shRNA | Short hairpin ribonucleic acid |
| siRNA | Small interfering ribonucleic acid |
| STELA | Single telomere length analysis protocol |
| SV40 | Simian vacuolating virus 40 or Simian virus 40 |
| TANK1/2 | Tankyrase 1/2 |
| TRAP | Telomere repeat amplification protocol |
| TRF | Telomere restriction fragment |
| TRF1/TRF2 | Telomere repeat-binding factor 1/2 |
| μg | Microgram |
| μM | Micromolar |
| UT | Untreated |
| VEGF | Vascular endothelial growth factor |

# Chapter One

## Introduction and Literature Review

Breast cancer is the second most common cancer among women in the US after skin cancer. While early detection and improved therapy has led to an overall decline in breast cancer mortality, metastatic disease remains largely incurable, indicating a need for improved therapeutic options for patients. Breast cancer patients are often treated with therapeutic regimens that can be cytotoxic and result in harmful secondary effects. Treatment methods include the use of radiation, chemotherapy, and molecular targeted therapy; however resistance often develops quickly in more advanced breast cancer, resulting in tumor recurrence and/or metastatic disease (Polyak 2007). There are six major hallmarks of cancer, with limitless replicative potential (cellular immortality) being one of these six acquired capabilities (Hanahan and Weinberg 2000). Cells are believed to have a counting mechanism that regulates cellular lifespan, which is defined by repetitive DNA sequences called telomeres found at the end of chromosomes. Telomeres shorten with each round of cell division due to the end replication problem (Hanahan and Weinberg 2000). In cancer, however, telomere length is maintained, resulting in the immortal phenotype. The research presented within this dissertation will focus on targeting this hallmark in breast cancer cells and its implications in breast cancer therapy in combination with three other conventional cancer therapies. Using these combination therapies is likely to improve treatment regimens, as targeting multiple pathways involved in cancer progression is likely to increase patient survival over single drug therapy.

**Telomeres and their role in replicative cellular aging and cancer**

Telomeres are specialized structures at the end of chromosomes consisting of 5-12 kb of repeated $(TTAGGG)_n$ sequences in humans and are essential for cell survival (Figure 1.1). Telomeres act to protect the ends of chromosomes from recombination, end-to-end fusions, and recognition as damaged DNA. Telomeres contain a short, single stranded 3'-overhang of approximately 3 kb that forms a loop structure (t-loop) by inserting into double stranded telomeric DNA. This loop structure protects the 3' end of the DNA from degradation or unregulated elongation by telomerase (discussed below). Telomeres are lost from the lagging strand of DNA with each cell cycle in normal cells and shorten until one or more become critically short, or dysfunctional, triggering a cell growth arrest, termed replicative senescence (Figure 1.1; Kim et al. 2002, Blackburn 2001, Cong et al. 2002, Hahn 2003).

Telomere structure and stability is maintained through many telomere associated proteins (Hahn 2003, Kim et al. 2002). Two structurally related DNA binding proteins, TRF1 and TRF2, bind double stranded telomeric DNA and play an important role in maintaining telomere length. Overexpression of wild-type TRF1 resulted in telomere shortening, whereas mutant TRF1 induces telomere elongation (van Steensel and de Lange 1997, van Steensel et al. 1998). TRF2 has been shown to assist in the formation of the t-loop and maintain genomic stability. Expression of dominant negative TRF2 has been shown to result in end-to-end chromosome fusions and senescence (Hahn 2003, Bryan and Cech 1999). A third DNA binding protein, POT1, binds to single stranded telomeric DNA and helps maintain the t-loop structure (Kim et al. 2002, Hahn 2003, Bryan and Cech 1999). Several telomere associate proteins have also been shown to be a

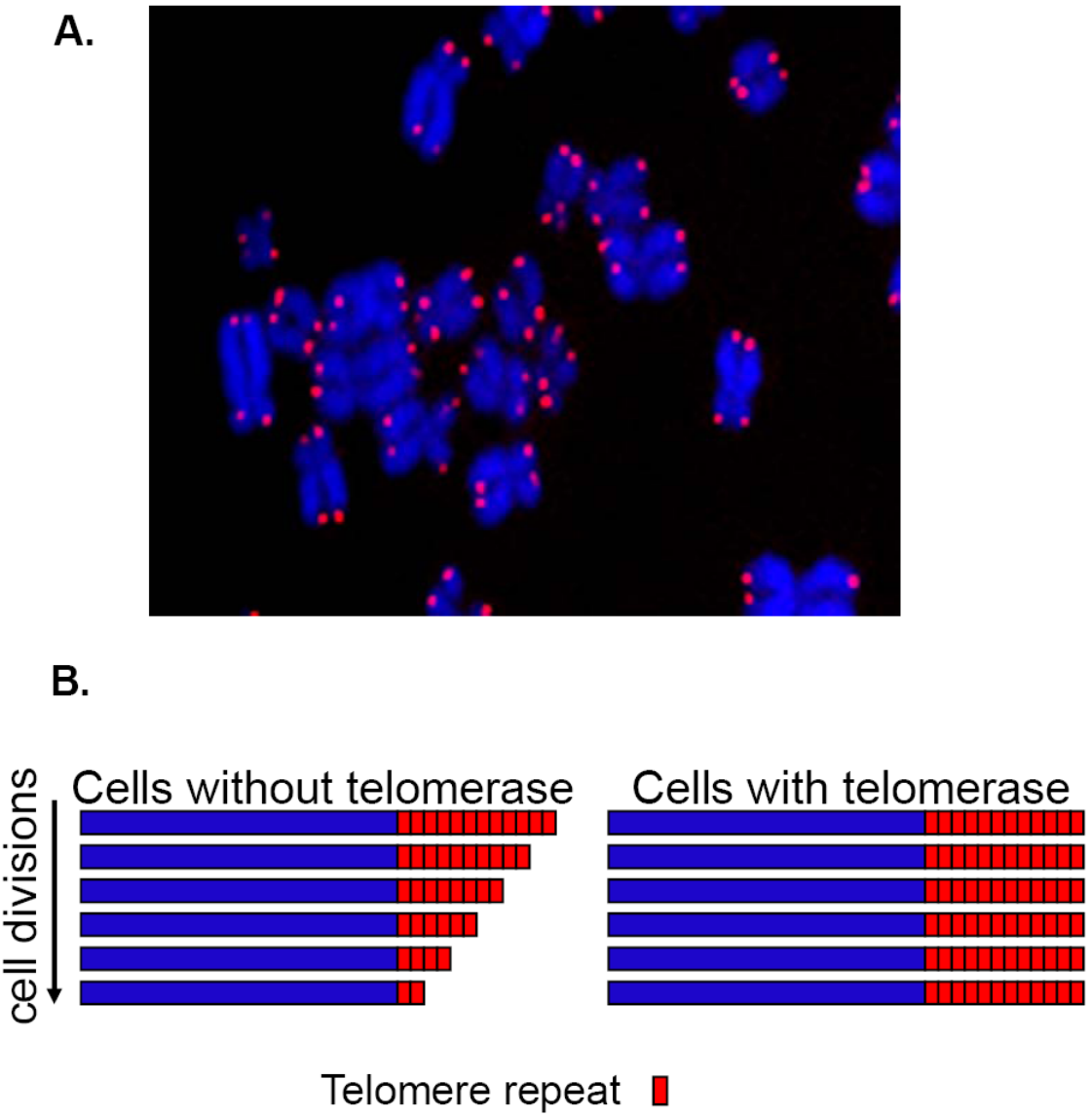


**Figure 1.1. Telomeres act as protective caps at the end of chromosomes.** (A) Telomeres are located at the ends of chromosomes. In this fluorescence in situ hybridization (FISH) figure, the chromosomes (DNA) are stained with DAPI (blue). Telomeres are depicted as red signals at the ends of each chromosome arm. Figure courtesy of the Herbert laboratory (unpublished data). (B) Telomeres shorten during each round of cell division. In cells with telomerase activity, telomere length is maintained, providing cells with the ability to proliferate indefinitely.

part of the DNA damage response pathway (Hahn 2003, Kim et al. 2002). This includes two DNA repair enzymes, TANK1 and TANK2, which seem to help regulate the activity of TRF1. The Ku complex also localizes to the telomeres and is involved in the processing of the single-strand overhangs, as well as protecting the telomere from degradation and recombination (Kim et al. 2002, Hahn 2003, Bryan and Cech 1999).

DNA is constantly bombarded by exogenous and endogenous damaging agents that can induce mutations, genomic instability, and ultimately lead to cancer development. In most cases, however, cells become senescent due to telomere dysfunction before they acquire enough mutations to become cancerous, indicating that growth arrest induced by short telomeres may be an intrinsic anti-cancer mechanism (Shay et al. 2001, Hahn 2003, Kim et al. 2002). Cell growth arrest and cell death are seen in response to dysfunctional telomeres, although this may be at least partially dependent on functional p53 and Rb pathways. In cells with mutant Rb, but functional p53, cell death is more likely to occur in response to critically short telomeres. If both pathways are inactive, however, cells can bypass senescence and proliferate until telomeres reach critically short lengths, at which time cells undergo crisis (Kim et al. 2002). Most cells will die at this stage; however, a few cells will be able to escape crisis and continue to proliferate uncontrollably. The ability of cells to escape this crisis stage is thought to be one of the major hallmarks of cancer development, leading to evasion of telomere-based growth limitations (Shay and Wright 2002, Hanahan and Weinberg 2000). Importantly for cancer development, cells that escape crisis are highly susceptible to end to end fusions, breakage fusion bridge (BFB) cycles, and genomic instability due to telomere

dysfunction, increasing the chances of activation of telomere maintenance pathways (i.e., telomerase reactivation).

**Telomerase and cancer**

One critical hallmark of cancer is the ability of cells to proliferate indefinitely or bypass senescence (Hanahan and Weinberg 2000). The most common method for cells to escape replicative senescence is the reactivation of telomerase to maintain telomeres. Human telomerase is a ribonucleoprotein complex consisting of a catalytic reverse transcriptase component (hTERT) and an RNA template (hTR) that act to elongate existing telomeres by synthesizing de novo telomeric repeats onto the 3'-overhang end using the hTR template as a template for extension (Figures 1.2 and 1.3; Shay et al. 2001, Hahn 2003, McEachern et al. 2000). In humans, telomerase is expressed during embryonic development but is repressed in most adult tissues. In cancer development, however, telomerase activity is reactivated through unknown mechanisms (Cong et al. 2002, McEachern et al. 2000, Kim et al. 2002). hTERT alone has been shown to be sufficient for cellular immortalization and allowing transformed cells to escape from crisis (Bodnar et al. 1998).

Importantly, telomerase reactivation can cooperate with the aberrant expression of oncogenes and tumor suppressor genes to induce neoplastic transformation of normal human epithelial cells (Cong et al. 2002). Interestingly, telomerase-proficient stem cells still exhibit telomere shortening, indicating that at least in some cases, telomerase activity is not sufficient to prevent telomere erosion and senescence (Kim et al. 2002). Despite the high percentage of cancer cells expressing telomerase activity, a small minority of

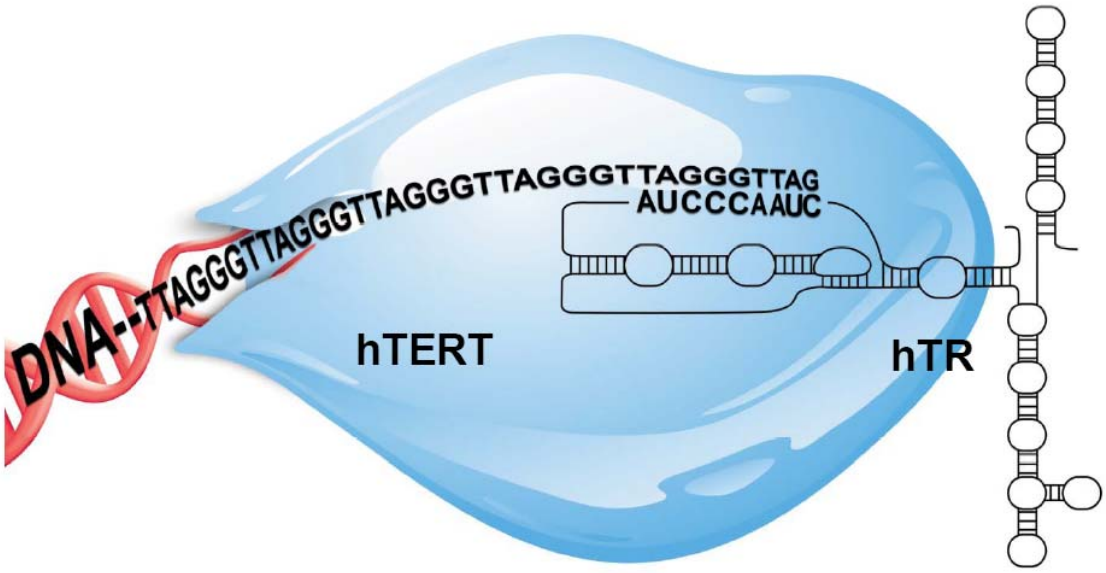


**Figure 1.2. The telomerase complex.** The telomerase complex consists of a catalytic protein subunit (hTERT) and an RNA template component (hTR). The intrinsic RNA template region recognizes and binds to telomeric DNA sequences (consisting of TTAGGG repeats, approximately 5-12 kb in length in humans) to maintain telomere length.

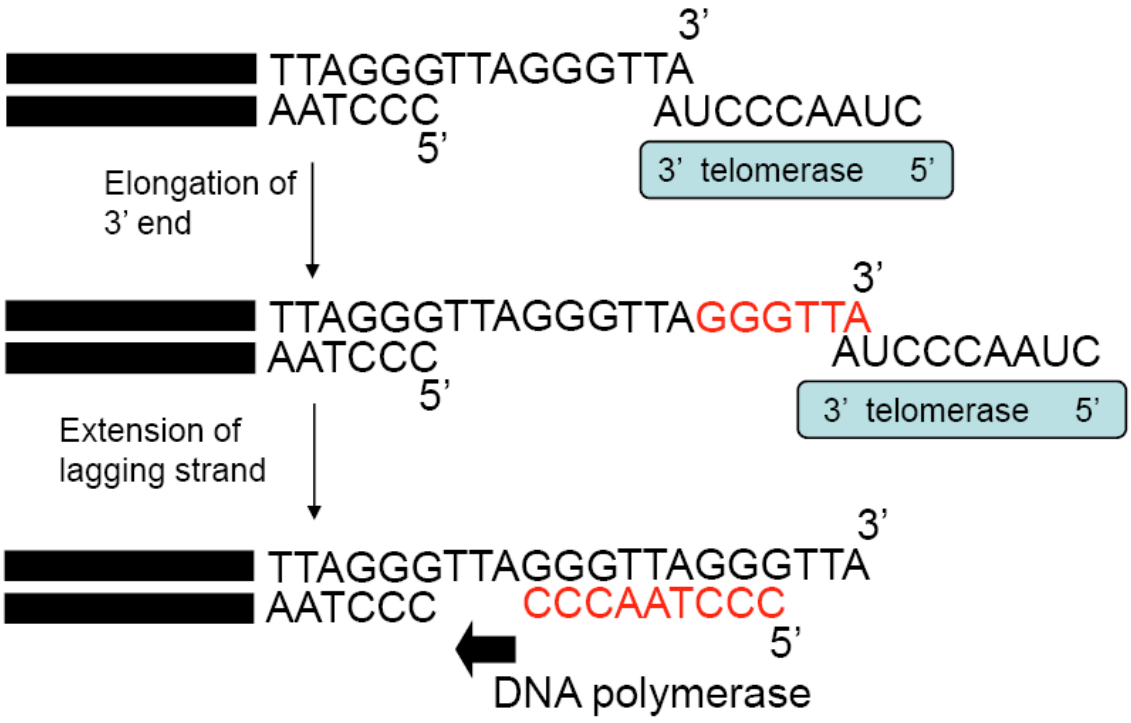


**Figure 1.3. Telomerase maintains telomere length.** The RNA template region of telomerase (hTR) recognizes and binds to telomeric DNA and extends the 3' end of the parental DNA strand via the catalytic activity of hTERT. DNA polymerase can then act on the 5' end of the lagging strand to maintain telomere integrity. Adapted from Alberts et al. *Molecular Biology of the Cell*, 2002.

cells maintain stable telomeres without the expression of hTERT or telomerase. This telomere stabilization is achieved through a second form of telomere maintenance termed alternative lengthening of telomeres (ALT), which is dependent on recombination. ALT-positive cells are mainly found in sarcomas and *in vitro* SV40-transformed fibroblasts. Interestingly, telomere maintenance through ALT and telomerase reactivation has been proposed to coexist, indicating that these mechanisms are not mutually exclusive (Hahn 2003).

The telomerase RNA component, hTR, is a member of a class of small nucleolar RNA molecules and is universally expressed in all tissues. Despite differences in the primary sequence of the hTR gene between species, the secondary structure is highly conserved, indicating the importance of the RNA structure in telomerase function (Cong et al. 2002). siRNAs targeting hTR have been shown to decrease telomerase activity and inhibit xenograft tumor formation without the shortening of the overall telomere population (Zimmermann and Martens 2007, Shammas et al. 2005). Additionally, depletion of wild-type hTR using a mutated template hTR (mt-hTR) results in the synthesis and incorporation of "mutant" telomeric DNA, which can subsequently affect the binding of protective telomere-associated proteins and telomere structure (Feng et al. 1995, Goldkorn and Blackburn 2006, Cerone et al. 2006b). Mt-hTR has also been shown to decrease cell viability and increase apoptosis independently of initial telomere length, as well as inhibit tumorigenesis in mice and increase the sensitivity of cancer cells to chemotherapeutic agents (Saretzki 2003, Kim et al. 2001, Cerone et al. 2006b, Goldkorn and Blackburn 2006). The catalytic subunit of telomerase, hTERT, is only found in cells that express telomerase activity, indicating that hTERT is the rate limiting component of

the telomerase enzyme (Cong et al. 2002, Hahn 2003). As with hTR, the sequence of hTERT is highly conserved between species. To support the importance of hTERT in telomere maintenance, it has been shown that introducing exogenous hTERT into primary cells results in the induction of telomere maintenance and escape from senescence. Conversely, introducing a single amino acid mutation into the primary hTERT sequence leads to telomere shortening and senescence (Cong et al. 2002).

Telomerase activity can be detected in 85-90% of human tumors, but not in most normal tissues, making telomerase an attractive target for cancer therapy (Kim et al. 1994). A correlation between increasing telomerase activity from early to late stage tumors has been observed (Kim et al. 2001, Watanabe et al. 2002). Furthermore, telomere lengths in ductal carcinoma in situ (DCIS) and invasive breast cancer are shorter than in normal surrounding tissue (Meeker et al. 2004). Maintenance of telomeres by telomerase is essential for the survival of tumor growth and for metastasis, allowing for stabilization of genomic instability triggered by short telomeres (Bryan and Cech 1999, Herbert et al. 2001, Meeker et al. 2004).

**Importance of telomere maintenance and telomerase in cell survival**

Telomere dysfunction is associated with diseases of aging, poor wound healing, and vascular disease (Kim et al. 2002). In addition to its role in cancer, telomerase plays a role in other human diseases as well. Mutations in either hTR or hTERT are associated with several diseases, including dyskeratosis congenita, bone marrow failure, and idiopathic pulmonary fibrosis (Garcia et al. 2007). Mutations in genes encoding hTERT and hTR alter the level of telomerase activity and inhibit the proliferative capacity of

cells in a range of tissues, as sufficient telomerase activity in somatic cells is critical for human health and viability (Collins and Mitchell 2002). Collectively, the involvement of telomerase in the development of these diseases indicates that telomerase may be far more important in regulating human growth and development than has previously been known.

As stated above, mutations in hTR and hTERT, or in the associated protein dyskerin, result in aplastic anemia, premature organ failure, and dyskeratosis congenita (DKC), which further implicates the essential role of telomerase in cell and organismal survival (Collins and Mitchell 2002, Hahn 2003). In normal cells, the protein dyskerin is a nuclear protein that is responsible for early steps in RNA regulation and is involved in the processing of hTR. Loss of association between dyskerin and telomerase leads to the loss of normal telomere maintenance (Hahn 2003). A number of different mutations in both hTR and TERT have been found in patients with bone marrow failure syndromes, leading to a reduction in telomere length. The lack of telomerase activity and short telomeres in bone marrow cells may be a marker of progenitor cell depletion in bone marrow failure syndromes. Idiopathic pulmonary fibrosis (IPF) is a progressive, fatal lung disease characterized by lung scarring and abnormal lung tissue function. The mutations resulting in IPF are different from those found in DKC, although DKC patients do often present with pulmonary fibrosis. While the mutations between these three conditions differ, there are underlying similarities between DKC, IPF, and bone marrow failure syndromes (Garcia et al. 2007).

The use of mouse models has become an informative method for understanding how telomeres and telomerase function in mammals. There are, however, significant

differences in telomere biology between mouse and human. Laboratory mouse telomeres are substantially longer than human telomeres, suggesting that the effects of telomerase inhibition may take longer than would be seen in a human. Additionally, telomerase is poorly repressed in mice, with expression found in most tissues (Hahn 2003). These findings led to the generation of mouse models that lack the RNA component of telomerase (mTR), which provided the *in vivo* proof that loss of one of the telomerase complex components resulted in lack of telomerase activity and telomere shortening (Blasco et al. 1997). Due to the length of murine telomeres, however, the mTR knockout mice did not demonstrate phenotypes associated with telomerase/telomere dysfunction until the sixth generation, when telomeres had significantly shortened. At this point, knockout mice displayed defects in normal cellular function in highly proliferative tissues, indicating that telomere shortening limits the proliferation of cells *in vivo*. When the mTR knockout was bred with mice harboring mutations in cell cycle checkpoint genes or with mice that were exposed to known chemical carcinogens, mice were less prone to develop cancer (Blasco et al. 1997, Hahn 2003). However, when combined with mutations in tumor suppressor genes such as p53, an increase in cancer rates was observed particularly epithelial cancers. The increase in cancer incidence was associated with an increase in genomic instability in these tumors (Hahn 2003). Taken together, this research provided insight into the importance of telomere and telomerase function in human disease, particularly epithelial cancers such as breast cancer.

**Breast cancer**

Breast cancer is the most frequently diagnosed cancer in women, with an estimated 180-200,000 new cases expected to be diagnosed in 2008 in the United States, and one million cases expected worldwide. About 40,000 women and 450 men will die from the disease in 2008. A large number of risk factors are associated with the development of breast cancer, including race, age at menses or menopause, age at first pregnancy, the use of birth control pills, and lifestyle choices (alcohol consumption, exercise, and diet). Women over the age of 55 or with close blood relatives with breast cancer have a higher risk of developing the disease themselves. Genetics also play a role in breast cancer risk, with approximately 5-10% of breast cancers thought to be due to inherited genetic mutations. Breast cancer cannot be prevented, although limiting the exposure to known risk factors may lower the chance of developing cancer (American Cancer Society, www.cancer.org). The high incidence of breast cancer emphasizes the lack of complete understanding about the cause of this disease and how to successfully prevent and cure development and recurrence (Hinestrosa et al. 2007).

The development of breast cancer involves progression through defined pathological and clinical stages, starting with ductal hyperproliferation, followed by subsequent evolution towards in situ and invasive carcinomas, and finally into metastatic disease (Polyak 2007). The biology of breast cancer development, however, remains poorly understood. There are many prognostic factors that indicate potential disease outcomes, including presence of metastases, histologic grade, expression of steroid and growth factor receptors, expression of oncogenes, and suppression of tumor suppressor genes. However, these factors alone only provide limited information about the biology

of the disease. The heterogeneity of breast tumors and the large number of factors involved in the development of tumors emphasizes the importance of conjointly studying multiple genetic alterations together. This has led to the classification of distinct subtypes based upon gene signatures. There are currently five distinct subtypes of breast cancer based on gene expression arrays: basal-like, luminal A, luminal B, HER2+, and normal breast-like. Understanding these differences is vital to developing targeted cancer-preventative and -therapeutic interventions (Sørlie et al. 2001, Sørlie et al. 2005, Polyak 2007). The research presented within this dissertation will focus upon the two subtypes with the shortest disease-free and overall survival times, the basal-like and HER2+ subtypes.

Basal-like breast cancers are often described as "triple negative" due to lack expression of estrogen receptor (ER), the progesterone receptor (PR), and amplification of the HER2 receptor. Because of this, patients with basal-like breast cancer face a poor prognosis and are not responsive to therapies targeting these receptors. In addition, basal-like cancers often have an elevated frequency of p53 mutations, high levels of genomic instability, and are highly invasive (Shakya et al. 2008). HER2 (human epidermal growth factor receptor 2) is a member of the ErbB/EGFR family of transmembrane receptor tyrosine kinases that plays a role in tumor cell growth, invasion, and metastasis (Slamon et al. 2001, Ross et al. 2004, Hudis 2007). HER2 amplification is found in approximately 10-20% of breast cancers and is associated with a more aggressive disease, greater likelihood of recurrence, poorer prognosis and decreased survival compared to women with HER2-negative breast cancer (Ross et al. 2004, Menendez et al. 2005, Sledge 2004).

In addition, HER2 overexpression is associated with resistance to chemo- and endocrine therapies (Menendez et al. 2005, Burstein 2005, Tan et al. 1997).

**Cancer therapy: current approaches and new advances**

Over the past twenty to thirty years, there have been significant advances in diagnosing and treating breast cancer. Early diagnosis remains one of the best factors for successfully treating breast cancer. Cancer prevention has also come under investigation, with much research aimed at determining who is at risk for cancer and how to prevent it from developing in these individuals. Approximately 20% of women with a history of early breast cancer will develop metastatic disease (Stevanovic et al. 2006) and metastatic tumors are responsible for approximately 90-100% of cancer related deaths. These numbers highlight the importance of research into understanding tumor progression and recurrence so that advanced breast cancer can be treated successfully. This understanding includes determining gene signatures of breast cancer subtypes and recognizing the response of each subtype to a certain type of therapy. Knowing how an individual tumor will respond to treatment will lead to the development of individualized therapeutic regimens, thus maximizing results while minimizing exposure time to toxic therapeutics. One of the most important areas of research is in understanding the response to therapy, and how resistance develops in a large number of cancers, in order to help prevent or overcome resistance (Polyak 2007).

Most women with breast cancer will undergo surgery to remove as much of the cancer as possible. Early stage breast cancer is largely curable. Typically, after surgical removal of the tumor mass, breast cancer patients are treated with radiation to induce

lethal DNA damage and cell death in remaining cancer cells. Additional therapy involves the use of chemotherapeutic drugs that are highly toxic and lack selectivity. Despite high cure rates in early stage disease, resistance to therapy often develops quickly in advanced disease. Current therapies are aimed to prolong survival, control symptoms, and maintain quality of life (Orlando et al. 2007, Barni and Mandalà 2005). Metastatic breast cancer is generally incurable with a low number of patients achieving long-term survival after standard chemotherapy (Welch et al. 2000, Stevanovic et al. 2006). Although there are a large number of agents available for the treatment of metastatic disease, overall survival has changed little during the last half century. The research presented herein will focus on three different types of cancer therapy; ionizing radiation, chemotherapy (specifically paclitaxel, a microtubule stabilizing drug), and targeted therapy using trastuzumab, a monoclonal antibody against the HER2 receptor. A more detailed introduction to each method of treatment will be given in the following chapters.

*Ionizing radiation*

Ionizing radiation (IR) is an important cancer therapy that utilizes high-energy rays to kill cancer cells by inducing lethal DNA damage and double-stranded breaks (Bernier et al. 2004). IR is often used in conjunction with either surgery or chemotherapy to shrink a tumor or inhibit re-growth. In early-stage breast cancer, radiation significantly reduces the risk of local recurrences (Haffty et al. 2006). In cases of invasive disease, adjuvant chemotherapy precedes IR, as these two therapeutic techniques are not generally used together due to added toxicities. Studies in mammals and yeast have shown that mutations in genes responsible for DNA double-strand break repair resulted in increased

IR sensitivity (Goytisolo et al. 2000). Although radiation therapy is well tolerated, several adverse effects have been reported. These effects can include secondary cancers, skeletal complications, radiation-induced heart disease, and lung disease (Hirbe et al. 2006, Munshi 2007). There are two primary ways in which radiation therapy can be given: external radiation, in which a tumor is subjected to a beam of radiation, or by placing radioactive pellets into the normal tissue next to the cancer (American Cancer Society, www.cancer.org). Internal radiation therapy is also known as brachytherapy, which allows for delivery of a high dose of radiation to a small area. It is useful in cases that need a high dose of radiation or a dose that would be more than the normal tissues could stand if given externally. There is ongoing research into methods to deliver radiation more effectively, limiting the toxicity to normal tissues surrounding the tumor. This includes research into agents that sensitize cancer cells to radiation, or protect normal cells from damage induced by radiation (American Cancer Society, www.cancer.org).

*Chemotherapy*

Chemotherapy is an important treatment option for many cancers. Chemotherapy is the use of systemic drugs, making treatment particularly useful for cancers that have metastasized (American Cancer Society, www.cancer.org). This approach is beneficial for shrinking large tumors so that they are small enough to be removed by lumpectomy instead of mastectomy. These drugs, however, can also damage normal cells, which can leads to serious side effects. More than 100 drugs are currently available for chemotherapy, either alone or in combination with other drugs or treatments. These agents vary widely in their chemical composition, how they function, their usefulness in

treating specific forms of cancer, and side effects. Alkylating agents (nitrogen mustards, nitrosoureas**)** damage DNA to prevent the cancer cell from reproducing through modifications to the bases in the DNA backbone. Intercalating agents (cisplatin, carboplatin) stabilize DNA structure so that it cannot be replicated. Antimetabolites (5-fluorouracil, capecitabine) interfere with DNA and RNA replication by substituting altered bases for the normal building blocks of RNA and DNA, thus inhibiting synthesis. Anthracyclines are compounds that interfere with enzymes involved in DNA replication. For example, topoisomerase inhibitors interfere with enzymes that help separate the strands of DNA during replication. The main chemotherapy drug that will be discussed in this study is paclitaxel, a mitotic inhibitor. Mitotic inhibitors stop mitosis by stabilizing microtubules or inhibiting enzymes from making proteins required for cell division.

Paclitaxel is one of the most extensively utilized anti-cancer agents, with clinical efficacy in a wide range of cancers (Wang et al. 2000, Barni and Mandalà 2005). Paclitaxel acts to stabilize microtubules, thus inhibiting cell proliferation at the metaphase/anaphase boundary (Rowinsky 1997, Jordan and Wilson 1996, Mo et al. 2003). The disruption of normal microtubule function halts mitosis and interferes with several critical interphase functions, ultimately leading to cell death (Rowinsky 1997). However, the success of paclitaxel in the clinical setting can often be inhibited by drug resistance, which severely limits the effectiveness of chemotherapy. While the exact mechanisms of resistance remain unclear, resistance is considered to be mediated by altered drug uptake, variations in tubulin structure, and evasion of apoptotic pathways (Fojo and Menefee 2007). Importantly, the design of new therapeutic regimens is

necessary, due to the fact that paclitaxel has negative effects on normal, as well as cancer cells.

*Molecular targeted therapy*

Molecular targeted therapy is a technique that blocks the growth of cancer cells by interfering with specific molecules/gene products needed for tumor growth. These targeted cancer therapies may be more specific and effective than current chemotherapeutic treatments and less harmful to normal cells. There are several types of targeted therapeutics, most of which are monoclonal antibodies and small molecule inhibitors. Important targets include the growth factors and their receptors, such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and HER2. The research presented within this dissertation will focus in on trastuzumab, a monoclonal antibody against the extracellular portion of HER2.

Trastuzumab (Herceptin™) binds the extracellular portion of HER2 and is thought to induce antibody-dependent cellular cytotoxicity, prevent receptor activation and signaling, inhibit angiogenesis, and induce apoptosis (Ross et al. 2004, Hudis 2007). Trastuzumab has become an important therapeutic option for patients with HER2-positive breast cancer and is widely used for treating advanced metastatic disease (Sledge 2004, Ross et al. 2003). Importantly, trastuzumab works well in combination with many chemotherapeutic agents (Slamon et al. 2001, Nicolini et al. 2006, Pegram et al. 2004). Trastuzumab administration, however, can be associated with adverse side effects including ventricular dysfunction, cardiac failure, infusion reactions, and pulmonary events (Sledge 2004, Pegram et al. 2004). In addition, not all HER2 overexpressing

breast cancer cells respond to trastuzumab treatment and resistance develops rapidly in a large number of patients (Menendez et al. 2005, Burstein 2005, Tan et al. 1997, Nahta et al. 2007a).

The ability to escape cell death has important implications in cancer development and treatment. Successful treatment with therapeutics is largely dependent on the ability of an agent to trigger cell death, typically through the induction of apoptosis, although this may not be the only form of cell death associated with cancer therapy (Eom et al. 2005, Okada and Mak 2004). Mutations in genes associated with the induction of death give cancer cells a growth advantage, allowing these cells to become invasive (Okada and Mak 2004). The migration of cancer cells to distant sites represents the most dangerous aspect of cancer, with metastatic breast cancer being responsible for approximately 90% of breast cancer related deaths. Median survival after appearance of metastases is approximately 2-3 years. Metastatic disease is largely incurable and the goal of standard therapies is to alleviate symptoms and prolong symptom-free survival (Welch et al. 2002, Barni and Mandalà 2005).

Conventional therapeutics have proven to be initially effective in most cancer patients, although many relapse (Li et al. 2008). Tumor re-growth and disease relapse appear to be due to the development of resistance to therapy, or insufficient primary therapy, which decreases sensitivity, or to the existence of a subset of intrinsically resistant cancer cells. Therefore, it is important to investigate novel therapeutics that target potentially resistant cells as well as those cells capable of metastasizing. Furthermore, understanding the mechanisms underlying the response to therapeutics is vital for devising new strategies for the treatment of cancer that have longer lasting

effects on cancer cells while limiting the effect to normal cells. One prevalent area of research is the discovery of biomarkers that have the potential to guide treatment in patients to provide the greatest possible chance for survival. Biomarkers have been defined as any measurable factor that signals for malignancy or the potential for malignancy, or predicts cancer behavior, prognosis, or response to therapy (Hinestrosa et al. 2007). One potential biomarker is telomerase, as activity increases during cancer progression (Hoos et al. 1998, Herbert et al. 2001). Reactivation of telomerase can therefore be associated with disease development and progression. Importantly, telomerase activity is measured through easily interpreted assays, which is an important aspect of biomarker assessment in breast cancer treatment.

**Targeting telomerase and telomere maintenance: a novel anticancer strategy**

Telomerase activity can be detected in 85-90% of human tumors, but not in most normal cells, making telomerase an attractive target for cancer therapy, regardless of tumor histology (Kim et al. 1994). It is possible to directly or indirectly target telomerase activity by inhibiting hTERT or hTR expression or function, or by targeting telomerase interactions with the telomere (Figure 1.4). Additionally, taking advantage of hTERT reactivation in cancer cells enables the use of vaccines that signal the immune system to specifically destroy cells expressing hTERT antigens (Shay and Wright 2006, Vonderheide et al. 2002, Nair et al. 2000, Vonderheide 2008). The most widely studied telomerase inhibitors are agents that target hTERT, hTR, telomerase-associated proteins, and the accessibility of active telomerase to telomeres.

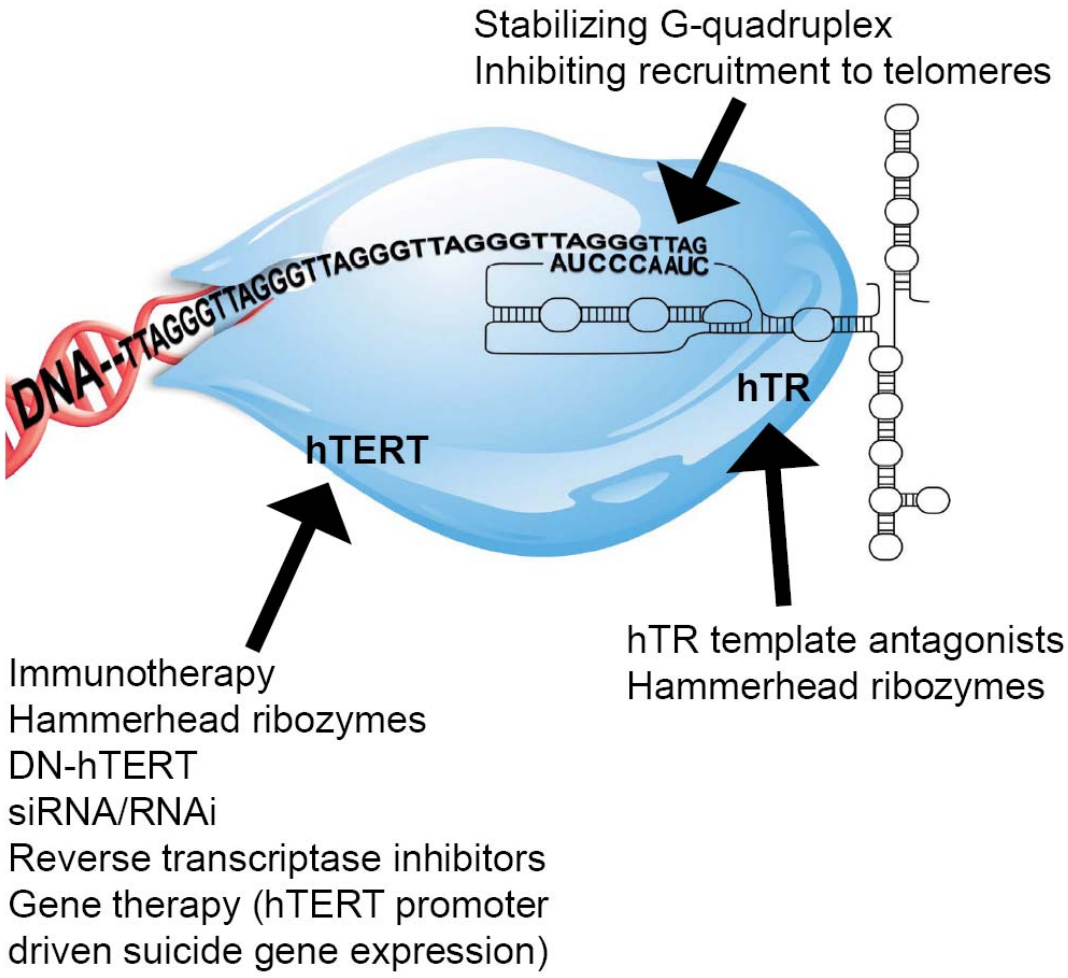


**Figure 1.4. Telomerase offers multiples targets for inhibition of enzyme function.** Inhibition of telomerase activity can be achieved through a variety of methods. This includes inhibiting transcription or translation of hTERT and hTR, inhibiting the function of the telomerase complex, and inhibiting association between telomerase and the telomere.

There are several ways to target the catalytic function of telomerase through inhibition of hTERT expression or activity. The expression of a dominant negative hTERT (DN-hTERT) in immortalized or cancer cells results in telomerase inhibition and progressive telomere shortening, followed by cell growth inhibition and death due to telomere dysfunction (Hahn 1999, Zhang et al. 1999). The use of small interfering RNAs (siRNAs) also inhibits telomerase activity and sensitizes cancer cells to irradiation and to chemotherapy (Masutomi et al. 2005, Nakamura et al. 2005). There are a variety of non-nucleoside agents that have anti-telomerase activity as well, resulting in decreased proliferation and the induction of a senescence-like phenotype after long-term treatment due to progressive telomere shortening (Saretzki 2003, Kelland 2005). This class of agents includes reverse transcriptase inhibitors (i.e., azidothymidine, or AZT, a compound used in HIV treatment) that have anti-telomerase activity; however, these effects are not specific for telomerase over other polymerases. Additionally, these compounds generally require high doses to effect cell growth and viability, and often result in nonspecific cellular toxicity (Kelland 2005, Strahl and Blackburn 1994, Strahl and Blackburn 1996). One of the most widely studied compounds of this class is BIBR1532 (2-[(E)-3-naphthalen-2-yl-but-2-enoylamino]-benzoic acid), a highly specific, non-competitive catalytic inhibitor that is mechanistically similar to inhibitors of HIV reverse transcriptase (Zimmermann and Martens 2007, Pascolo et al. 2002). The long lag phase associated with this type of inhibition (up to 100 days) is expected for a classical telomerase inhibitor, but may not be clinically relevant for effective cancer treatment (Kelland 2005, Zimmermann and Martens 2007, Saretzki 2003).

Targeting hTR rather than hTERT can be advantageous, as hTERT has been implicated to have extracurricular activities in addition to its well characterized role in telomere maintenance (Chang and DePinho 2002). The focus of the research presented herein will involve a specific antisense oligonucleotide telomerase template antagonist, GRN163L (Figure 1.5a). This compound acts to inhibit telomerase activity through binding hTR, thus interfering with the telomerase complex binding to the telomere (Figure 1.5b). Telomerase template antagonists also inhibit the enzymatic activity of telomerase rather than inhibiting protein translation, and thus would not interfere with other functions of the telomerase complex (for example, involvement in DNA repair). The sequence of the hTR template region is known (5'-CUAACCCUAAC-3'), simplifying the design of oligonucleotide-based inhibitors. The knowledge of this sequence also makes the development of mismatch oligonucleotides possible. GRN163L is a second generation, lipid-conjugated N3'→P5' thio-phosphoramidate oligonucleotide containing a palmitoyl lipid group at the 5' terminal end of its sequence. This type of modification and chemical backbone enhances the stability of the oligonucleotide against nuclease digestion, enhances binding to proteins, and improves cellular uptake (Herbert et al. 2005). GRN163L has been shown to efficiently inhibit telomerase activity in immortalized and cancer cells, indicating its potential for use as a cancer therapeutic (Djojosubroto et al. 2004, Herbert et al. 2005). Recently, published reports have found dramatic effects of GRN163L, compared to earlier generations of telomerase template antagonists, on cancer cell growth inhibition before the bulk of the telomeres would have theoretically reached critical shortening, as measured by Southern hybridization methods (Dikmen et al. 2005, Hochreiter et al. 2006, Gellert et al. 2006). The mechanisms

underlying the response to GRN163L are not completely understood; therefore, it is of interest to further elucidate the mechanisms of GRN163L for the development of new cancer therapeutic regimens.

**The use of telomerase inhibition in cancer therapy**

The original hypotheses for targeting telomerase in cancer have been supported by a large body of work demonstrating that telomerase inhibition resulted in the erosion of telomeric DNA, eventually leading to apoptosis or senescence (Hahn et al. 1999, Zhang et al. 1999, Herbert et al. 1999, Herbert et al. 2005, Gellert et al. 2006 for review, Shay 2003 for review). This effect is expected to be relatively specific to cancer cells with few side effects for somatic cells and stem cells, as cancer cells typically have shorter telomeres than those found in normal tissue (Shay and Wright 2007, Gellert et al. 2005, Shay and Wright 2006). *In vivo* studies have demonstrated that telomerase inhibition not only inhibits the growth of primary tumors, but also inhibits cancer metastases to the lung (Hochreiter et al. 2006, Jackson et al. 2007).

As described in this chapter, inhibition of telomerase activity can be achieved in a number of ways (Figure 1.4). At the present time, few of these agents have reached clinical trials. Original *in vitro* studies utilizing telomerase inhibitors demonstrated a lag phase before critical telomere shortening and cell growth inhibition (Hahn et al. 1999, Zhang et al. 1999, Herbert et al. 1999). Recent reports, however, have demonstrated that the new generation of telomerase inhibitors, particularly the lipid-conjugated oligonucleotides, may target cancer cell growth more efficiently than previously expected (Herbert et al. 2005, Hochreiter et al. 2006, Jackson et al. 2007). These findings may

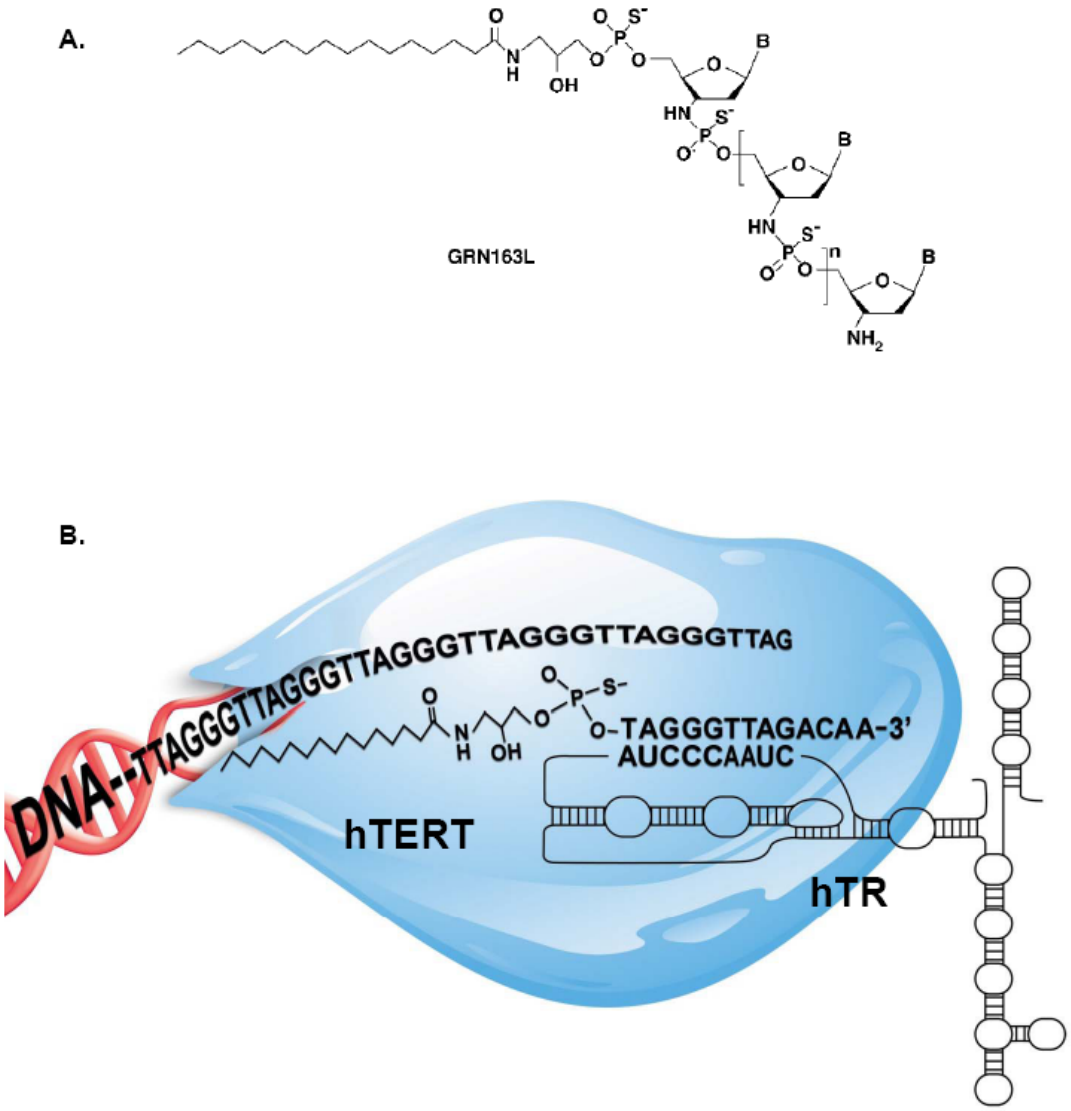


**Figure 1.5. GRN163L binds to hTR, inhibiting telomerase from recognizing its telomeric substrate.** (A) Structure of GRN163L. GRN163L is a 13-mer lipidated N3'→P5' thiophosphoramidate oligonucleotide containing a palmitoyl group at the 5' terminal end of its sequence (5' -TAGGGTTAGACAA-3'). (B) GRN163L is complementary to the intrinsic template region (3'-CAAUCCCAAUC-5') of the RNA component of telomerase, hTR. Binding of GRN163L to hTR inhibits association with telomeric DNA.

provide an increased chance for therapeutic value with low doses of drug, as well as a better therapeutic window for the use of other therapies in order to limit the amount of toxicity in normal cells. Telomerase inhibitors were originally proposed to be used in addition to other therapeutic regimens to prevent recurrent disease or metastasis after primary therapy. The current knowledge about the efficacy of telomerase inhibition is promising for the use of these compounds as cancer therapeutics. However, the mechanisms underlying the response to telomerase inhibition remain unclear. Additional studies are needed to fully comprehend how to appropriately and effectively use these compounds. An important consideration for the use of telomerase inhibition based therapeutics is the emergence of cell populations utilizing other methods of telomere maintenance, such as the ALT pathway. Although cells exploiting the ALT pathway have not been observed in human cancer cell lines after treatment with telomerase inhibitors, this possibility remains an important potential in the development of therapeutic resistance, particularly if the use of telomerase inhibitors will be chronically required to control primary cancer cell growth and metastasis (Hahn 2003).

GRN163L, the primary telomerase inhibitor discussed in this research, has been in Phase I/II clinical trials for patients with chronic lymphocytic leukemia (CLL) since 2005 and for solid tumor malignancies since 2006 (www.clinicaltrials.gov). Phase I trials have revealed that weekly administration of GRN163L is well tolerated by patients with no dose-limiting toxicities or serious adverse side effects yet reported. More importantly, there has been a beneficial effect of treatment in multiple patients with CLL, with stable disease and tumor lysis syndrome both being reported. Based on recent results from those studies and recent observations on myeloma cancer stem cells, a fourth clinical trial for

GRN163L was initiated in late 2007 for multiple myeloma patients. Importantly for this research, a Phase I/II trial with GRN163L in combination with paclitaxel and bevacizumab (anti-angiogenesis agent) has just been initiated for patients with locally recurrent or metastatic breast cancer.

**Research aims and significance**

Published reports have shown a significant correlation between telomerase activity and tumor size, lymph node status, and disease stage in breast cancer. Telomerase activity increased in primary carcinomas with ongoing tumor progression (Hoos et al. 1998, Herbert et al. 2001). Furthermore, telomere lengths in cancer cells are shorter than in the surrounding normal tissue (Meeker et al. 2004). These findings support the hypothesis that telomerase is essential for cancer cell survival. The telomerase template antagonist GRN163L has been shown to efficiently inhibit telomerase activity in cancer cell lines, reduce breast tumor growth and metastasis, and limit adhesion to culture substrates (Herbert et al. 2005, Hochreiter et al. 2006, Jackson et al. 2007).

The mechanisms of cancer cell growth inhibition via GRN163L, however, are not completely understood. In addition to inhibition of telomerase activity, telomere maintenance, and cell proliferation, preliminary results indicate that exposure to GRN163L alters cellular structure and stability in cancer cells. The overall goal of this research project was to further elucidate the mechanisms of GRN163L to support the central hypothesis that the telomere dysfunction, structural and proliferative changes in breast cancer cells induced by GRN163L can synergize with other forms of conventional

cancer therapy to inhibit the tumorigenicity of breast cancer cells both *in vitro* and *in vivo*. The aims of this research were accomplished by: 1) determining the effects of combining telomere dysfunction induced by GRN163L with a DNA damage inducer (irradiation); 2) elucidating the mechanisms underlying the cellular response to GRN163L and the effect of combination therapy with the mitotic inhibitor paclitaxel; and 3) testing the hypothesis that a telomerase inhibitor can augment the effects of trastuzumab in breast cancer cells with HER2 amplification. The combination of telomerase inhibition via GRN163L and irradiation (IR) was shown to significantly enhance the effects of radiation on breast cancer cell growth *in vitro* and primary tumor growth *in vivo* (Chapter Two; Gomez-Millan et al. 2007). The increased sensitivity to IR was due to induced telomere dysfunction, suggesting a relationship between extended telomerase inhibition, telomere length, and inhibition of DNA damage-induced repair. Furthermore, this research demonstrates that in addition to the known telomere dysfunction effects from continuous telomerase inhibition, GRN163L induces a rapid inhibitory effect on cell growth and invasion. This rapid response synergistically inhibits the growth of breast cancer cells *in vitro* and *in vivo* with paclitaxel and trastuzumab, supporting the use of this compound in the clinical setting. Combining GRN163L with these three therapies can not only aid in determining whether the effects on cell growth inhibition can be synergistic, but can also provide insights into the mechanism of action of GRN163L. In addition, the results of this work not only compliment the development of mechanism-based combination treatments with GRN163L, but also improve the understanding of the role of telomerase (activity) in cancer. Furthermore, by elucidating the effects of GRN163L alone and in combination with other therapeutics, biomarkers

can be designed for monitoring the response to treatment or disease progression. The use of multiple agents in combination often results in therapeutic synergy, which may be able to overcome drug resistance in a large number of cancers. Using these combination therapies is likely to improve treatment regimens, as targeting multiple pathways involved in cancer progression is likely to increase patient survival over single drug therapy. However, current combination therapies often rely on methods that are toxic to both normal and cancer cells. The use of GRN163L and the combination treatments proposed in this study could thus reduce the negative side effects of chemotherapeutic drugs and mortality due to metastatic disease.

## Chapter Two

## Telomerase Template Antagonism and Telomere Dysfunction via GRN163L Increases Radiation Sensitivity in Breast Cancer Cells

This work is reprinted in part from *Int J Radiat Oncol Biol Phys*, Jaime Gomez-Millan, Erin M. Goldblatt, Sergei M. Gryaznov, Marc S. Mendonca, and Brittney-Shea Herbert, "Telomere Dysfunction Induced by GRN163L Increases Radiation Sensitivity in Breast Cancer Cells", Vol. 67:897–905 (2007) with permission from Elsevier.

### Abstract

Telomerase activity can be detected in 85-90% of human tumors, which stabilizes telomeres and prevents apoptosis or senescence due to genomic instability. There has been a link between telomerase, telomeres, and DNA repair, as DNA repair proteins can be found at the telomeres, and dysfunctional telomeres induce a DNA damage response. Irradiation (IR) induces lethal DNA damage and double-stranded breaks, thus suggesting that the combination of telomerase inhibition and IR may be synergistic. Our lab utilizes the telomerase template antagonist GRN163L, which is complementary to the template region of the telomerase ribonucleic acid component (hTR). The objective of this study was to determine whether exposure to GRN163L enhances radiation sensitivity in human breast cancer cells through inhibition of telomerase activity and gross telomere dysfunction. Results demonstrate that GRN163L inhibits telomerase activity and shortens telomeres, as well as alters cell cycle phase distribution, of MDA-MB-231 breast cancer cells. Additionally, telomere dysfunction induced by GRN163L enhances the effects of IR *in vivo*, with a significant decrease in tumor growth of MDA-MB-231 breast cancer cells in mice when exposed to GRN163L plus IR. These results indicate that GRN163L is

a promising adjuvant treatment in combination with radiation therapy that may improve the therapeutic index by enhancing the radiation sensitivity.

**Introduction**

Telomeres are repetitive $(TTAGGG)_n$ sequences found at the end of chromosomes (Figure 1.1, page 3) that act as protection from chromosomal recombination, end to end fusions, and recognition as damaged DNA (Blackburn 2001, Cong et al. 2002). Telomerase is a ribonucleoprotein complex that acts to maintain telomeres by replacing the sequences lost in during each round of DNA replication (Figure 1.2, page 6). Telomerase activation can be detected in 80-90% of cancer cells, whereas most somatic human cells do not have telomerase activity (Cong et al. 2002, Shay and Bacchetti 1997). This makes telomerase inhibition an attractive treatment for cancer therapy. Targeting the RNA template of telomerase (hTR) using small antisense oligonucleotides prevents the activity of the telomerase enzyme (Shay and Wright 2006). The most well-studied compound in this class is GRN163L, a N3'→P5' thio-phosphoramidate that stably binds hTR (Figure 1.5, page 25), is resistant to degradation, and has both high affinity and specificity for targets (Herbert et al. 2005). This method of action makes GRN163L a unique telomerase inhibitor as it blocks the biochemical activity of telomerase as opposed to inhibiting hTERT protein expression. This compound is a potent inhibitor of telomerase activity and tumorigenicity *in vitro*, and has been shown to be effective in reducing tumor growth *in vivo* (Dikmen et al. 2005, Herbert et al. 2005, Djojosubtruto et al. 2005, Gellert et al. 2006, Hochreiter et al. 2006). One common problem with using small molecule inhibitors of hTERT or hTR is that many require long treatment times,

and response is dependent upon initial telomere lengths (Sarezki 2003, Kelland 2005, Zimmermann and Martens 2007). Although this indicates that telomerase inhibitors may not be used for single agent therapy, there have been numerous reports that suggest that telomerase inhibition can sensitize cells to chemotherapy or irradiation (Ward and Autexier 2005, Cerone et al. 2006a, Djojosubruto et al. 2005, Kelland 2005, Gellert et al. 2005, Gomez-Millan et al. 2007, Zimmermann and Martens 2007).

Ionizing radiation (IR) is an important therapeutic modality that induces DNA damage and double-stranded breaks that are lethal to the cells when the damage cannot be repaired (Bernier et al. 2004). Although radiation therapy is well tolerated, several adverse effects have been reported. This can include skeletal complications, radiation induced heart disease, and lung disease (Hirbe et al. 2006, Munshi 2007). IR after lumpectomy is widely accepted as a standard of care for early stage breast cancer. In early-stage breast cancer, radiation significantly reduces the risk of local recurrences (Haffty et al. 2006). In cases of invasive disease, adjuvant chemotherapy precedes IR, as these two therapeutic techniques are not generally used together due to added toxicities. However, delayed IR may increase the risk of relapse in some subsets of patients. In a retrospective study performed in 2006, Haffty et al. report that concurrent IR and chemotherapy resulted in improved relapse rates and lower toxicity than sequential IR and chemotherapy (Haffty et al. 2006). This study, however, was performed using a chemotherapeutic regimen that is no longer routinely used, demonstrating the need to study the use of IR with new therapeutic agents.

There is a link between telomeres and DNA damage, as components of DNA damage response pathways have been found at the telomeres. Additionally, previously

published reports indicate a possible role of telomerase in DNA repair independent of its role in maintaining telomere length. Ectopic expression of hTERT in normal cells enhanced DNA repair, and these cells also had a lower mutation rate (Sharma et al. 2003, Shin et al. 2004). This suggests that the association of telomerase inhibition and IR may be synergistic. Nakamura et al. demonstrated that siRNA specific for hTERT increased sensitivity of cancer cells to IR or DNA damage inducing chemotherapeutic agents (Nakamura et al. 2005). The purpose of the study herein was to determine the effects of combining GRN163L-induced telomere dysfunction and IR. The goal was to determine whether the enzymatic inhibition of telomerase by GRN163L enhances sensitivity of breast cancer cells to IR, and whether this enhancement depends on the induction of telomere dysfunction induced by GRN163L. This was the first study that investigated the interactions between IR and telomerase inhibition via GRN163L in human breast cancer cells.

**Results**

*GRN163L efficiently inhibits telomerase activity and reduces telomere length in MDA-MB-231 breast cancer cells*

Telomerase activity and processivity was measured using the polymerase chain reaction (PCR) based telomere repeat amplification protocol (TRAP) by introducing telomerase in cell lysates to an artificial "telomere". If telomerase is present and functional in cell lysates, the enzyme will be able to elongate and amplify the telomeric substrate. To test the effects of GRN163L on telomerase activity in breast cancer cells, MDA-MB-231 cells were treated with GRN163L for 9-42 days and passaged weekly. To

ensure continual telomerase inhibition, cells were treated with fresh drug every third day. Telomerase activity was measured at three different time points representing early, mid, and late passages after initiation of treatment with GRN163L. The TRAP assay showed an absence of telomerase activity after 9, 20, and 42 days compared to untreated cells, indicating that resistance to GRN163L did not develop (Figure 2.1).

To confirm that telomerase inhibition results in progressive telomere shortening, the average telomere length was measured after 9, 20, and 42 days of treatment using the telomere/terminal restriction fragment (TRF) assay. To measure telomeric DNA, genomic DNA was isolated from MDA-MB-231 cells, and digested with a mixture of restriction enzymes to isolate telomeric DNA (TTAGGG/CCCTAA sequences). This DNA was used in a modified Southern gel hybridization with a radiolabeled telomeric probe. The average telomere size was determined by the mean of the fragment lengths per sample. As expected, there was a progressive reduction in the average telomere length with continued exposure to GRN163L over time. In the cells treated for 42 days, the average telomere length was reduced from 3.0 to 2.5 Kb (Figure 2.2). Mismatch oligonucleotide treatment did not exhibit any effect on telomere length (data not shown). The measured telomere length using the Southern hybridization-based TRF assay may underestimate the actual loss of telomeric DNA, as this assay may not accurately measure subtelomeric regions or critically short telomeres. However, similar decreases in telomere length have been shown to have biological effects on cell growth (Herbert et al. 1999). Additionally, published reports demonstrate that a small number of short telomeres may

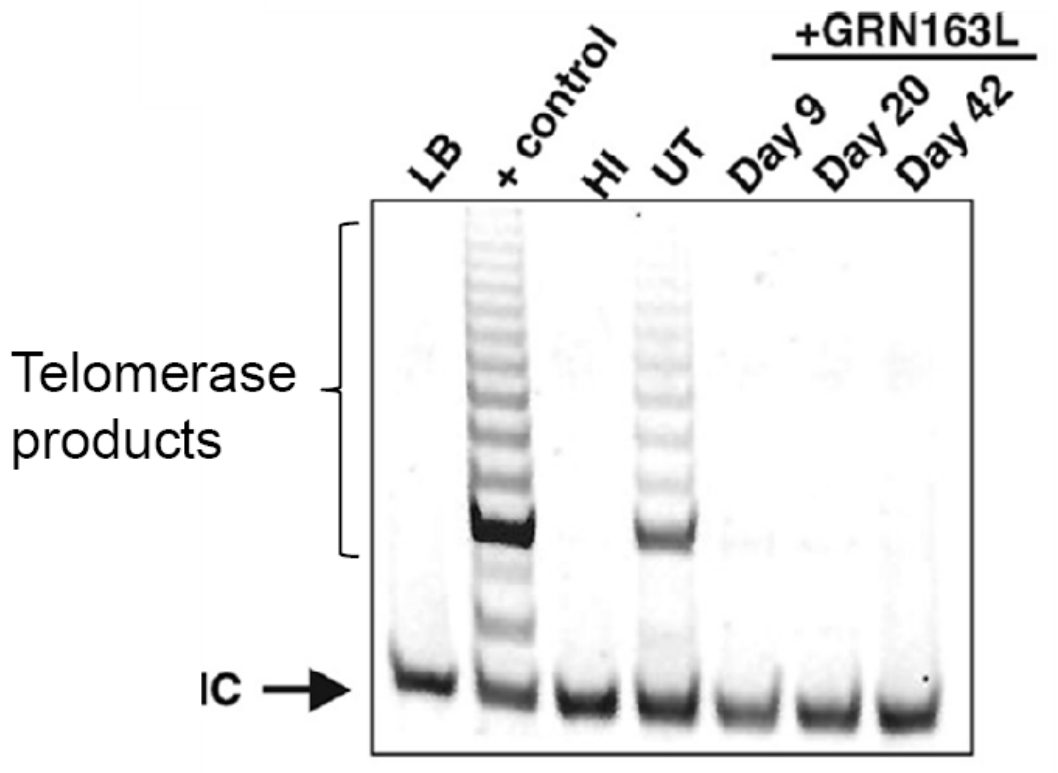


**Figure 2.1. Measurement of telomerase activity in cells treated with GRN163L.** Telomerase activity was measured via the TRAP assay with 500 cell equivalents per lane using a fluorescently labeled oligonucleotide primer. Five-hundred cell equivalents from MCF-7 cells served as positive control (+ control) for the assay. Lysis buffer alone (LB) and heat inactivated (HI) MCF-7 cells served as negative controls. Telomerase activity, as represented by the 6-bp incremental ladder of telomerase products above the internal standard control (IC) band, remained inhibited throughout treatment compared to untreated (media only) MDA-MB-231 cells (UT).

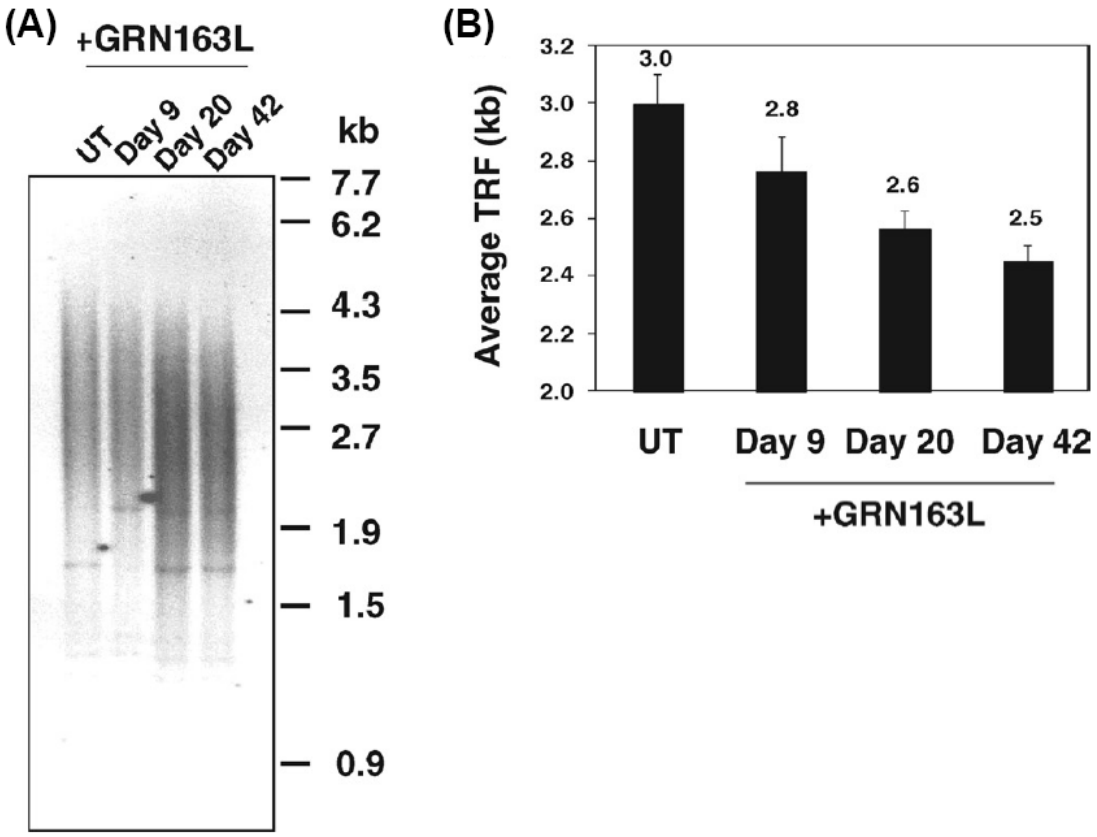


**Figure 2.2. Measurement of telomere length in cells treated with GRN163L.** (A) Average telomere length was measured in untreated (media only) MDA-MB-231 cells and in cells treated with GRN163L for 9, 20, or 42 days using a modified in-gel Southern hybridization of digested genomic DNA. (B) Average telomere length (in kb) decreased 0.5 kb in size for cells treated for 42 days with GRN163L compared with untreated cells. TRF = telomere/terminal restriction fragment.

be responsible for cell viability rather than the average telomere length (Hermann et al. 2001).

*GRN163L reduces cell proliferation and induces a G2 cell cycle shift in MDA-MB-231 cells*

With continuous treatment of GRN163L and progressive telomere shortening, MDA-MB-231 breast cancer cells exhibit a reduction in cell proliferation and population doubling levels compared to controls (Chapter Three; Hochreiter et al. 2006, Gomez-Millan et al. 2007). The decrease in cell proliferation and plating efficiency may be explained by an inhibition in proper cell cycling. Arrests in any phase of the cell cycle can inhibit proper proliferation rates and therefore explain the decrease in cell growth and replication. Cells treated with GRN163L for 9-42 days were collected for cell cycle analysis. Untreated MDA-MB-231 cells had a phase distribution of 45.5% in G1, 32.6% in S, and 21.9% in G2. There was a significant increase in G2 was shown in cells treated for 9 and 20 days compared with controls (Table 2.1). After 9 days treatment with GRN163L, the percentage of cells in G2 increased to 40.9%, while the percentage of cells in G1 decreased to 31.4%. A similar trend was found after 20 days of treatment, with 40.9% of cells in G2 and 28.5% of cells in G1. Interestingly, there is a similar cell cycle phase distribution between controls and cells treated for 42 days. Cells treated for 42 days had a distribution of 47.1% in G1, 29.9% in S, and 23.15% in G2.

**Table 2.1 Cell cycle phase distribution after treatment with GRN163L**

| | G1 | S | G2/M |
|---|---|---|---|
| **Untreated** | 45.5% | 32.6% | 21.9% |
| **9 days** | 31.4% | 28.6% | 40% |
| **20 days** | 28.5% | 25.4% | 46.1% |
| **42 days** | 47.1% | 29.9% | 23.15% |

MDA-MB-231 cells were treated with GRN163L for 9-42 days prior to collection for cell cycle analysis. Cells were stained with propidium iodide prior to determining cell cycle distribution using FACScan. Results were analyzed using the ModFit LT program to determine percentage of cells in each cell cycle phase (Verity Software House Inc).

*GRN163L sensitizes breast cancer cells to irradiation* in vivo

Gomez-Millan et al. have demonstrated that while MDA-MB-231 cells are sensitive to radiation, treatment with GRN163L can significantly enhance radiation sensitivity (Gomez-Millan et al. 2007). This effect was demonstrated through clonogenic assays to measure proliferative capability after treatment with GRN163L and IR. While exposure to GRN163L for 9-20 days did not affect colony formation ability, cells with telomere dysfunction induced by GRN163L after 42 days of treatment had significantly reduced colony formation ability. Additionally, it was reported that the combination of GRN163L and IR can inhibit DNA repair, as evidenced by decreased survival after combination therapy versus either GRN163L or IR alone. Again, this was determined to be due to telomere dysfunction after long-term treatment with GRN163L (Gomez-Millan et al. 2007).

It has been suggested that radiotherapy treatment can accelerate repopulation of proliferative tumor cells during the course of the treatment. This acceleration has been suggested as one of the primary causes of tumor recurrence after radiation therapy (Bentzen and Thames 1991). To determine whether the addition of GRN163L can improve the effectiveness of IR on tumor growth *in vivo*, combination therapy on the growth of xenograft MDA-MB-231 tumors were investigated. MDA-MB-231 cells cultured with or without mismatch oligonucleotide (MM) for seven weeks were injected into the left and right flanks, respectively, of 14 mice. In 14 separate mice, MDA-MB-231 cells cultured with GRN163L for 7 weeks were injected into the right flanks. Mice were divided into treatment groups (n=7 per group): untreated (UT), mismatch (MM)-alone, IR-alone, MM+IR, GRN163L-alone, and GRN163L + IR. Once tumors reached

~50 $mm^3$ (13 days post injection), mice receiving GRN163L were treated with 30 mg/kg (indicated in Figure 2.3 by white arrows). The following day, mice were treated with a clinically relevant dose of 6 Gy irradiation (indicated in Figure 2.3 by the gray arrow). A second *in vivo* dose of GRN163L was administered 4 weeks post-injection. GRN163L-treated MDA-MB-231 cells displayed a lag in tumor growth compared with the xenografts of untreated or mismatch control-treated cells (Figure 2.3; $p = 0.0005$ and 0.00019, respectively). MDA-MB-231 tumors subjected to a clinically relevant dose of 6 Gy irradiation regressed within 1 week post-irradiation and maintained a lag in tumor growth compared with MDA-MB-231 untreated, mismatch-alone, and GRN163L-alone treated xenografts. More importantly, the combination of GRN163L and IR resulted in a highly statistically significant difference in tumor growth compared to mice receiving IR alone or IR + MM (Figure 2.3; $p = 1.8 \times 10^{-6}$ and $1.3 \times 10^{-4}$, respectively). GRN163L was well tolerated by the mice, and the animals did not show any health or behavioral problems. After 80 days, all 7 mice (100%) in the GRN163L plus IR group survived compared with a 14-52% survival in the other groups.

**Discussion**

GRN163L, a telomerase template antagonist currently in clinical trials, binds to hTR (Figure 1.5, page 25) and inhibits the enzymatic activity of telomerase, resulting in telomere shortening as a cell divides. The results presented here demonstrate that exposure to GRN163L progressively shortens the average telomere length, resulting in a reduction in the proliferation rate of cancer cells and a decrease in plating efficiency. Additionally, this is the first time the effects of GRN163L and radiation have been studied in breast cancer cells. The combination of telomere dysfunction induced

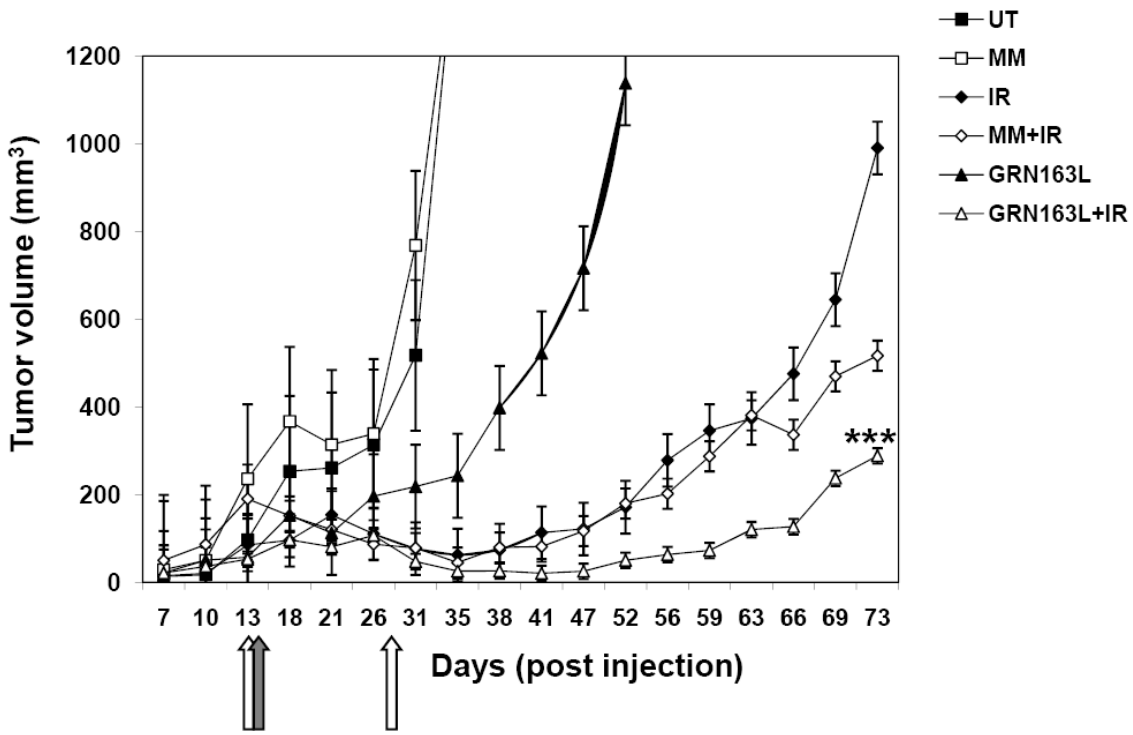


**Figure 2.3. Efficacy of GRN163L in combination with irradiation in an MDA-MB-231 xenograft tumor model.** Tumor growth rate of untreated (UT), mismatch-treated (MM), or GRN163L-treated MDA-MB-231 cells with or without the combination of irradiation (IR) is shown (n = 7 per group). Cells were pre-treated for seven weeks with oligonucleotides prior to injection. Data are presented as mean tumor volume ± SE. White arrows indicate administration of GRN163L. Gray arrow indicates date of irradiation treatment. (*) Represents a statistical difference between the group receiving ionizing radiation alone or MM + IR vs. the group receiving the combination of GRN163L and ionizing radiation ($p = 1.8 \times 10^{-6}$ and $1.3 \times 10^{-4}$, respectively). Ionizing radiation alone vs. IR + MM, $p = 0.267$.

by GRN163L significantly enhances the effects of radiation on breast cancer cell growth *in vitro* (data not shown; Gomez-Millan et al. 2007) and primary tumor growth *in vivo* (in the present study).

The cell cycle is highly important in determining sensitivity to IR, as the response to DNA damage is the activation of cell cycle checkpoints (Pawlik and Keyomarsi 2004, Samuel et al. 2002). Cells are most radiosensitive in the G2 phase of the cell cycle, less sensitive during G1, and least sensitive during S phase. Many chemotherapeutic drugs arrest cells in G1 or G2, thus helping to explain the increasing sensitivity to IR after treatment (Pawlik and Keyomarsi 2004). After 9-20 days of treatment with GRN163L, there was an increase in the percentage of cells in the G2 phase of the cell cycle, suggesting difficulty in fully progressing through the cell cycle. This effect could be a result of an overall lengthening of the G2 phase, or the induction of tetraploid cells with a limited ability to proliferate. However, we did not observe an increase in radiosensitivity in cells treated with GRN163L for 9-20 days prior to irradiation despite the increase in the percentage of cells in G2 (data not shown; Gomez-Millan et al. 2007). It is possible that the increase in the number of cells in G2 alone was not significant enough to sensitize the entire population to IR and that a significant amount of telomere dysfunction was needed to induce DNA damage, thus explaining why we do not find significant benefit of GRN163L on sensitivity to IR at these time points (data not shown; Gomez-Millan et al. 2007).

In cells exposed to GRN163L for 42 days, cell cycle distribution is similar to untreated cells. There is, however, a significant drop in the plating efficiency (PE) and an increase in doubling time (data not shown; Gomez-Millan et al. 2007), indicating a

significant reduction in the proliferative capacity of treated cells. In addition to the substantial reduction in PE compared with control cells, there was telomere shortening, which could help explain the decrease in proliferation and PE, as short telomeres can be recognized as damaged DNA. Critically short telomeres may not be accurately measured in the TRF assays (providing average telomere lengths) if present in small numbers; however, these telomeres can be measured through fluorescence in situ hybridization (FISH). Using this method has allowed for the association of critically short telomeres and DNA damage foci in a cell. These short telomeres can trigger growth arrest or cell death without affecting the average telomere size. These observations are also supported by published reports demonstrating that a small number of short telomeres are responsible for cell viability rather than the average telomere length (Zou et al. 2004, Ouellette et al. 2000, Hermann et al. 2001). It is also possible that telomere dysfunction induced by GRN163L prior to IR induces mitotic catastrophe, which is a type of cell death that occurs during mitosis as a result of DNA damage or abnormal spindle formation (Eom et al. 2005). Mitotic catastrophe is thought to result from a combination of deficient cell-cycle checkpoints and cellular damage, resulting in failure of cell cycle arrest before or at mitosis. This would trigger cell division with faulty chromosome segregation, resulting in cells with abnormal DNA content (Eom et al. 2005, Castedo et al. 2004). The loss of cells due to induction of mitotic catastrophe after 42 days of treatment with GRN163L could explain the reduction in plating efficiency.

DNA repair is active during the G2 phase of the cell cycle, where double strand breaks are recognized and repaired. At this time, functional telomeres may be recognized by DNA repair machinery and would be accessible to repair enzymes. This association

with DNA repair proteins may be necessary for telomere maintenance (Verdun et al. 2005). This response, however, does not typically lead to general DNA damage responses, therefore not resulting in a continued cell cycle arrest. After 9-20 days of treatment with GRN163L, telomeres have most likely not reached a critically short state, thus remaining functional. Thus, the increase seen in the percentage of cells in G2 may be due to cells undergoing DNA repair. After 42 days of treatment with GRN163L, telomeres are more likely to have reached a critically short length, thus limiting the association of DNA repair proteins. At this time point, the cell cycle distribution is similar to that of untreated cells; however, there is a significant benefit from the addition of telomere dysfunction induced by GRN163L and IR. This synergistic response is most likely due to overwhelming the cells with DNA damage (induced by telomere dysfunction and IR), as wells as inhibiting telomerase from participating in DNA repair (Sharma et al. 2003, Shin et al. 2004). This hypothesis supports the idea that the change in cell cycle distribution observed after 9-20 days of treatment may not be contributing to GRN163L induced IR sensitivity. The loss of cells due to mitotic catastrophe could explain the similarities in the cell cycle distribution between untreated control cells and cells treated with GRN163L for 42 days; i.e., cells that could not repair damage and therefore cycle properly may have been eliminated from the population, leaving only cells that could bypass cell-cycle checkpoints. Additionally, it has been hypothesized that DNA double-strand breaks induce repair through homologous recombination (HR), using the material from a partner chromosome as a template to repair the damaged region. Indeed, telomerase deficiency has been reported to increase the rate of chromosomal relocations via homologous recombination in yeast, further implicating the role of

telomerase in global genomic stability. Furthermore, telomerase-deficient mice are hypersensitive to IR and demonstrate a delay in DSB repair that may reflect a defect in HR (Meyer and Bailis 2008). These findings support the data presented herein; telomerase inhibition may inhibit DNA repair via HR, resulting in cell death due to DNA damage. The cell cycle distribution seen after 42 days of treatment with GRN163L could therefore reflect the population of cells still able to repair DNA damage and cycle properly.

hTERT has a reported role in recruiting DNA repair proteins to damaged DNA sites in fibroblasts (Sharma et al. 2003, Shin et al. 2004). Ectopic expression of hTERT enhances repair of IR induced DNA damage in fibroblasts independently of telomere length (Sharma et al. 2003). Conversely, hTERT suppression in fibroblasts alters chromatin structure and DNA damage response to IR and sensitize cells to IR (Nakamura et al. 2005, Masutomi et al. 2005). These studies, taken together, suggest a role for telomerase in DNA damage repair independent of its role in maintaining telomeres. Telomere dysfunction induced by GRN163L sensitized breast cancer cells to radiation *in vitro* and inhibited DNA repair after the induction of telomere dysfunction (data not shown; Gomez-Millan et al. 2007). This is confirmed in the present studies by the fact that combination of GRN163L and IR significantly reduces tumor volume *in vivo*, suggesting that critically short telomeres synergize with IR to inhibit primary tumor growth. These results also support previous studies demonstrating that in mice lacking the telomerase RNA component, cells with short telomeres are more sensitive to radiation and that telomere dysfunction enhances the effects of IR in gastrointestinal stem cells (Goytisolo et al. 2000, Wong et al. 2000). Mice treated with GRN163L or IR alone

demonstrated an initial lag in tumor growth, followed by progressive disease. This would be expected, as treatment was only administered once (for IR) or twice (for GRN163L). Mice receiving both GRN163L and IR demonstrated a significantly reduced tumor volume for the bulk of the experiment, with tumor growth remaining static for an additional thirty days prior to observation of increased tumor volume. This indicates that the effect of GRN163L is reversible with the discontinuation of treatment, although the induction of telomere dysfunction severely inhibited tumor growth long after termination of therapy.

In summary, treatment of MDA-MB-231 breast cancer cells with GRN163L results in the induction of telomere dysfunction *in vitro* and synergistically enhances radiation sensitivity *in vivo* at clinically relevant doses. These results suggest a relationship between extended telomerase inhibition, telomere length, and impairment of DNA damage induced repair. The combination of telomerase inhibitors and IR may allow for the use of lower doses of IR, a reduction in the overall time of treatment, and a decrease in secondary effects. Although treatment with GRN163L for shorter periods of time did not show any synergism with IR, the decrease in proliferation observed could also have a potential effect when combined with IR in clinical settings. Thus, the *in vivo* results with MDA-MB-231 xenografts support GRN163L as a promising adjuvant cancer treatment in combination with radiation therapy. With GRN163L currently in clinical trials, it will be of interest to further study whether other tumor models respond to GRN163L in combination with radiation.

## Materials and Methods

*Cell culture and reagents*

MDA-MB-231 breast cancer cells were cultured at 37°C and 5% $CO_2$ in DMEM containing 10% cosmic calf serum and routinely checked for mycoplasma contamination. Cell cultures were supplemented as necessary with GRN163L or the mismatch control oligonucleotide. The telomerase template antagonists used in this study are complementary to the telomerase RNA component (hTR) template region for telomerase (5' -CUAACCCUAAC-3'). The lipid modified N3'→P5' thio-phosphoramidate oligonucleotide (GRN163L, 5' -Palm-TAGGGTTAGACAA-NH2-3') and the 5'-palmitoyl mismatch control oligonucleotide (5' -Palm-TAGGTGTAAGCAA-NH2-3') were prepared as previously described (Herbert et al. 2005). Cells were treated with 1-2.5 μM GRN163L as necessary. These doses effectively inhibit 100% telomerase activity within 48 hours of treatment (data not shown).

*Telomerase activity assay*

Telomerase activity was measured using the polymerase chain reaction– based Telomeric Repeat Amplification Protocol (TRAP) as previously described (Hochreiter et al. 2006, Herbert et al. 2006). Briefly, MDA-MB-231 cells were treated with GRN163L as described. Cells were collected and lysed in NP-40 lysis buffer to release the telomerase enzyme from cells. The telomerase in the cell lysate was combined with a primer resembling an artificial "telomere". After primer extension with a Cy5 fluorescently labeled telomerase template primer and polymerase chain reaction amplification, reaction products were run on a 10% nondenaturing acrylamide gel. The

gel was visualized, without drying, on a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Telomerase activity was demonstrated as a 6-bp telomerase-specific ladder above the 36-bp internal standard control band (IC).

*Telomere length assays*

Measurements of average telomere length in a population of cells were performed as described previously using the telomere/terminal restriction fragment (TRF) assay (Hochreiter et al. 2006, Oullette et al. 2000). Briefly, DNA was isolated and the proteins digested 4 hours followed by inactivation of proteinase K for 30 minutes at 70°C and dialysis at 4°C overnight. Genomic DNA was digested to completion with a mixture of restriction enzymes (1U/μg each of AluI, HaeIII, HinfI, MspI, and RsaI, Roche Boehringer Mannheim, Indianapolis, IN). The digested DNA was separated on a 0.7% agarose gel in 0.5X TBE. The gel was denatured for 20 minutes in 0.5 mol/L NaOH/1.5 mol/L NaCl, rinsed with distilled $H_2O$, 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 then probed with a radiolabeled telomeric probe for 16 hours at 42°C in 5X SSC buffer, 5X Denhardt's solution, 10 mmol/L $Na_2HPO_4$, and 1 mmol/L $Na_2H2P_2O_7$. The gel was then washed once for 20 minutes in 2X SSC, twice for 15 minutes each in 0.1X SSC at room temperature, and exposed to a phosphor screen (PhosphorImager).

*Cell cycle analysis*

MDA-MB-231 cells were treated with GRN163L for 9-42 days before collection for cell cycle analysis as previously described (Asai et al. 2003). Briefly, $5x10^5$ cells were collected and fixed in 70% ethanol. Fixed cells were washed in PBS and resuspended in equal volumes of 0.1 mg/mL propidium iodide and 0.5 μg/mL DNase-free RNase and incubated in the dark for 30 minutes on ice. Samples were filtered prior to analysis by FACscan and analyzed using the ModFit LT program (Verity Software House Inc).

*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 the institutional Laboratory Animal Resources Center and Institutional Animal Care and Use Committee. Five million ($5 \times 10^6$) MDAMB-231 cells cultured with or without 1 μM mismatch oligonucleotide (Herbert et al. 2005) for 7 weeks were injected into the left and right flanks, respectively, of 14 mice. In 14 separate mice, $5 \times 10^6$ MDA-MB-231 cells cultured with 1 μM GRN163L for 7 weeks were injected into the right flanks. Mice were then divided into six treatment groups (n = 7 per group): untreated (UT), mismatch (MM)-alone, IR-alone, MM+IR, GRN163L-alone, and GRN163L+IR. When tumors reached ~50 $mm^3$ (day 14 post-injection), the flanks of mice in the GRN163L= IR (n = 7), IR-alone, and MM+IR groups (n = 7) were subjected to 6 Gy irradiation. Tumors were measured twice weekly, and mice were euthanized when tumors reached ~2 $cm^3$. Tumor volume was calculated as (length x $width^2$)/2 (in $mm^3$), and data were presented

as mean tumor volume ± SE. At Days 13 and 31 post-injection, mice in the GRN163L alone (n = 7) and GRN163L + IR groups (n = 7) were given 30 mg/kg GRN163L intraperitoneally (IP) to ensure inhibition of telomerase activity. Statistical analysis was performed using Microsoft Excel and GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA). ANOVA was used to determine statistical difference between treatment groups, where $p < 0.05$ was considered statistically significant.

## Chapter Three

## The Telomerase Template Antagonist GRN163L Alters MDA-MB-231 Breast Cancer Cell Growth and Structure, and Augments the Effects of Paclitaxel

The work presented in this chapter has been prepared for submission as "The Telomerase Template Antagonist GRN163L Alters MDA-MB-231 Breast Cancer Cell Structure and Augments the Effects of Paclitaxel" by Erin M. Goldblatt, Erin Gentry, Sergei Gryaznov, and Brittney-Shea Herbert.


### 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 85-90% of human tumors, which stabilizes telomeres and prevents apoptosis or senescence. The objective of the present study was to elucidate the mechanisms underlying the rapid response to the telomerase template antagonist GRN163L in MDA-MB-231 breast cancer cells and whether GRN163L could be used in mechanism-based combination therapy. *In vitro*, GRN163L induces altered cell morphology and reduced growth rates without a significant effect on breast cancer cell viability within the first 14 days. Combination treatment of GRN163L and paclitaxel significantly decreased viability compared to paclitaxel alone or a mismatch oligonucleotide plus paclitaxel. Additionally, *in vivo* MDA-MB-231 tumor formation was significantly inhibited with GRN163L and paclitaxel, and this combination significantly reduced invasion of MDA-MB-231 cells *in vitro*. These data support a rationale for potentially combining GRN163L with paclitaxel for the treatment of breast cancer in the clinical setting.

## 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 are highly toxic and lack selectivity, and resistance often develops quickly. Additionally, approximately 20% of women with a history of early breast cancer will develop metastatic disease (Stevanovic et al. 2006). Metastatic tumors are responsible for approximately 90% of cancer related deaths, making the development of new treatments that inhibit metastasis highly important in cancer therapy. Current therapies are aimed to prolong survival, control symptoms, and maintain the quality of life for breast cancer patients (Orlando et al. 2007, Barni and Mandalà 2005).

Telomerase activity can be detected in 85-90% of human tumors, but not in normal somatic cells, making telomerase an attractive target for cancer therapy (Kim et al. 1994). Telomerase inhibition results in the erosion of telomeric DNA, genomic instability, and eventually to apoptosis or senescence (Herbert et al. 2005, Gellert et al. 2006, Shay 2003). Previous studies from our laboratory have shown that *in vitro*, the telomerase template antagonist GRN163L reduces colony formation ability and replicative capacity of breast cancer cells within two weeks. In addition, using *in vivo* human xenograft models in mice, GRN163L reduces human breast tumor volume and metastases within 30 days (Hochreiter et al. 2006). This reduction in breast cancer metastasis may be explained by GRN163L inhibiting tumor cells migration, adherence, or growth at distant sites (Hochreiter et al. 2006, Jackson et al. 2007). Previous reports have demonstrated that inhibiting telomerase activity alone or in combination with current therapeutic techniques can inhibit tumor cell growth *in vitro* (Ward and Autexier

2005, Cerone et al. 2006a, Gomez-Millan et al. 2007). These studies demonstrated that the augmented combination effect with telomerase inhibitors is through telomere dysfunction.

Microtubules are the major components of the cytoskeleton, and are vital for the maintenance of cell shape, signaling, and proliferation, making them an important target for anti-cancer drugs (Jordan and Wilson 2006, Tommasi et al. 2007). Paclitaxel is one of the most extensively utilized anti-cancer agents, with clinical efficacy in a wide range of cancers (Wang et al. 2000, Barni and Mandalà 2005). Paclitaxel acts to stabilize microtubules, thus inhibiting cell proliferation at the metaphase/anaphase boundary (Rowinsky 1997, Jordan et al. 1996, Mo et al. 2003). 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 altered drug uptake, variations in tubulin structure, and evasion of apoptotic pathways (Fojo and Menefee 2007).

During cancer progression, cells can develop the ability to become invasive, which allows access to transportation through blood and lymphatic vessels (Barkan et al. 2008, Azios et al. 2007, Acconcia et al. 2006). Under normal conditions, cells require attachment to a solid surface before they can grow. Adhesion is mediated by the secretion of molecules found in the extracellular matrix (ECM), such as collagen, laminin, and fibronectin (Barkan et al. 2008). Integrins are cell-surface matrix receptors that act to sense whether or not cells are attached to these matrix proteins. Integrins cluster to form focal adhesions, which affect the organization of the cytoskeleton and activate intracellular signaling pathways, including signals for proliferation, survival, and

migration (Parsons 2003, Cary and Guan 1999). 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 (Acconcia et al. 2006). The formation of focal adhesions helps in the organization of the cytoskeleton and activates intracellular signaling pathways, including signals for proliferation, survival, and migration (Parsons 2003, Cary and Guan 1999, Barkan et al. 2008).

Focal adhesion kinase (FAK) and Pyk2 are two important proteins associated with cellular morphology, growth, proliferation, and migration. The exact roles of Pyk2 and FAK in cancer remain unclear, and published reports suggest possible antagonistic cellular functions for Pyk2 and FAK (Gelman 2003). Induction of Pyk2 expression has been shown to inhibit cell cycle progression and stimulates cell migration, while FAK expression can stimulate cell proliferation. Suppression of adhesion-induced FAK activation has been shown to inhibit the migration and invasion of cancer cells, possibly due to a reduction in protease secretion (Parsons 2003, Cary and Guan 1999, Schlaepfer et al. 2004, Barkan et al. 2008). Interestingly, Pyk2 is capable of compensating for some, but not all, FAK-regulated functions in FAK-knockout cells (Sieg et al. 2000, Gelman 2003).

Metastatic breast cancer is a highly heterogeneous disease; therefore the development of new therapeutic regimens is becoming exceedingly important. New treatment options have emerged with the introduction of novel agents, but success is still hindered by the development of resistance to therapy. The use of multiple agents in combination often results in therapeutic synergy, which may be able to overcome drug

resistance in a large number of cancers. However, current combination therapies rely on methods that are toxic to both normal and cancer cells. GRN163L, a telomerase template antagonist currently in clinical trials, has previously been shown to be specific to cancer cells, with little effect on normal cells (Hochreiter et al. 2006), making this an appealing compound to use in combination therapies.

The overall goal of this present study was to determine the cellular response to GRN163L to understand how this compound may augment breast cancer therapy. GRN163L was found to induce 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 nM/sub-nM concentrations of GRN163L and paclitaxel resulted in a significant synergistic combination index 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 was observed *in vivo,* indicating a potential use for GRN163L in adjuvant therapy for breast cancer.

**Results**

*Treatment with a single dose of GRN163L inhibits telomerase activity for six days in MDA-MB-231 breast cancer cells*

To confirm GRN163L as an effective telomerase inhibitors in breast cancer cell lines, MDA-MB-231 cells were treated with 2.5 μM GRN163L or mismatch (MM) oligonucleotide and collected after 1-6 days to determine the length of inhibition after a single treatment. As shown in Figure 3.1, GRN163L effectively inhibited telomerase

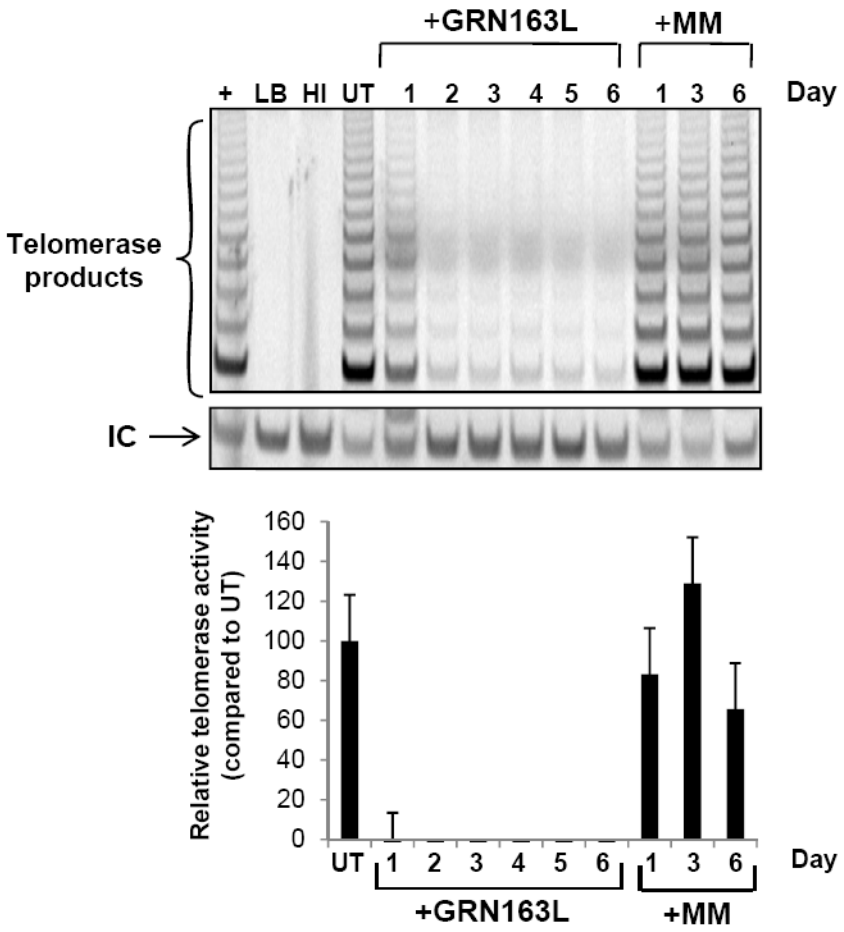


**Figure 3.1. GRN163L effectively inhibits telomerase activity over six days after a single treatment.** MDA-MB-231 breast cancer cells were treated with 2.5 μM GRN163L or mismatch oligonucleotides and collected after 1-6 days to determine the length of inhibition after a single treatment. Telomerase activity was analyzed using the Telomerase Repeat Amplification Assay (TRAP). Results shown are the telomerase products depicted as a ladder. Relative telomerase activity was calculated as the ratio of telomerase products (ladder) intensity to the intensity of the internal control (IC) band and normalized to the untreated (media only) sample. A positive control cell extract (TRAPeze® Telomerase Detection kit) and NP-40 lysis buffer (LB) were used as a positive and negative control, respectively. UT= untreated sample; HI= heat inactivated control samples; MM= mismatch control treated samples.

activity in MDA-MB-231 cells through six days after a single treatment. The mismatch (MM) control oligonucleotide had no significant effect on telomerase activity.

*Treatment with GRN163L alters the morphology of breast cancer cells*

Previous *in vivo* experiments have demonstrated a reduction in metastasis within 30 days exposure to GRN163L (Hochreiter et al. 2006). This may be due to an inhibition of breast cancer cell migration and/or growth at distant sites. To test the cellular responses to GRN163L, MDA-MB-231 breast cancer cells were treated with 19.5 nM-2.5 μM GRN163L or 10 μM mismatch oligonucleotides, and the cellular morphology was monitored daily. Non-tumorigenic MCF10A cells were used to determine whether the response to GRN163L was cancer cell specific. In addition, MDA087 immortalized breast fibroblasts, which utilize the alternative lengthening of telomeres (ALT) pathway and do not express telomerase activity, were also tested to determine if the cellular responses to GRN163L required telomerase. As shown in Figure 3.2, the morphology of MDA-MB-231 cells was altered by treatment with GRN163L, resulting in a rounding-up effect on adherent cells as well the extension of fillipodia-like structures. This effect was observed with 0.3125-2.5 μM GRN163L, but treatment with lower doses did not result in altered morphology. In contrast, normal mammary epithelial cells did not demonstrate a morphological response to treatment with GRN163L. Interestingly, MDA087 breast cancer cells utilizing the ALT pathway also demonstrated a morphological 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 activity. Additionally, the morphological response to GRN163L was tested in

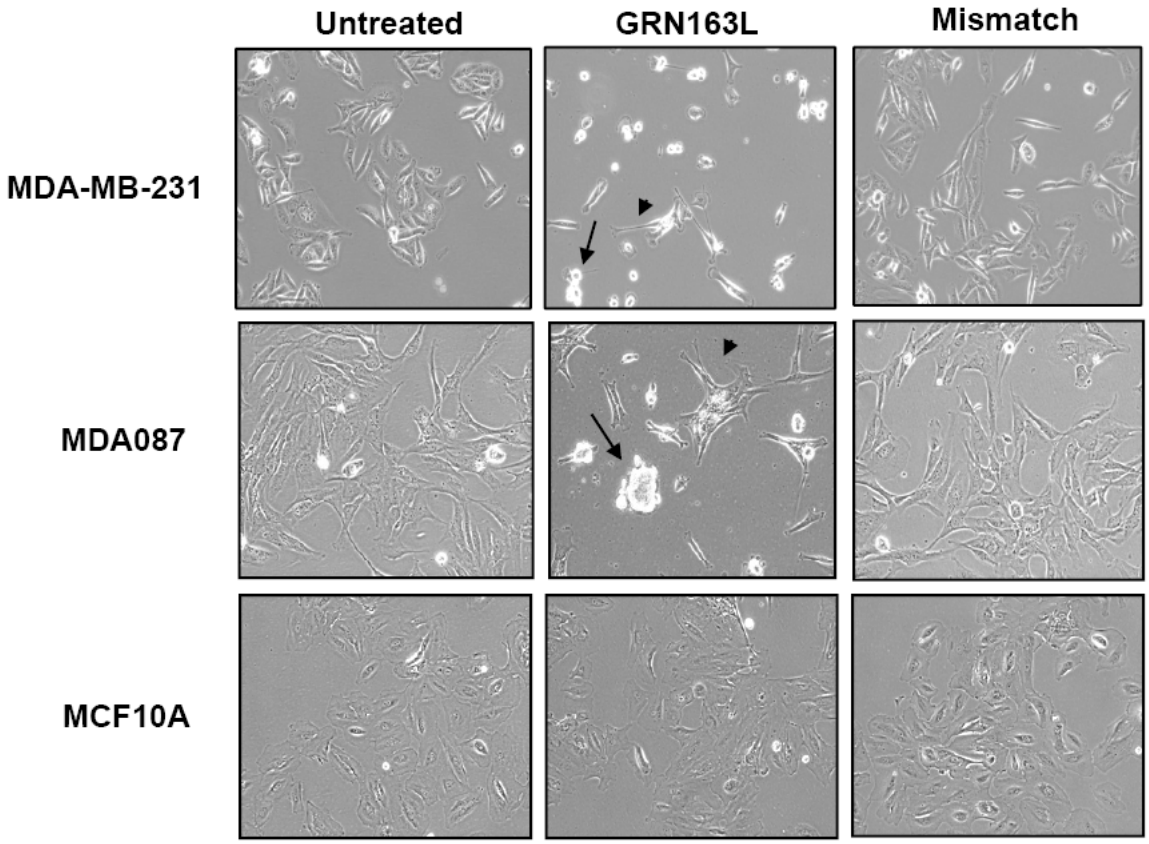


**Figure 3.2. Morphology changes in response to treatment with GRN163L.** $5x10^4$ MDA-MB-231 breast cancer cells were plated in 6-well dishes with 2.5 μM GRN163L or mismatch (MM) oligonucleotide and monitored every 12 hrs for alterations in morphology using a light microscope. Within 24 hours, treatment with GRN163L induced morphologic changes in MDA-MB-231 breast cancer cells and MDA087 telomerase-negative, immortalized breast fibroblasts with no effect in non-tumorigenic MCF10A cells. Cells are shown after 72 hours of treatment. Arrows point to rounded cells, arrowheads point to fillipodia-like structures.

a wide array of breast cancer cell lines, including luminal and HER2+ breast cancer cells. All cell lines tested demonstrated a cell-type specific change in morphology in response to GRN163L (data not shown). Interestingly, the effects of GRN163L on cell adhesion and architecture is reversible with the addition of ECM proteins to cell culture dishes (Goldblatt et al. unpublished observations; Jackson et al. 2007). This reversibility indicates that GRN163L may be interfering with the ability of breast cancer cells to make or secrete ECM proteins, or the binding of integrins to the ECM.

*GRN163L significantly inhibits cell proliferation in MDA-MB-231 cells*

To determine the effect of GRN163L on cell growth, MDA-MB-231 cells were plated onto tissue culture dishes in triplicate. As shown in Figure 3.3a, treatment with GRN163L dramatically inhibited breast cancer cell growth over time. Treatment with mismatch oligonucleotide had no effect on cell growth, indicating that the effect on cell growth is not related to the use of lipidated oligonucleotides but is intrinsic to the telomerase template antagonist. The total population doublings per week were calculated to determine the rate of growth inhibition. As shown in Figure 3.3b, the average time for one population doubling increased from 1.5 days in untreated cells, to 4.25 days in cells treated with GRN163L ($p = 4.34 \times 10^{-7}$). As expected, the mismatch oligonucleotide again had no effect on population doubling rate compared to untreated cells ($p = 0.22$).

To further understand the growth inhibition induced by GRN163L, cell proliferation was measured using a commercial BrdU incorporation assay. MDA-MB-231 cells were treated with 0.5-2.5 μM GRN163L or 2.5 μM mismatch oligonucleotide for 72 hours. These doses all induced a morphological response to GRN163L and

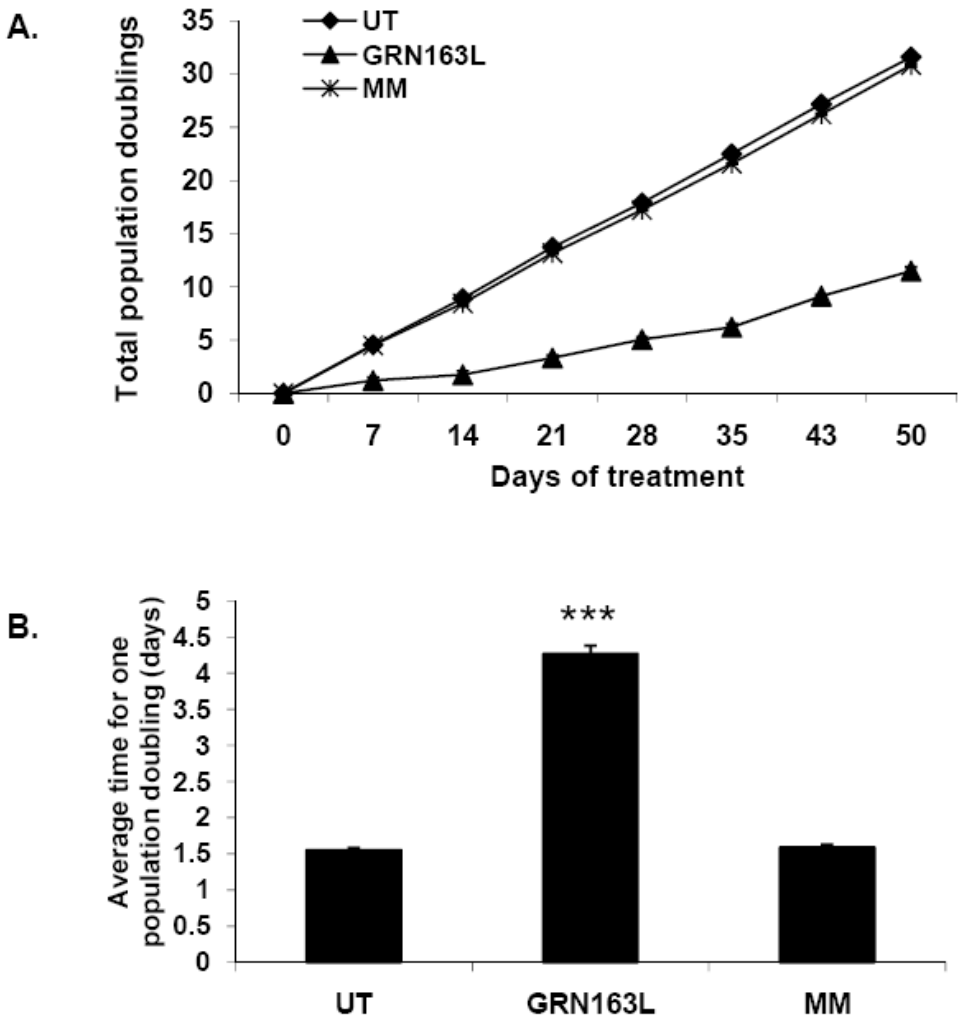


**Figure 3.3. Effect of GRN163L on cell growth.** (A) $5x10^4$ MDA-MB-231 cells were plated in 6-well plates in triplicate. Cells were treated with 2.5 μM GRN163L or mismatch (MM) oligonucleotide, replenishing the drug every third day. After 7 days, cells were collected and counted to determine population doublings. Each sample was counted in triplicate. Triplicate samples were combined before replating to avoid selection for resistant cells. (B) Average time for one population doubling was determined for MDA-MB-231 cells in response to GRN163L or mismatch (MM) oligonucleotides. GRN163L significantly increases the average doubling time of MDA-MB-231 cells ($p = 4.34 \times 10^{-7}$). Mismatch oligonucleotide has no effect on doubling rate ($p = 0.22$).

reduced growth of MDA-MB-231 cells (Figure 3.3). Results demonstrate that cell proliferation is significantly inhibited even at the 0.5 μM dose of GRN163L (Figure 3.4; $p < 0.005$) and progressively decreases at higher doses. The mismatch control did not have a significant effect on BrdU incorporation ($p > 0.05$). Taken together with the growth inhibition, the decrease in BrdU incorporation indicates that the growth inhibition induced by GRN163L may be due to difficulty progressing through the DNA synthesis and cell division phases of the cell cycle. The reduction in proliferation as measured by BrdU incorporation correlates with the increase in doubling time after treatment with GRN163L. After 72 hours, there are 75% fewer cells in the GRN163L treated group versus controls (Figure 3.3) and an 80% reduction in BrdU incorporation (Figure 3.4). The results of these assays together could be explained through two possible scenarios. GRN163L alters cell morphology and adhesive ability (Figure 3.2). The altered morphology could interfere with proper cell division, and therefore lessen the number of cells undergoing DNA replication (explaining the decrease in BrdU incorporation). Alternatively, if GRN163L inhibits DNA synthesis, cell proliferation would decrease and population doubling time would increase.

*GRN163L significantly inhibits colony formation ability of breast cancer cells*

The plating efficiency (PE) of MDA-MB-231 cells after exposure to 1 μM GRN163L for ~5 days was studied to determine whether the change in doubling time is associated with an overall decrease in replicative potential and survival capacity. Treatment with GRN163L resulted in a significant decrease in PE, from $0.876 \pm 0.086$ in untreated cells to $0.216 \pm 0.062$ in cells treated with GRN163L ($p = 0.009$). The

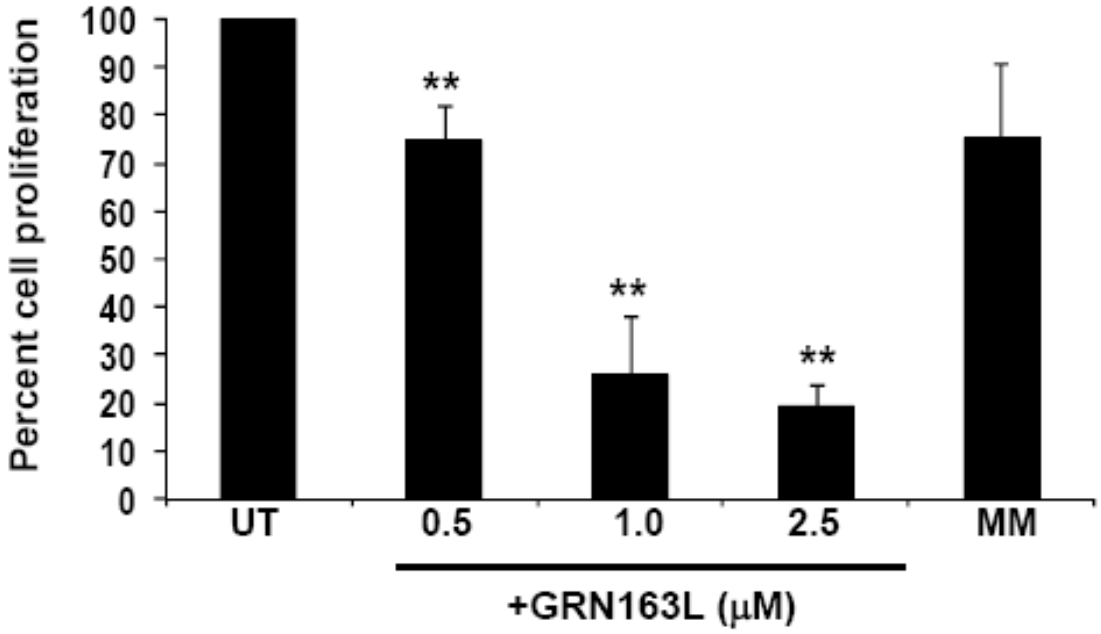


**Figure 3.4. GRN163L inhibits cell proliferation within 72 hours.** MDA-MB-231 cells were grown in 96-well plates with 0.5, 1, or 2.5 μM GRN163L or 2.5 μM mismatch oligonucleotides for 72 hours. 10 μM BrdU was added to culture media 12-18 hours prior to assay. BrdU incorporation was measured at 405 nm with a reference wavelength at 490 nanometers. UT = untreated (media only), MM = mismatch control treated. ** $p < 0.005$.

mismatch control oligonucleotide did not result in significant reduction in PE (Figure 3.5; $p = 0.08$). This decrease in proliferative potential occurs prior to bulk telomere shortening, indicating a rapid effect on the proliferative ability of MDA-MB-231 cells. This result has previously been seen with other breast cancer cell lines with treatment times of 2-3 passages ($\leq$ 2 weeks) (Hochreiter et al. 2006, Gellert et al. 2006).

*GRN163L does not induce apoptosis or reduce cell viability in MDA-MB-231 cells within the first two weeks*

One explanation for the reduction in cell growth and longer population doubling time in short-term GRN163L-treated MDA-MB-231 cells is the induction of cell death, particularly apoptosis. To test for the induction of apoptosis, MDA-MB-231 cells were treated with 2.5 μM GRN163L or mismatch oligonucleotides for 72 hours and analyzed for DNA fragmentation. To assess the induction of apoptosis, the amount of histone-associated DNA released into the cytoplasmic fraction of cell lysates was measured using the Cell Death Detection ELISA. Doxorubicin was used a positive control, as this chemotherapy drug is known to induce apoptosis. As shown in Figure 3.6, treatment with GRN163L did not induce cell death within 72 hrs. While GRN163L did not induce rapid cell death, there was an obvious reduction in cell growth that may be due to a reduction in cell viability. To determine the long term effects of GRN163L on cell viability, MDA-MB-231 cells were treated with 2.5 μM GRN163L or mismatch oligonculeotides, and collected and counted via the Trypan blue exclusion assay daily for fourteen days. Although there is a decrease in cell number, the remaining cells did not demonstrate

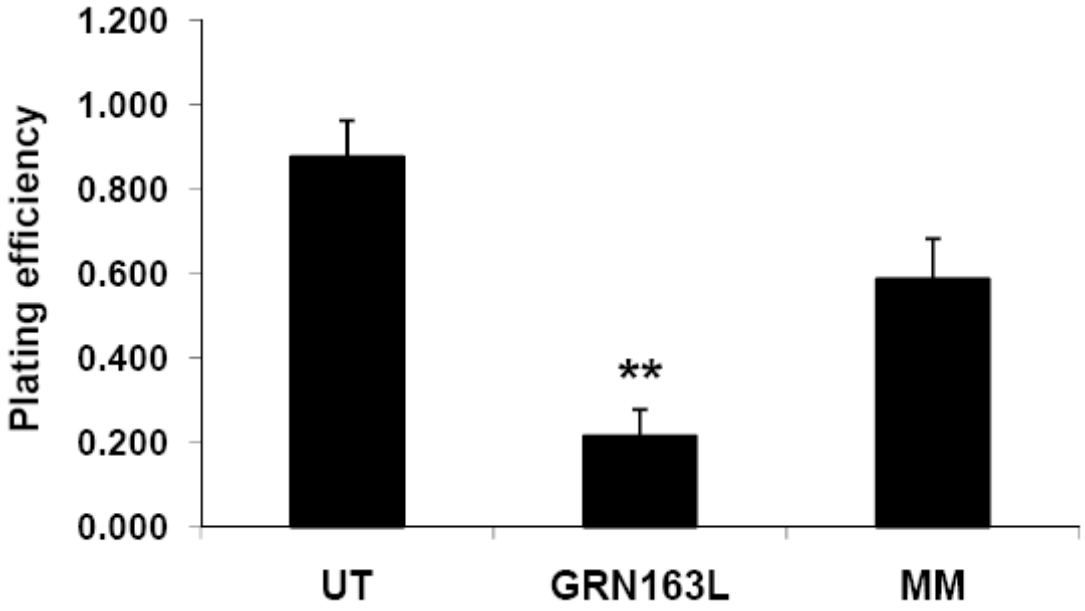


**Figure 3.5. GRN163L reduces proliferative potential of MDA-MB-231 cells.** Plating efficiency was determined by treating MDA-MB-231 cells with 1 μM GRN163L or control mismatch oligonucleotide (MM) for ~5 days prior to seeding at clonal density. Plating efficiency (PE) was calculated by dividing the average number of colonies by the number of cells initially plated. The PE decreased from 0.876 ± 0.086 in untreated (media only) cells to 0.216 ± 0.062 in cells treated with GRN163L (** p=0.009). The mismatch control oligonucleotide did not result in significant reduction in PE (p = 0.08). Data is the average of multiple experiments.

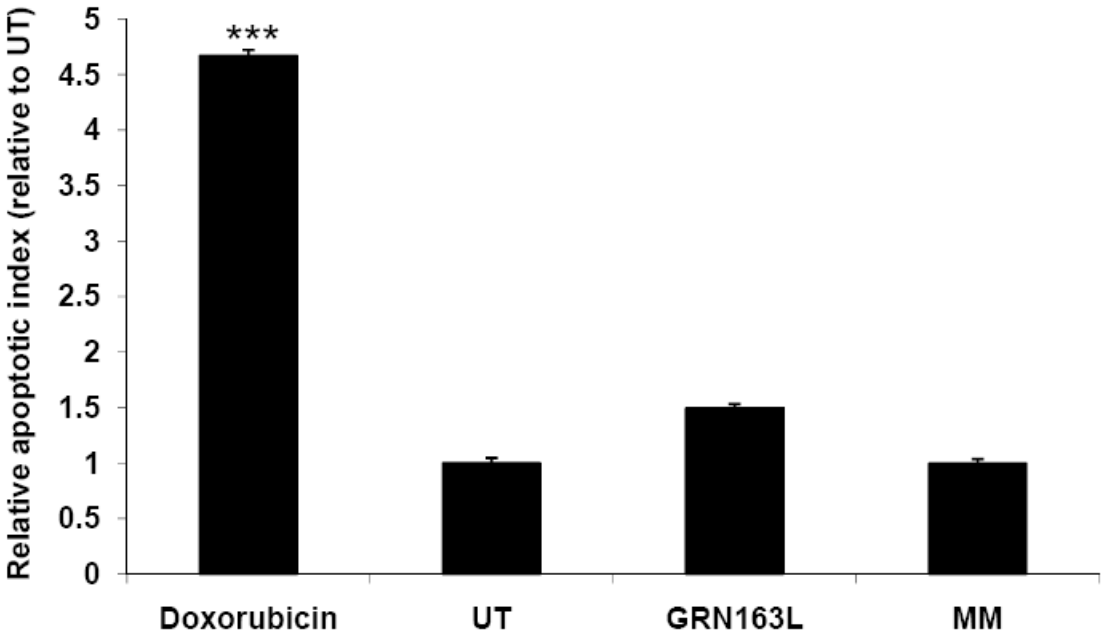


**Figure 3.6. GRN163L does not induce apoptotic cell death within 72 hrs of treatment.** $5x10^5$ MDA-MB-231 cells were plated in $10cm^2$ dishes with 2.5 μM GRN163L or mismatch (MM) oligonucleotide. 5 μM doxorubicin was used as a positive control. Cells were incubated for 72 hours prior to collection and use in the Cell Death ELISA (Roche). The absorbance was read at 405 nanometers with a reference wavelength of 490 nanometers. Results are representative of two experiments. UT= untreated (media only) control cells; *** $p < 0.0005$.

significantly decreased viability (Table 3.1). These results taken together indicate that the growth inhibition seen in MDA-MB-231 breast cancer cells for the first two 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, the actin filaments and focal adhesions were microscopically examined to determine whether this telomerase template antagonist can disrupt structural organization of breast cancer cells. The regulation of focal adhesion and actin filament organization is important for cell migration (Sieg et al. 1999). MDA-MB-231 cells were treated with 2.5 μM GRN163L or mismatch oligonucleotides in chamber slides, and grown for 72 hours, prior to fixing and staining. Results are shown in Figure 3.7. 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, possibly helping to maintain cell shape (Sieg et al. 1999). 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 (Sieg et al. 1999). Additionally, cells treated with GRN163L display fillipodia-like structures Furthermore, under normal physiological conditions, cells form focal adhesions in multiple places along the cell membrane, primarily located at the ends of actin

**Table 3.1. GRN163L does not significantly inhibit MDA-MB-231 cell viability.**

| | Untreated | GRN163L | Mismatch |
|---|---|---|---|
| **Day 1** | 92.18 | 86.65 | 89.17 |
| **Day 2** | 90.00 | 85.43 | 85.45 |
| **Day 3** | 90.61 | 88.93 | 91.63 |
| **Day 4** | 92.25 | 84.55 | 94.21 |
| **Day 5** | 95.83 | 85.71 | 94.62 |
| **Day 6** | 94.10 | 87.10 | 92.59 |
| **Day 7** | 96.93 | 83.78 | 97.26 |
| **Day 10** | 95.24 | 87.50 | 96.61 |
| **Day 14*** | 85.37 | 85.15 | 88.44 |

MDA-MB-231 cells were treated with 2.5 μM GRN163L or mismatch oligonucleotides every third day. Cell viability was measured daily using the Trypan blue exclusion assay and reported in percent values. Cell viability decreased by 10-15% after treatment with GRN163L. Mismatch oligonucleotide had no effect on viability. * After 14 days, cells were overly confluent, resulting in decreased viability of untreated (media only) and mismatch control cells.

filaments (Sieg et al. 1999). As shown in Figure 3.7, untreated and mismatch control treated cells demonstrated 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 cell spreading disruption effects observed in breast cancer cells in response to GRN163L.

*GRN163L alters the expression of proteins involved in cellular structure and function*

The morphology and structural changes induced by treatment with GRN163L indicates that the expression of proteins involved in signaling cellular attachment and intracellular organization may be altered. In an effort to understand the mechanism underlying the response to treatment with GRN163L, the protein levels of β- and γ-tubulin were measured by Western immunoblotting to determine changes in two of the main components of microtubules. Additionally, FAK and Pyk2 were measured, as these proteins play a role in microtubule organization, cell spreading, growth, and proliferation (Parsons 2003, Cary and Guan 1999, Barkan et al. 2008, Zhao et al. 2000, Lipinski 2003). Changes in any of these proteins could result in the changes in MDA-MB-231 cell morphology and growth. As shown in Figure 3.8, the levels of β- and γ-tubulin, as well as basal FAK, did not significantly shift in response to treatment with GRN163L. The expression of Pyk2, however, increased in a cyclical fashion after 12 and 72 hours treatment with GRN163L.

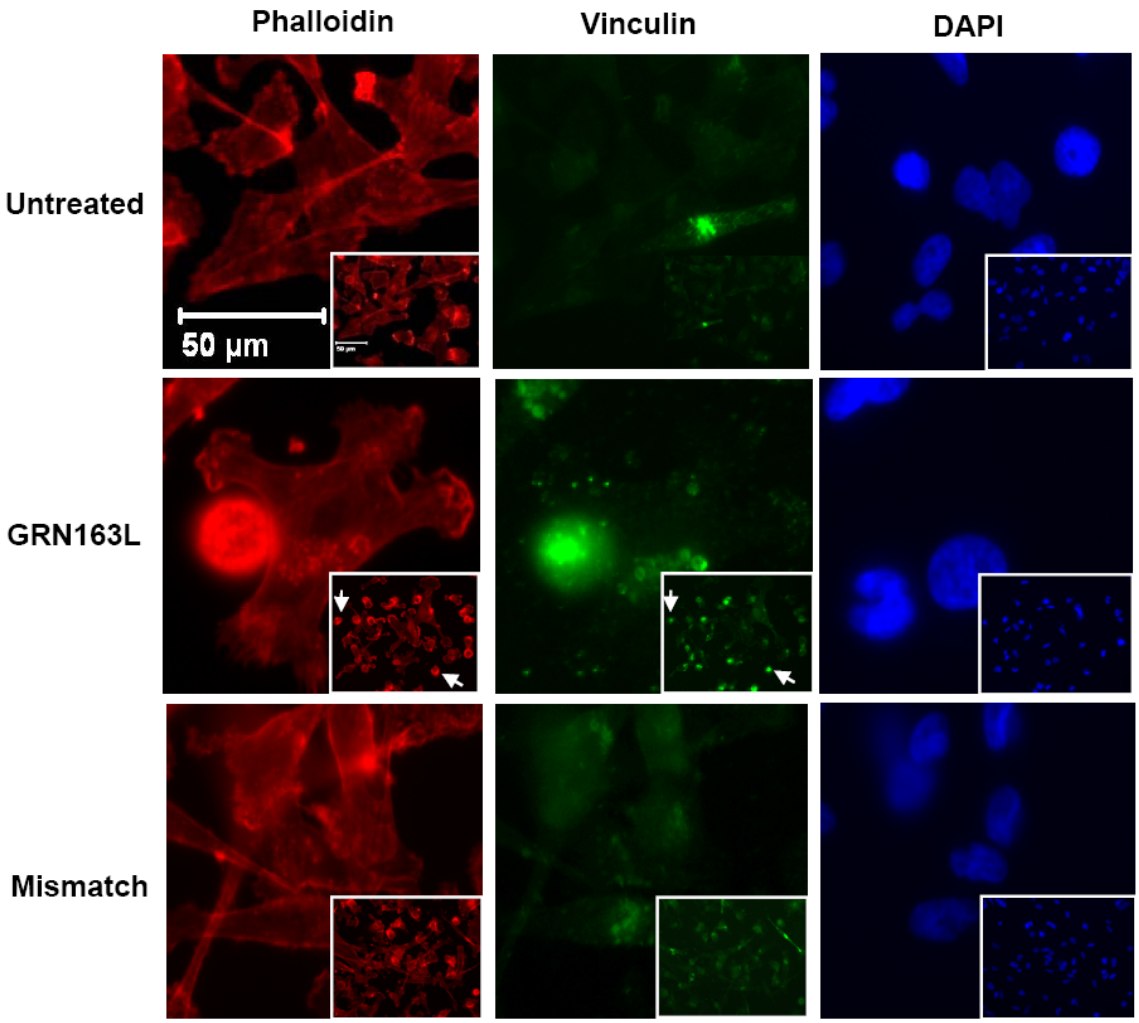


**Figure 3.7. Effects of GRN163L on focal adhesion formation and actin filament organization.** MDA-MB-231 cells were plated on chamber slides with 2.5 µM GRN163L or mismatch oligonucleotides for 72 hours. Cells were fixed and stained with phalloidin (actin filaments) or vinculin (focal adhesions). GRN163L disrupts cell adhesion as evidenced by punctate vinculin staining rather than diffuse staining seen in untreated (media only) and mismatch control treated samples. GRN163L also disrupts cell structure, as seen by altered actin filament organization. Panels are representative cells from a larger field (insets). Scale bars (50 µm) depicted in top left panel represents scale for all panels and insets. Arrow heads point to rounded up cells with punctuate vinculin staining.

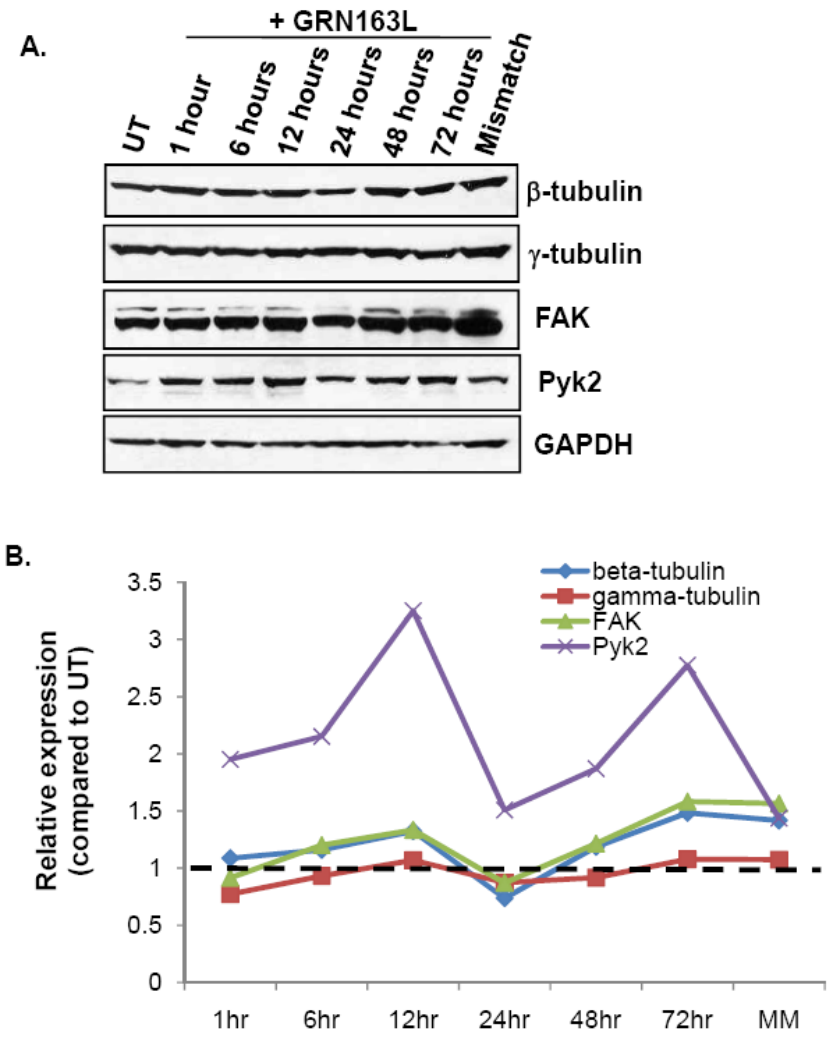


**Figure 3.8. GRN163L effects the expression of signaling pathway proteins involved in cellular structure and function.** MDA-MB-231 cells were treated with 2.5 μM GRN163L or mismatch oligonucleotides for the depicted times. Western immunoblotting for the indicated adhesion and morphology proteins was performed (A). Densitometry levels were determined by comparing intensity of FAK, Pyk2, γ-tubulin, and β-tubulin to GAPDH and relative intensity levels for each sample were plotted and normalized to untreated (media only) samples (B). Untreated and mismatch (MM) control treated cells were analyzed after 72 hours.

proliferation. Speculatively, the upregulation of Pyk2, a protein involved in reshaping cellular architecture, may be induced to aid the cell in reshaping its actin filaments. Restructuring the actin filaments into a more regular pattern could potentially allow the cell to continue replicating in a normal fashion.

*GRN163L synergistically enhances the effects of paclitaxel on inhibiting cell proliferation in 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 0.005-1.25 μM GRN163L either alone or with 0.5-12.5 nM paclitaxel for five days. This range of doses encompasses treatments that do not induce morphological responses, as well as those that are known to affect cell morphology and growth. Cells were collected and counted to determine the effect of individual or combination therapy on cell growth. MDA-MB-231 cells displayed a dose-dependent response to both paclitaxel and GRN163L in terms of cell growth inhibition (Figure 3.9). The mismatch control oligonucleotide did not exhibit significant inhibitory effects on cell growth. On the other hand, the combination of GRN163L and paclitaxel resulted in a significant shift in the growth inhibition curve compared to either agent alone (Figure 3.9, upper panel). Low doses of GRN163L were used to rule out any possible cytotoxicity induced by the lipid-modified oligonucleotide. The synergistic effect of combination treatment was true even at low nM doses of GRN163L, where telomerase is can still be inhibited, albeit at a lower level. Importantly, the mismatch control did not alter the growth inhibition effects of

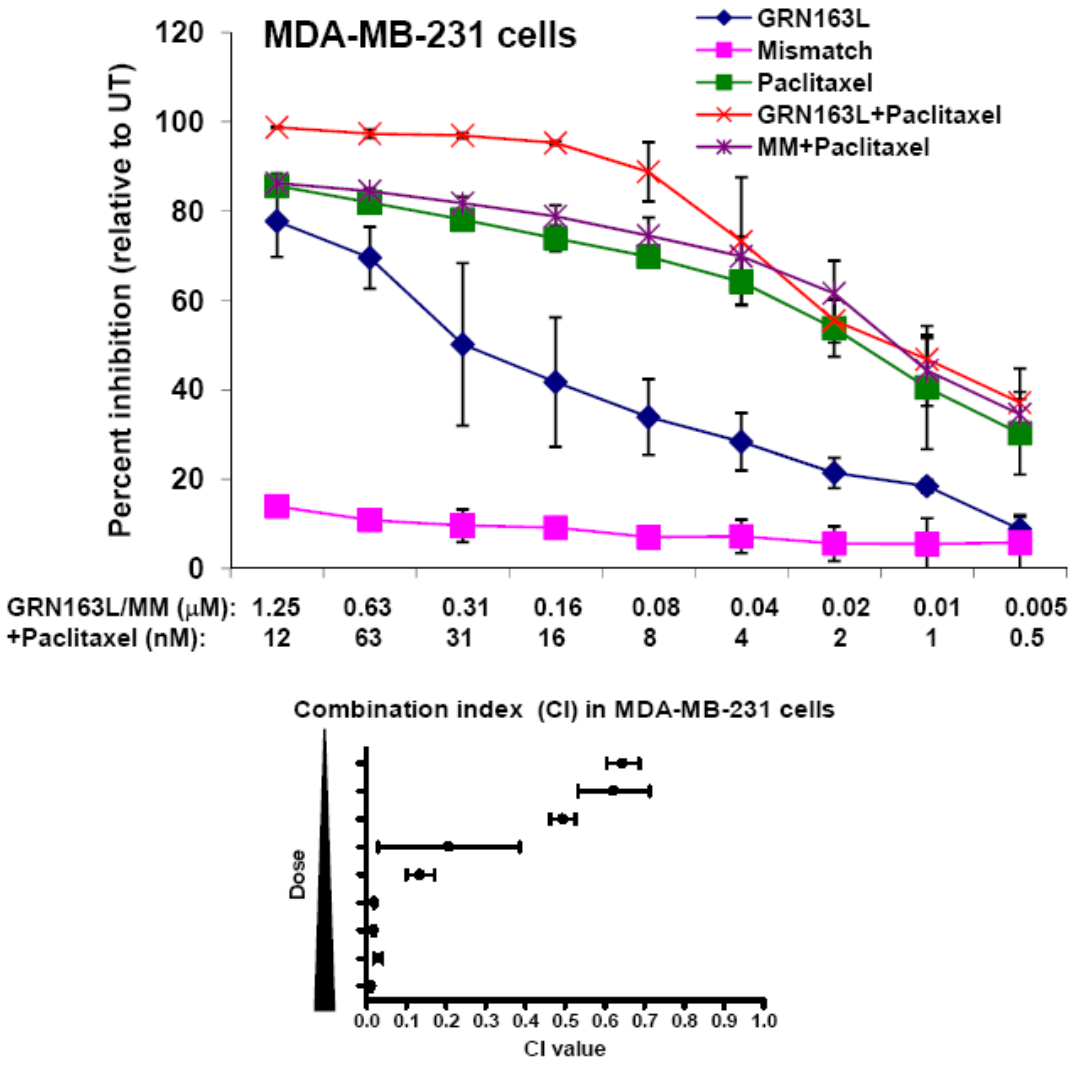


**Figure 3.9. GRN163L synergistically augments the effects of paclitaxel in MDA-MB-231 breast cancer cells.** MDA-MB-231 cells were treated with 1:2 serial dilutions of GRN163L or a mismatch oligonucleotide alone (MM), paclitaxel alone, or the combination of GRN163L/MM with paclitaxel at a 1:10 ratio. Cells were collected after five days to determine cell number (and viability). Percent growth inhibition was plotted versus concentration for each treatment group (top panel). Calcusyn software (Biosoft, Cambridge UK) was used to ascertain the combination index (CI) value for each combination ratio (Dose) to determine whether combination treatments have an additive or synergistic effect (lower panel; ±SD). A CI value of CI <1.0 denotes synergistic interaction (more than additive), a CI of 1.0 demonstrates summation (additive), and a CI >1.0 indicates antagonistic interaction (less than additive).

paclitaxel. The combination index (CI) as developed by Chou and Talalay (Chou and Talalay 1984) was determined for the dual therapy to determine whether combination therapy was synergistic (CI < 1.0), additive (CI = 1.0), or antagonistic (CI > 1.0). As shown in Figure 3.9 (lower panel), GRN163L acted synergistically with paclitaxel to inhibit cell growth of MDA-MB-231 cells at all combination doses tested.

*GRN163L has no effect on the growth inhibition induced by paclitaxel in non-tumorigenic MCF10A cells*

To determine whether the effects of lipid-conjugated telomerase template antagonists and paclitaxel on cancer cell growth could also be observed in non-tumorigenic breast epithelial cells MCF10A cells were treated in the same manner as in the experiments with the MDA-MB-231 cells. As shown in Figure 3.10, GRN163L and the mismatch oligonucleotide control alone had no significant effect on the proliferation of MCF10A cells. Paclitaxel alone did inhibit cell growth at high doses, which would be expected after treatment with this mitotic inhibitor. The addition of GRN163L or mismatch oligonucleotide, however, had no significant effect on cell growth inhibition induced by paclitaxel.

*The combination of GRN163L and paclitaxel significantly inhibit tumor growth* in vivo

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. MDA-MB-231 breast cancer cells were injected into the mammary fat pads of 6- to 8-weekold mice. Mice were treated with 30 mg/kg GRN163L

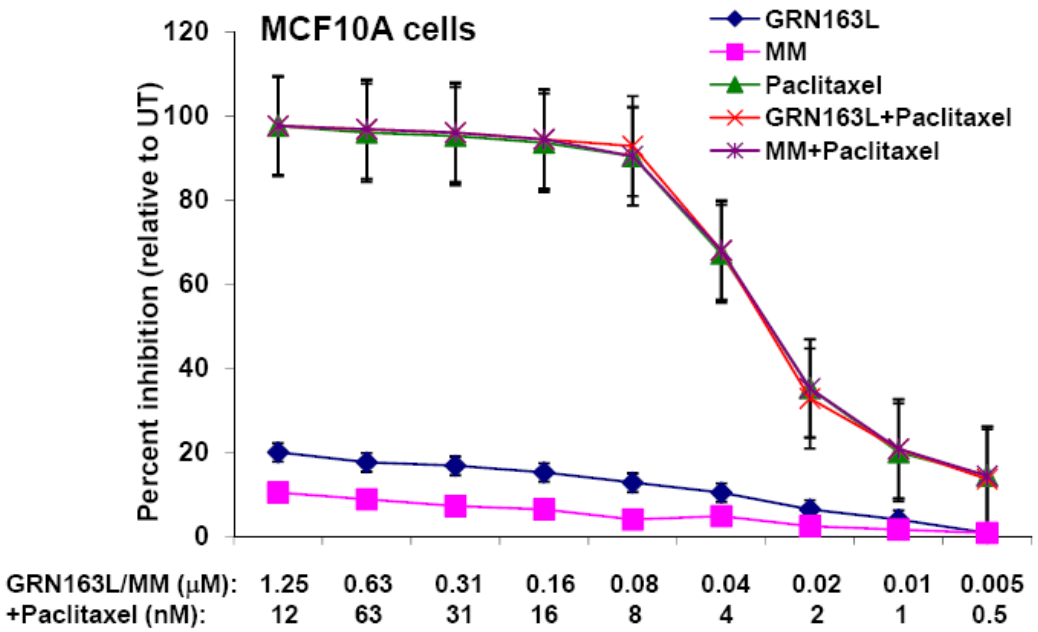


**Figure 3.10. GRN163L does not change the effect of paclitaxel in MCF10A cells.** MCF10A cells were treated with 1:2 serial dilutions of GRN163L or a mismatch oligonucleotide alone (MM), paclitaxel alone, or the combination of GRN163L/MM with paclitaxel at a 1:10 ratio (paclitaxel: GRN163L/MM). Cells were collected after five days to determine cell number (and viability). Percent growth inhibition was plotted versus concentration for each treatment group (top panel).

three times weekly and 15 mg/kg paclitaxel once weekly intraperitoneally (IP). Figure 3.11 depicts the overall tumor growth over the course of the experiment. All groups displayed an initial lag in tumor growth followed by progressive disease. The combination of GRN163L + paclitaxel, however, did significantly decrease tumor volume over PBS control treated animals ($p = 0.0072$). GRN163L was well tolerated by the mice, and the animals did not show any health or behavioral problems. At the end of this experiment, mice treated with GRN163L and paclitaxel displayed better survival rates than control animals (93% survival vs. 80% in control animals) or animals treated with either drug alone (81% in GRN163L treated animals, 86% in paclitaxel treated animals) due to mice experiencing excess tumor burden.

*The combination of GRN163L and paclitaxel significantly inhibits cell invasion over either drug alone*

GRN163L has been shown to inhibit cell migration/invasion and metastasis (Hochreiter et al. 2006, Jackson et al. 2007). The data presented in this dissertation, however, demonstrates an effect of GRN163L on cell growth at a much lower concentrations than previous studies (nM vs. μM), indicating that these lower doses may also inhibit the invasive potential of breast cancer cells. To test this theory, the ability of MDA-MB-231 cells to migrate through an artificial membrane after treatment with GRN163L or mismatch oligonucleotides was determined. MDA-MB-231 breast cancer cells were treated with 0.156-0.3125 μM GRN163L or mismatch oligonucleotides alone or in combination with 16-31nM paclitaxel for five days. Following treatment, cells were plated into matrigel-coated invasion chambers in serum free media for 24 hours. The

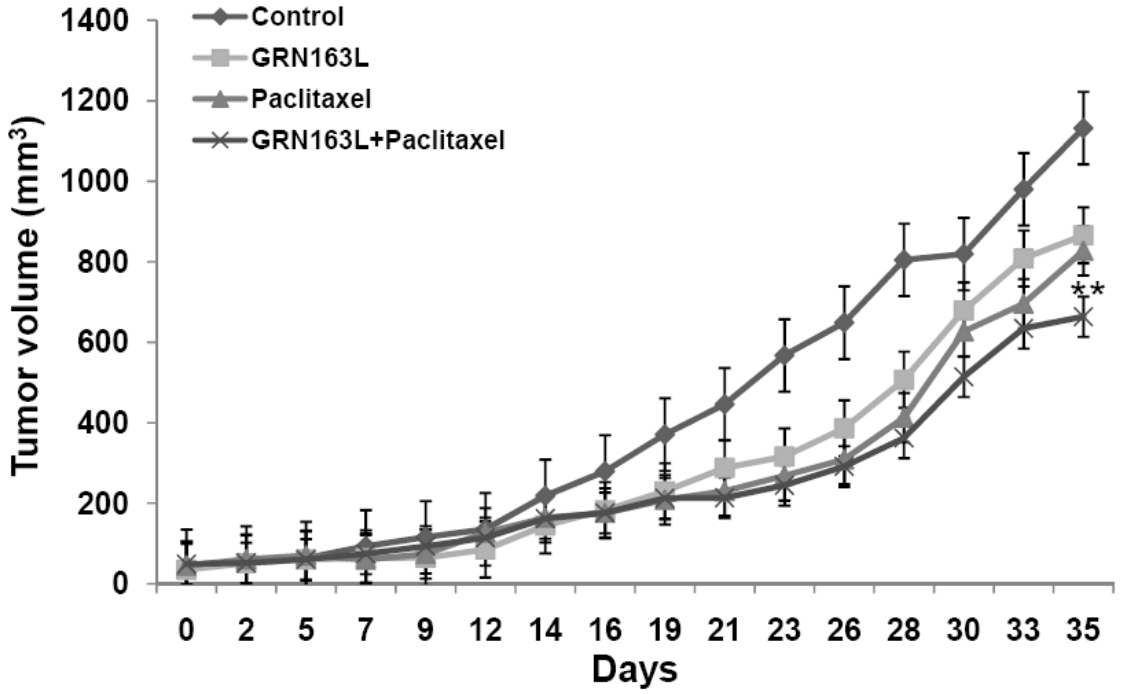


**Figure 3.11. Efficacy of GRN163L in combination with paclitaxel *in vivo*.** Tumor growth rate in mice treated with PBS control, GRN163L alone (30 mg/kg), paclitaxel alone (15 mg/kg), or the combination of GRN163L and paclitaxel is shown (n=30 tumors per group). Data are presented as mean tumor volume ± SE. Statistical differences were calculated using the Student's t-test (Microsoft excel). There was no statistical difference in the average tumor size between UT and GRN163L ($p = 0.16$) or paclitaxel ($p = 0.07$). There was also no significant different between GRN163L and paclitaxel ($p = 0.41$), or either drug alone versus the combination of both drugs ($p = 0.13$ and $p = 0.15$, respectively). There was, however, a significant difference in the average tumor size of control mice versus mice treated with GRN163L ± paclitaxel (** $p = 0.014$).

bottom well of the invasion chamber contained media with serum as a chemoattractant to stimulate cell migration. As shown in Figure 3.9 (page 72), these doses of GRN163L inhibit cell growth by 40-50%, and paclitaxel inhibited cell growth by approximately 80%. The combination of GRN163L and paclitaxel at these doses inhibited cell growth by 100%. As shown in Figure 3.3 (page 60), the doubling time of MDA-MB-231 cells increases to 4.25 days after treatment with GRN163L. Therefore, measuring growth after five days of treatment with GRN163L and paclitaxel takes into account the growth delay induced by GRN163L alone when determining the effect of combination therapy. Equal numbers of control and treated cells were assayed for invasive potential. As shown in Figure 3.12, GRN163L and paclitaxel alone inhibit cell invasion compared to 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 to either drug alone ($p = 0.002$ vs. GRN163L alone; $p = 0.017$ vs. paclitaxel alone). The combination of mismatch oligonucleotide and paclitaxel had no significant effect on the inhibition of invasion induced by paclitaxel alone ($p = 0.23$).

**Discussion**

The data presented here reinforces the use of GRN163L in the treatment of cancer, particularly breast cancer. This compound is known to be an effective telomerase inhibitor; the mechanisms underlying response were, however, unclear. As shown in this chapter, GRN163L likely disrupts normal focal adhesion formation and actin filament organization, thus disrupting normal cell adhesion and structure. This response

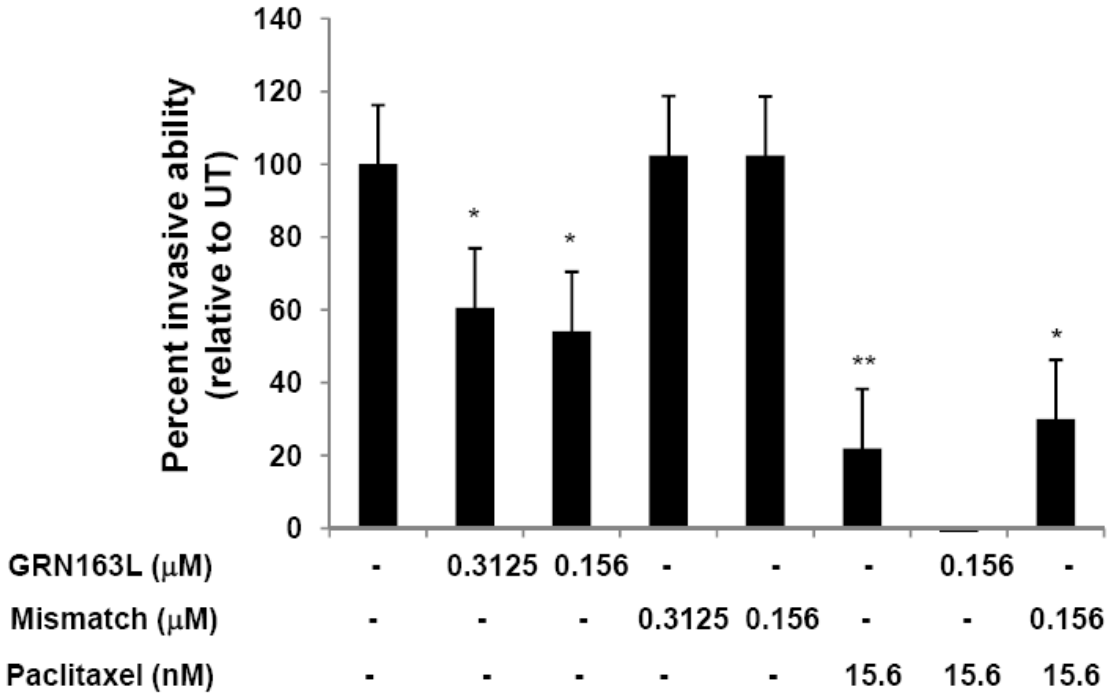


**Figure 3.12. The combination of GRN163L and paclitaxel significantly inhibits invasive potential of breast cancer cells *in vitro*.** MDA-MB-231 cells were treated with 0.156-0.3125 μM GRN163L or mismatch oligonucleotides with or without 15.6-31 nM paclitaxel for five days prior to measuring invasive ability. Cells were plated on matrigel-coated invasion chambers for 24 hours. Culture media containing 10% cosmic calf serum was used as a chemoattractant. BJ fibroblasts were used as a negative control (data not shown). Results are the average of four independent experiments. * = significantly different from untreated (media only) controls ($p < 0.05$); ** = significantly different from untreated controls and paclitaxel treatment ($p < 0.05$). Invasion was completely inhibited with 31 nM paclitaxel, and the addition of GRN163L or mismatch oligonucleotides had no further effect (data not shown).

significantly delays the growth of MDA-MB-231 cells without a reduction in cell viability or the induction of apoptosis. Importantly, this effect of GRN163L sensitized breast cancer cells to paclitaxel, an important chemotherapeutic used in the treatment of breast cancer. Additionally, the combination of GRN163L and paclitaxel significantly decreased tumor volume over PBS control treated animals ($p = 0.014$) and increased survival rates (data not shown). However, this effect on tumor growth was not significant when compared to paclitaxel or GRN163L alone ($p > 0.05$). Although the effect of combination therapy on the rate or incidence of metastasis was not tested *in vivo*, *in vitro* invasion assay results suggest that GRN163L plus paclitaxel significantly reduce the invasive potential of MDA-MB-231 cells.

Microtubules are the major components of the cytoskeleton, consisting of long, tube-shaped protein polymers. Microtubules are vital for the maintenance of cell shape, intracellular transport, cell signaling, and cell division, making them an important target for anti-cancer drugs (Jordan and Wilson 2006, Tommasi et al. 2007). Paclitaxel is one of the most extensively utilized anti-cancer agents that binds to microtubules and increases polymerization, inhibiting cell proliferation at the metaphase/anaphase boundary (Wang et al. 2000, Barni and Mandalà 2005, Rowinsky 1997, Jordan et al. 1996, Mo et al. 2003). Despite the clinical efficacy of the taxanes, there are tissue specificity and drug resistance issues associated with this class of chemotherapy drugs. Mechanisms underlying resistance remain unclear, as does the explanation as to why paclitaxel is an effective therapy for certain types of cancers (Jordan and Wilson 2006, Tommasi et al. 2007). This also indicates the need for new forms of therapy that can target resistant cells. Additionally, the toxicity of chemotherapeutic drugs indicates the need for new

combinations of therapies that will shorten treatment times to reduce the negative side effects associated with treatment.

The results presented herein indicate that the reduction in metastasis seen previously in response to GRN163L (Hochreiter et al. 2006, Jackson et al. 2007) could be due to a loss of proliferative potential of breast cancer cells, as demonstrated by a reduction in growth rates and DNA synthesis. While the response to GRN163L does not induce rapid cell death via apoptosis or decreased cell viability over two 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 oligonucleotides (Aoki et al. 2007). As discussed in Chapter Two, 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 (Eom et al 2005, Castedo et al. 2004).

Under normal conditions, cells require attachment to a solid surface before they can grow. Adhesion is mediated by the secretion of molecules found in the extracellular matrix (ECM), such as collagen, laminin, and fibronectin (Barkan et al. 2008). The formation of focal adhesions helps in the organization of the cytoskeleton and activates intracellular signaling pathways, including signals for proliferation, survival, and migration (Parsons 2003, Cary and Guan 1999, Barkan et al. 2008). During cancer progression, cells develop the ability to become invasive, which allows for transportation through blood and lymphatic vessels (Barkan et al. 2008, Azios et al. 2007, Acconcia et al. 2006). 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 (Acconcia et al. 2006). The response to GRN163L may disrupt this process, thus inhibiting the metastatic potential of migrating cancer cells. Indeed, the cytoskeleton plays a major role in the transition from a dormant state to a state of migration, with quiescent cells maintaining a more rounded shape. Metastatic cells often have highly regular actin filaments, indicating a strong attachment to the ECM (Barkan et al. 2008). In addition to the effect on cell growth, we demonstrate that GRN163L has an effect on cell architecture, resulting in altered actin filament organization and focal adhesion formation. These changes could lead to a reduction in migratory potential of breast cancer cells, thus reducing metastatic disease. A reduction in migration after treatment with GRN163L has been demonstrated in lung cancer cells (Jackson et al. 2007), although the mechanisms underlying this effect remain to be elucidated. That study, however, used a higher dose of GRN163L to inhibit migration and invasion than the doses used in these experiments. The results shown in Figure 3.9 (page 72) suggest that the combination of GRN163L and paclitaxel allows an effective growth inhibition at low nanomolar doses of both drugs, which would lead to reduced toxicitiy in patients.

The effect of telomerase inhibition in sensitizing cancer cells to chemotherapy drugs and irradiation (IR) is well documented, but has been tested by inducing massive telomere shortening prior to seeing sensitization, indicating a long lag time (Cerone et al. 2006a, Djojosubroto et al. 2005, Gomez-Millan et al. 2007, Ward and Autexier 2005). The data presented here shows that telomerase template antagonists can significantly increase the sensitivity of breast cancer cells to paclitaxel within five days, without a

significant effect on non-tumorigenic cells. In addition, GRN163L plus paclitaxel resulted in reduced or delayed MDA-MB-231 tumor growth *in vivo*. The combination of GRN163L and paclitaxel may, therefore, be effective in the treatment of breast cancer. In addition, the effect of this combination on the invasive potential of breast cancer cells *in vitro* and metastasis *in vivo* would therefore be important for breast cancer patients. Interestingly, we did not see an inhibition of tumor growth *in vivo* after treatment with GRN163L or paclitaxel, alone or in combination. This is not an unprecedented result, as previously published reports have shown that the combination of docetaxel, another microtubule inhibitor, and parthenolide, an NF-κB inhibitor, did not significantly decrease tumor volume over either drug alone (Sweeney et al. 2005). Lung metastasis, however, was significantly inhibited by combination therapy. The results of the invasion assay presented here indicate that the combination of GRN163L and paclitaxel may, therefore, be effective in the treatment of metastatic disease.

Telomerase inhibition has classically demonstrated a long lag phase before critical telomere shortening and cell growth inhibition (Hahn et al. 1999, Zhang et al. 1999, Herbert et al. 1999). The research presented here, however, shows a rapid response to GRN163L, with significant effects on MDA-MB-231 breast cancer cell growth and morphology within five days. Taking advantage of this rapid response by treating cells with GRN163L and paclitaxel resulted in a highly significant decrease in cell growth, indicating that shorter exposure times may be possible to reduce secondary effects of treatment with toxic therapeutics (IR, chemotherapy, or molecular targeted therapy). The effect of GRN163L on cell adhesion and morphology is reversible (data not shown, Jackson et al. 2007), with the return of telomerase activity, telomere elongation and

maintenance, and normal cell architecture. 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 (Wright et al. 1996, Forsyth et al. 2002, Shay and Wright 2002). These results further support the use of telomerase inhibitors in combination with other therapeutic regimens for the treatment of breast cancer. Taken together with previously published reports demonstrating that GRN163L inhibits primary breast cancer tumor growth *in vivo* (Hochreiter et al. 2006, Gomez-Millan et al. 2007), these results provide a rationale for the use of this drug in breast cancer clinical trials. Therefore, treatment with telomerase template antagonists in therapeutic regimens could have beneficial outcome not only on primary tumor growth due to the effects of telomere shortening, but also in therapeutic regimens for sensitization to therapy and prevention of recurrence.

## Materials and Methods

### *Cell culture*

MDA-MB-231 and MDA-087 breast cancer cells and BJ fibroblasts (American Type Culture Collection) were cultured in DMEM media containing 10% cosmic calf serum (HyClone, Logan, UT). Non-tumorigenic MCF10A cells were cultured in MEGM (Cascade Biologicals). All cells were cultured at 37°C and 5% $CO_2$ and routinely checked for mycoplasma contamination.

*Reagents*

The telomerase template antagonist used in this study is complementary to the telomerase RNA component (hTR) template region for telomerase (5'-CUAACCCUAAC-3'). The lipid-modified N3'→P5' thio-phosphoramidate oligonucleotide (GRN163L, 5'-Palm-TAGGGTTAGACAA-NH2-3') and the 5' -palmitoyl mismatch control oligonucleotide (5'-Palm-TAGGTGTAAGCAA-NH2-3'), was prepared as previously described (Herbert et al. 2005). Paclitaxel was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved as a 10 mM stock in dimethyl sulfoxide (DMSO).

*Telomerase activity assay*

Telomerase activity was measured using the polymerase chain reaction– based Telomeric Repeat Amplification Protocol (TRAP) as previously described (Chapter Two; Hochreiter et al. 2006, Wright et al. 1995). After primer extension with a Cy5 fluorescently labeled telomerase template primer and polymerase chain reaction amplification, the TRAP reaction products were run on a 10% nondenaturing acrylamide gel. The gel was visualized, without drying, on a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Telomerase activity was demonstrated as a 6-bp telomerase-specific ladder above the 36-bp internal standard control band (IC).

*Cell Growth and Viability Assays*

In order to determine how treatment with telomerase template antagonists or trastuzumab affect cell growth and viability, $5x10^4$ cells were plated with 2.5 μM

GRN163L or mismatch oligonucleotides in 12-well dishes. Cells were collected weekly and counted via a Beckman Coulter Counter to determine cell number. Triplicate samples were combined prior to replating to avoid selection of resistant cells. To determine the effects of immediate treatment on cell viability, 10 μl cell suspension was mixed with 10 μl Trypan blue and counted via hemacytometer.

*BrdU assay*

Cell proliferation was measured using the 5'-Bromo-2'-deoxy-uridine Labeling and Detection Kit (Roche Applied Sciences, Indianapolis, IN) according to manufacturer's instructions. Briefly, 2 x $10^4$ MDA-MB-231 cells were plated in 96-well plates with 0.5, 1, or 2.5 μM GRN163L or 2.5 μM mismatch oligonucleotides for 72 hours. 10 μM BrdU was added for 18 hours prior to determination of cell proliferation. Absorbance was read at 490 nm. Student's t-test was performed using Microsoft Excel.

*Colony formation assays*

MDA-MB-231 cells were treated with 1 μM GRN163L for ~5 days prior to plating for colony formation assays. 2-6 x $10^2$ cells were plated in 25$cm^2$ flasks. Six 25$cm^2$ flasks were plated for each treatment group, and colonies allowed to grow for 12-14 days without changing the media or adding any drugs prior to staining with 20% Giemsa. Colonies with more than 50 cells were counted to determine the survival fraction of each group. Plating efficiency (PE) was calculated by dividing the average number of colonies by the number of cells initially plated. Experiments were repeated at least in triplicate. Student's t-test was performed using Microsoft Excel.

*Cell death assay*

Cell death was measured using the Cell Death Detection ELISA from Roche (Roche Applied Sciences, Indianapolis, IN) according to manufacturer's instructions. Briefly, MDA-MB-231 cells were treated with 2.5 μM GRN163L or mismatch oligonucleotides for 72 hours. 5 μM doxorubicin (Sigma Chemical Co., St. Louis, MO) was used as a positive control for the induction of apoptosis. Student's t-test was performed using Microsoft Excel.

*Focal adhesion and actin filament immunofluorescence*

Staining for focal adhesion formation and actin filament organization was done using the Actin Cytoskeleton and Focal Adhesion Staining Kit from Chemicon International (Temecula, CA) as per manufacturer's protocol. Briefly, 5 x $10^4$ MDA-MB-231 cells were plated in chamber slides and grown for 72 hours. Cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton-X-100. Staining was done in 3% bovine serum albumin (Sigma Chemical Co., St. Louis, MO).

*Western immunoblotting*

Cells were harvested after treatment and lysed in buffer containing 5% 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. 10-50 μg protein lysate was subjected to SDS-PAGE followed by a two hour transfer to PVDF 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: anti-β-tubulin antibody and anti-γ-tubulin (Sigma Chemical Co., St. Louis, MO), anti-Pyk2 and anti-FAK (Upstate Cell Signaling Solutions, Lake Placid, NY), and GAPDH (Chemicon International, Temecula, CA). Densitometry levels for each blot was determined using β-actin as a loading control; relative intensity levels for each sample were plotted and normalized to untreated (UT) samples.

*Cell Growth and Viability Assays to Determine the Combination Index*

In order to determine how treatment with telomerase template antagonists or paclitaxel affect cell growth and viability, $5x10^4$ cells were plated in 12-well dishes in triplicate and were treated with 1:2 serial dilutions of GRN163L (0.005-1.25 μM) or paclitaxel (0.5-12.5 nM) in culture media. Cells were then collected after five days to obtain cell number and viability as measured by the Trypan blue exclusion assay. Percent inhibition of cell growth compared to untreated cells was determined and plotted. In order to determine the effects of combination therapy on breast cancer cells, parallel studies were done in which cells were treated with 1:2 serial dilutions of GRN163L or mismatch oligonucleotides (0.005-2.5 μM) in combination with paclitaxel (0.5-12.5 nM), in which the two agents were at a 1:10 ratio of each other (paclitaxel: GRN163L). CalcuSyn software (Biosoft, Cambridge, United Kingdom) was used to determine the combined effect of multiple drug treatment via the Chou and Talalay method (Chou and Talalay 1984). This program analyzes the effect of combining drugs with different mechanisms of action in order to determine whether single drug therapy is improved upon the addition of a second compound. A combination index (CI) value of <1.0

denotes synergistic interaction (more than additive), a CI of 1.0 demonstrates summation (additive), and a CI >1.0 indicates antagonistic interaction (less than additive).

*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. $1x10^6$ MDA-MB-231 breast cancer cells were injected into the mammary fat pads of 6- to 8-weekold mice. Following a recovery period of 2 days, mice began receiving treatment of 30 mg/kg GRN163L (n = 30) or a PBS solvent control (n = 30) three times weekly intraperitoneally (IP) or 15 mg/kg paclitaxel (n=30) once weekly via IP, alone or in combination with 30 mg/kg GRN163L (n=30). Average weight of animals and size of tumors were similar among treatment groups. Tumor volume was calculated as (length x $width^2$) / 2 (in $mm^3$). Student's t-test was performed using Microsoft Excel.

*Cell invasion assay*

Matrigel invasion assays were performed using the Cell Invasion Assay kit from Chemicon International (Temecula, CA) as per manufacturer's protocol. MDA-MB-231 cells were treated with 0.156-0.3125 μM GRN163L or mismatch oligonucleotides or 16-31 nM paclitaxel for five days. In order to determine the effects of combination therapy on the invasive potential of MDA-MB-231 breast cancer cells, parallel studies were done in which cells were treated with 0.156-0.3125 μM GRN163L or mismatch

oligonucleotides in combination with paclitaxel (16-31 nM), in which the two agents were at a 1:10 ratio of each other. The invasion assay was performed using 1.5 x $10^5$ cells per well without further addition of drug. The bottom chamber of the invasion chambers contained media with 10% cosmic calf serum (HyClone, Logan, UT) as a chemoattractant. Invasion was measured 24 hours after plating. Stained cells were dissolved in 10% acetic acid and the absorbance read at 560 nm. Student's t-test was performed using Microsoft Excel.

## Chapter Four

## Telomerase Template Antagonists Augment the Effects of Trastuzumab in HER2-positive Breast Cancer Cells

This work is reprinted from *Breast Cancer Research and Treatment*; Erin M. Goldblatt, Priscilla A. Erickson, Erin R. Gentry, Sergei M. Gryaznov, and Brittney-Shea Herbert, "Lipid-conjugated telomerase template antagonists sensitize resistant HER2-positive breast cancer cells to trastuzumab," DOI: 10.1007/s10549-008-0201-4 (2008) with permission from Springer.

### Abstract

HER2 amplification is found in 10-20% of breast cancers and is associated with a more aggressive disease, greater likelihood of recurrence, and decreased survival compared to women with HER2-negative (HER2-) breast cancer. Trastuzumab is a monoclonal antibody that inhibits HER2 activity, making this compound an important therapeutic option for patients with HER2-positive (HER2+) breast cancer. However, resistance to trastuzumab develops rapidly in a large number of breast cancer patients. The objective of this study was to determine whether GRN163L, a telomerase template antagonist currently in clinical trials for cancer treatment, can augment the effects of trastuzumab in breast cancer cells with HER2 amplification. Results demonstrate that GRN163L is an effective telomerase inhibitor and shortens telomeres in HER2+ breast cancer cells. Additionally, combining GRN163L with trastuzumab synergistically inhibits HER2-positive breast cancer cell growth. More importantly, GRN163L restored the sensitivity of therapeutically resistant breast cancer cells to trastuzumab. These findings implicate that telomerase template antagonists have potential use in the treatment of cancers that have developed resistance to traditional cancer therapy.

## Introduction

HER2 (human epidermal growth factor receptor 2) is a member of the ErbB/EGFR family of transmembrane receptor tyrosine kinases that plays a role in tumor cell growth, invasion, and metastasis (Slamon et al. 2001, Ross et al. 2004, Hudis 2007). HER2 amplification is found in approximately 10-20% of breast cancers and is associated with a more aggressive disease, greater likelihood of recurrence, poorer prognosis and decreased survival compared to women with HER2-negative breast cancer (Ross et al. 2004, Menendez et al. 2005, Sledge 2004). In addition, HER2 overexpression is associated with resistance to chemo- and endocrine therapies (Menendez et al. 2005, Burstein 2005, Tan et al. 1997).

Trastuzumab (Herceptin™) is a monoclonal antibody that binds the extracellular portion of HER2 and is thought to induce antibody-dependent cellular cytotoxicity, prevent receptor activation and signaling, inhibit angiogenesis, and induce apoptosis (Ross et al. 2004, Hudis 2007). Trastuzumab has become an important therapeutic option for patients with HER2-positive breast cancer and is widely used for treating advanced metastatic disease (Sledge 2004, Ross et al. 2003). Importantly, trastuzumab works well in combination with many chemotherapy agents, resulting in better response to treatment, increased time to disease progression, and increased survival (Nicolini et al. 2006, Pegram et al. 2004, Ross 2003). Trastuzumab administration, however, can be associated with adverse side effects including ventricular dysfunction, cardiac failure, infusion reactions, and pulmonary events (Sledge 2004, Pegram et al. 2004). In addition, not all HER2 overexpressing breast cancer cells respond to trastuzumab treatment as resistance develops rapidly in a large number of patients (Menendez et al. 2005, Burstein 2005, Tan

et al. 1997, Nahta et al. 2007a, Nahta et al. 2007b). Importantly, the negative side effects of trastuzumab can be exacerbated by the effects of chemotherapy drugs or radiation.

It has been proposed that HER2 amplification is associated with increased telomerase (hTERT) expression and telomerase activity in breast cancer tissue samples and cell lines (Goueli et al. 2004), although this association remains controversial. Telomerase is essential for unlimited cellular proliferation and tumorigenesis and has been shown to be increased in almost all human malignancies as compared to benign lesions (Shay and Bacchetti 1997, Kim et al. 1994). Telomerase inhibition results in the erosion of telomeric DNA, genomic instability, and eventually to apoptosis or senescence (Herbert et al. 2005, Gellert et al. 2006, Shay 2003, Hochreiter et al. 2006, Dikmen et al. 2005). Previous studies from our laboratory have shown that the telomerase template antagonist GRN163L, currently in clinical trials, inhibits telomerase activity, shortens telomeres, and reduces colony formation ability and replicative capacity of breast cancer cells. We also showed that GRN163L reduces tumor growth and metastasis of human breast cancer cells *in vivo* (Hochreiter et al. 2006). Inhibiting telomerase activity may affect how breast cancer cells respond to other therapy by reducing the ability for continual cellular replication. This suggests that the use of telomerase inhibitors in therapy regimens may be clinically applicable in the treatment of breast cancers, including the HER2 subset.

The purpose of this study was to determine whether the telomerase template antagonist GRN163L can augment the effects of trastuzumab in breast cancer cells with HER2 amplification. The combined effects of HER2 inhibition by trastuzumab and telomerase inhibition resulted in synergistic inhibition of breast cancer cell growth. In

addition, GRN163L, alone and in combination with trastuzumab, inhibited the growth breast cancer cells that had acquired resistance to trastuzumab. These results provide new insight into new strategies into the treatment of this particular subset of breast cancers.

## Results

### *Lipid-conjugated thio-phosphoramidate telomerase template antagonists inhibit telomerase activity and shorten telomeres in HER2-amplified breast cancer cells*

HER2 amplification has been linked with an increase in hTERT expression and telomerase activity in breast tumor tissue and cell lines (Goueli et al. 2004), which indicates that the use of telomerase inhibitors in therapy regimens for HER2 breast cancers may be clinically applicable. To test the hypothesis that HER2+ breast cancers have elevated telomerase activity levels compared to HER2- breast cancers, the baseline telomerase activity was determined in both HER2+ and HER2- breast cancer cell lines (Figure 4.1). In addition, the baseline protein levels of HER2 were determined. MDA-MB-231 breast cancer cells, which do not have HER2 amplification, had one of the lowest baseline telomerase activity levels of all cell lines tested, as well as nondetectable levels of HER2 expression. Both the MCF7/HER2 cells, which have exogenous HER2 expression, and trastuzumab-resistant SKBR3-R cells exhibited the highest amount of telomerase activity compared to their parental MCF7 (an empty vector infected MCF7 cells) and SKBR3 cells, respectively.

To validate lipid-conjugated thio-phosphoramidates as effective telomerase inhibitors in breast cancer cell lines with HER2 overexpression, cells were

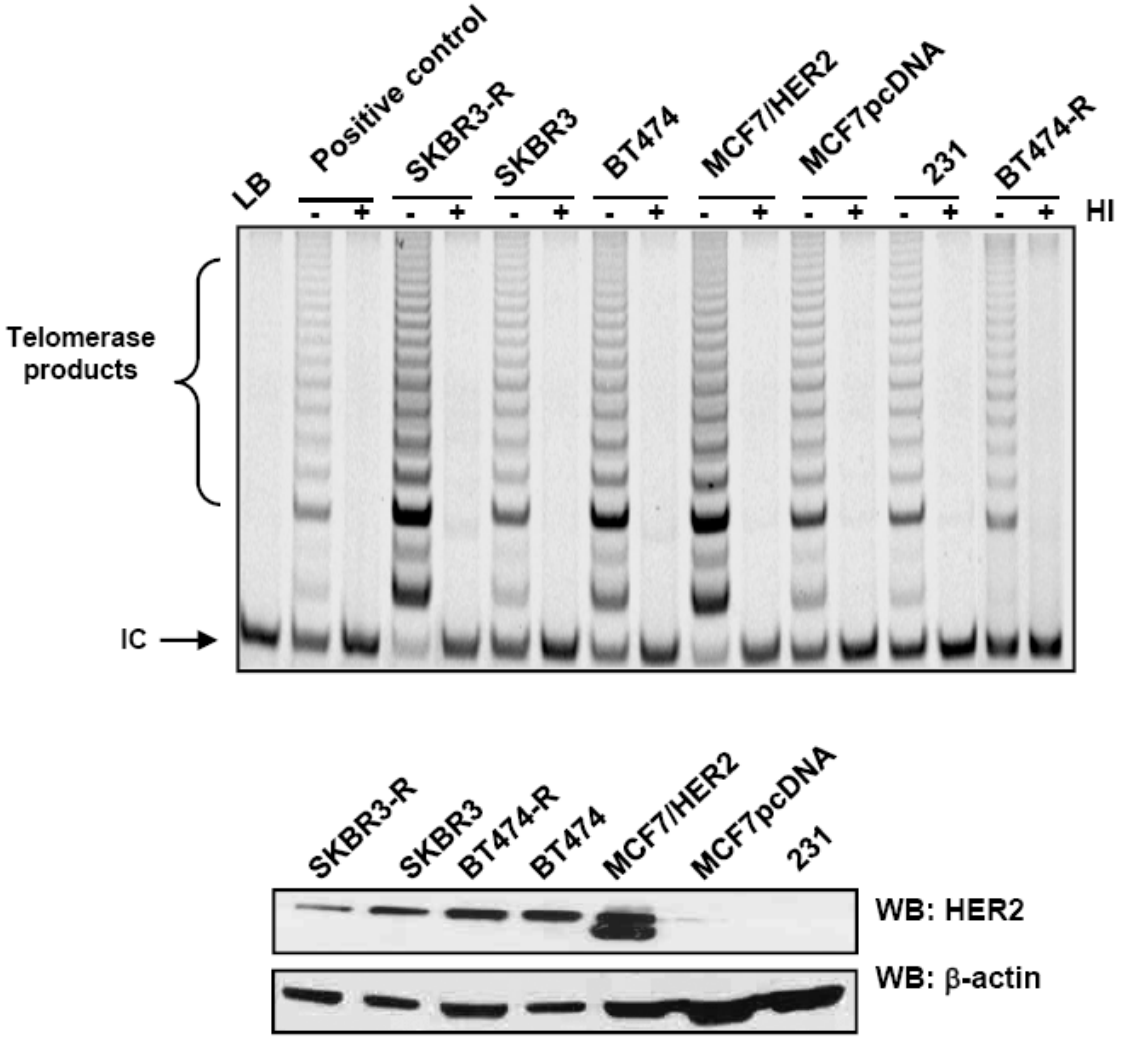


**Figure 4.1**. **Telomerase activity levels and HER2 levels.** Comparison of the baseline telomerase activity and HER2 protein levels in HER2+ vs. non-HER2 subtype breast cancer cells. Telomerase activity was analyzed using the Telomerase Repeat Amplification Assay (TRAP) with MDA-MB-231, SKBR3, BT-474, MCF7 pcDNA, or MCF7/HER2- breast cancer cells. Results shown are the telomerase products depicted as a ladder. A positive control cell extract (TRAPeze® Telomerase Detection kit) and NP-40 lysis buffer (LB) were used as a positive and negative control, respectively. HI= heat inactivated control samples; IC= internal control. Lower panel depicts HER2 expression by western immunoblotting where 15 μg protein was loaded for all cells with HER2 amplification, and 50μg protein was loaded for the non-HER2 subtypes (231 and MCF-7pcDNA cells). β-actin served as a loading control.

treated with a 1:10 serial dilution series of two telomerase template antagonists, GRN163L and 5'oleic163 (0.001-10 μM), for 24 hrs and collected for analysis of telomerase activity levels. Previous reports have shown that oleic acid, the main monounsaturated fat in olive oil, can regulate HER2 expression and activity (Menendez et al. 2005, Menendez et al. 2004, Menendez et al. 2007). This can in turn inhibit cell proliferation, induce apoptosis, and alter downstream targets of HER2 signaling in HER2+ breast cancer cells. These reports also showed that oleic acid worked synergistically with trastuzumab to enhance the effects of either drug alone (Menendez et al. 2004, Menendez et al. 2007). To this end, the inhibition of telomerase activity induced by 5'oleic163 was compared to the inhibition induced by GRN163L to determine whether the type of the lipid group (oleic versus palmitoyl) would make a difference in the level of telomerase inhibition in HER2+ breast cancer cells. The $EC_{50}$ for telomerase inhibition was in the low nM range for both compounds, indicating the universal potency of lipidated telomerase inhibitors (Herbert et al. 2005; data not shown). Both telomerase template antagonists inhibited telomerase activity in a dose-dependent manner regardless of HER2 status (Figure 4.2; Hochreiter et al. 2006).

We and others previously reported that GRN163L progressively shortens telomeres in a wide range of breast cancer cell lines (Herbert et al. 2005, Gellert et al. 2006, Hochreiter et al. 2006, Dikmen et al. 2005, Gomez-Millan et al. 2007). To confirm that treatment with telomerase template antagonists also efficiently shortens telomeres in HER2+ breast cancer cells, SKBR3 cells were grown for eight weeks in the presence of GRN163L, 5'oleic163, or mismatch oligonucleotides. Treatment with GRN163L and 5'oleic163 resulted in progressive telomere shortening, averaging ~100 bp per

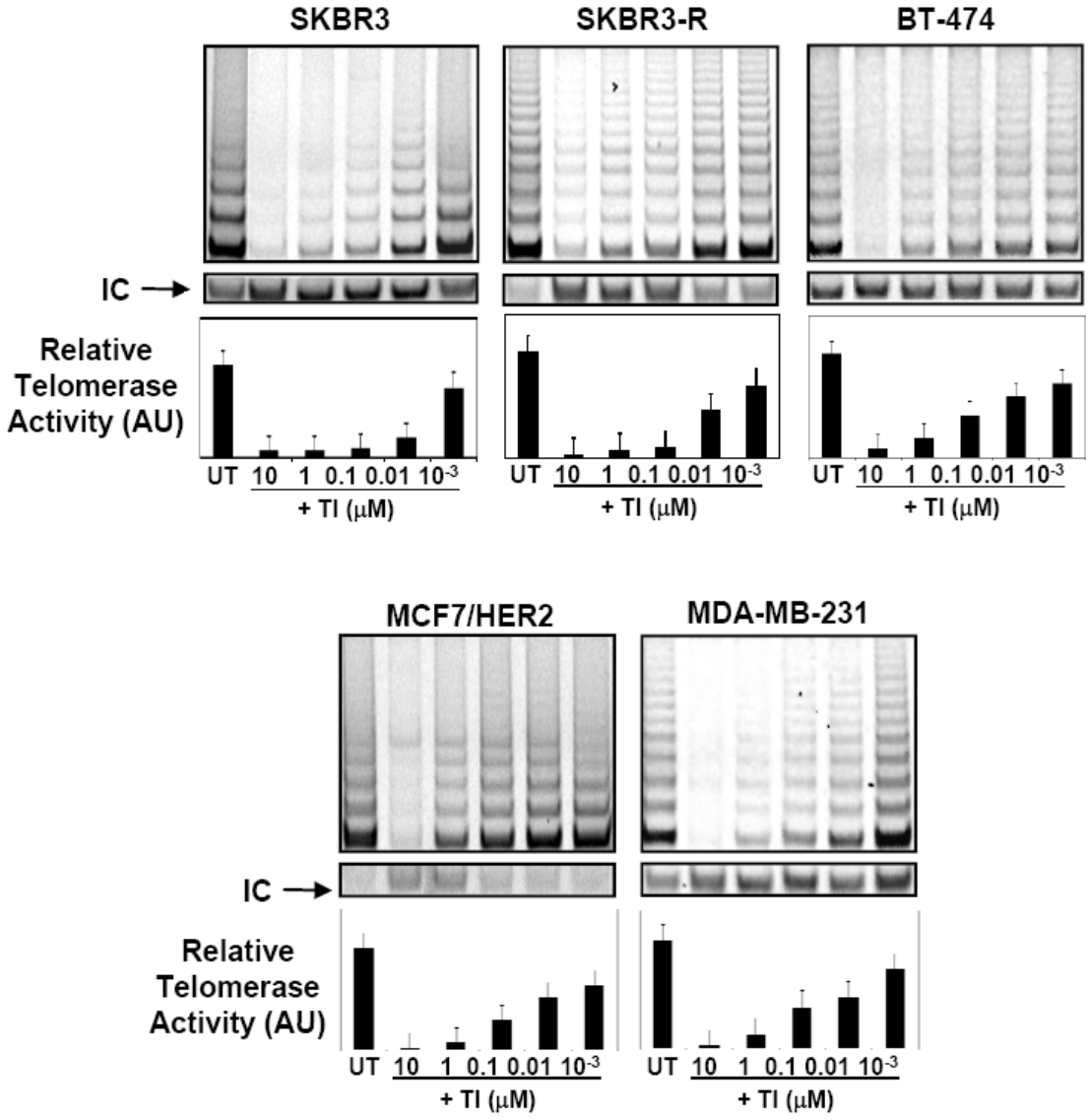


**Figure 4.2. Effects of lipid-conjugated telomerase template antagonists on telomerase activity in HER2+ breast cancer cells.** Telomerase template antagonists effectively inhibit telomerase activity in breast cancer cells in a dose-dependent fashion regardless of HER2 status. Breast cancer cells were treated with a 1:10 serial dilution of 0.001-10 μM 5'oleic163. Cells were collected after 24-72 hours for telomerase activity assays. MDA-MB-231, MCF7 pcDNA, and BT-474 samples demonstrate telomerase inhibition after 24 hours. MCF7/HER2 and SKBR3 cells, which have higher baseline telomerase activity, also take longer for inhibition (48-72 hours respectively). Relative telomerase activity was determined as in Figure 4.1 and is depicted as arbitrary units (AU). Results are representative of multiple, independent experiments.

population doubling compared with untreated or mismatch control treated cells (Hochreiter et al. 2006; data not shown), confirming that telomerase template antagonists are effective at inhibiting telomere maintenance and survival in HER2+ breast cancer cells.

*GRN163L significantly inhibits cell proliferation*

To determine the effect of treatment with GRN163L on HER2+ breast cancer cell growth, SKBR3 and BT474 cells were plated onto tissue culture dishes in triplicate. As shown in Figure 4.3a, treatment with GRN163L inhibited SKBR3 breast cancer cell growth over time. Treatment with mismatch oligonucleotide had no effect on cell growth, indicating that the effect on cell growth is not related to the use of lipidated oligonucleotides, but is intrinsic to the telomerase template antagonist. The total population doublings per week were calculated to determine the rate of growth inhibition. As shown in Figure 4.3b, the average time for one population doubling increased from 2.4 days in untreated cells, to 4 days in cells treated with GRN163L. As expected, the mismatch oligonucleotide again had no effect on population doubling time. Similar results were seen with BT74 and MCF7/HER2 cells (data not shown).

*Telomerase template antagonists synergistically enhance the effects of trastuzumab on inhibiting cell proliferation in HER2+ breast cancer cells*

To determine whether the GRN163L can augment the inhibitory effects of trastuzumab on cell growth in HER2+ breast cancer cells, SKBR3 cells were plated

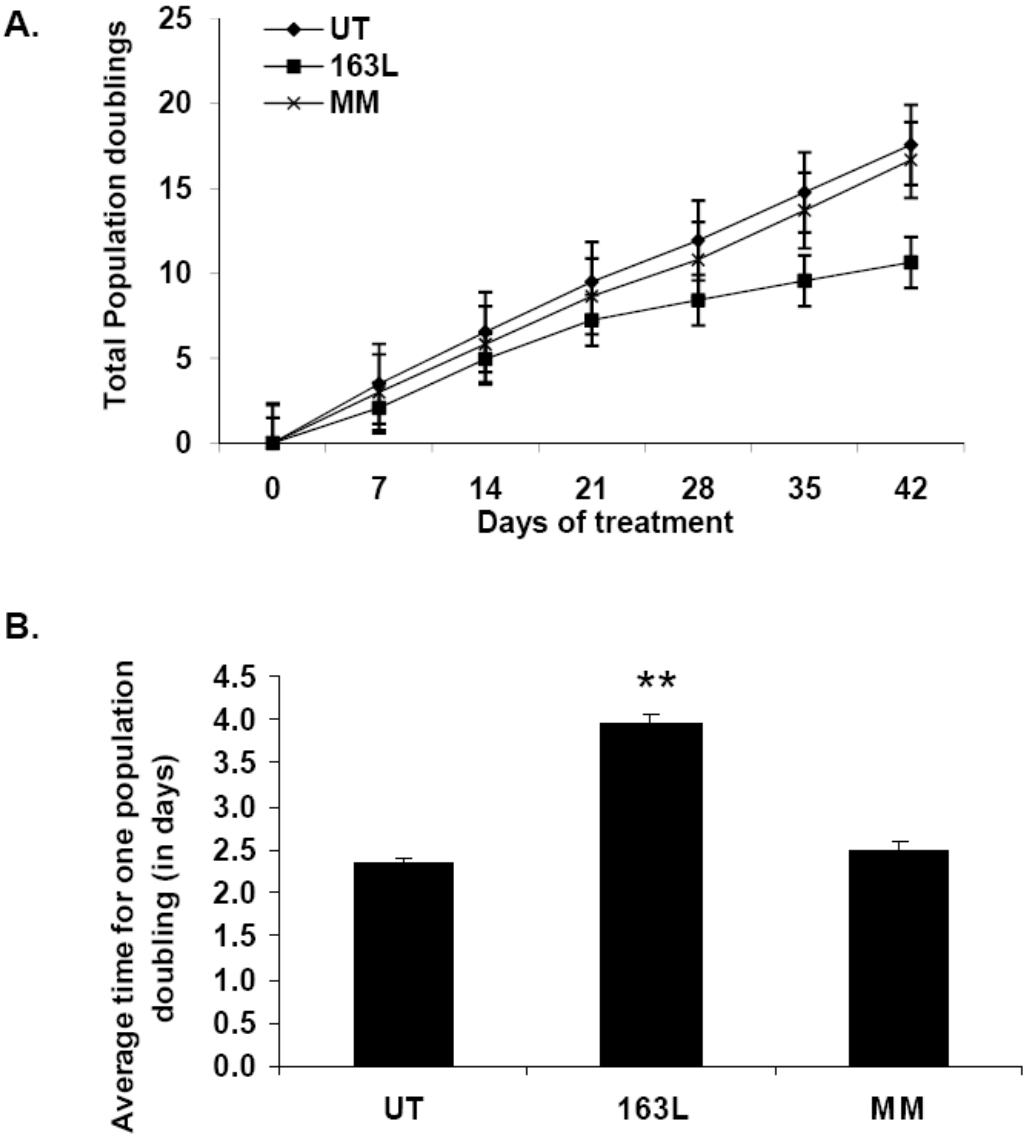


**Figure 4.3. Effects of GRN163L on HER2+ breast cancer cell growth.** (A) 5 x $10^4$ SKBR3 cells were plated in 6-well plates in triplicate. Cells were treated with 2.5 μM GRN163L or mismatch (MM) oligonucleotide, replenishing the drug every third day. After 7 days, cells were collected and counted to determine population doublings. Each sample was counted in triplicate. Samples were combined before replating to avoid selection for resistant cells. (B) Average doubling time (in days) was determined for in response to GRN163L or mismatch (MM) oligonucleotides. GRN163L significantly increases the average time for the population of SKBR3 cells to double once (in days, $p = 0.0015$). Mismatch oligonucleotide has no effect on doubling time ($p = 0.095$). Student's t-test was performed using Microsoft Excel.

with 0.0195-10 μM GRN163L either alone or with 0.005-2.5 μM trastuzumab for five days. Cells were collected and counted to determine the effect of individual or combination therapy on cell growth and viability. SKBR3 cells demonstrated a dose-dependent response to growth inhibition by trastuzumab and GRN163L (Figure 4.4). A lipidated, mismatch oligonucleotide control did not exhibit significant effects on cell growth inhibition. The combination of GRN163L and trastuzumab resulted in a significant shift in the growth inhibition curve compared to either agent alone (Figure 4.4, upper panel). Low doses of GRN163L were used to rule out any possible toxicity induced by the oligonucleotide. The synergistic effect of combination treatment was found even at low nM doses of GRN163L, where telomerase is still inhibited. In addition, the mismatch control did not alter the growth inhibition effects of trastuzumab. The statistical combination index (CI) was determined for the dual therapy to determine whether combination therapy was synergistic, additive, or antagonistic. As shown in Figure 4.4 (lower panel), GRN163L acted synergistically with trastuzumab to inhibit cell growth of SKBR3 cells at all combination doses tested. Furthermore, similar observations were made for SKBR3 cells which exhibited a large population of short, dysfunctional telomeres (Hochreiter et al. 2006) due to extended treatment with the telomerase inhibitors (Table 4.1). Similar results were obtained with a 5'oleic163, suggesting that the specific lipid group modification did not make a difference in HER2+ breast cancer cells (Table 4.2).

To determine whether the synergism between lipid-conjugated telomerase template antagonists and trastuzumab could be observed in other cell lines, BT474 (HER2+) breast cancer cells were treated as above to determine percent growth

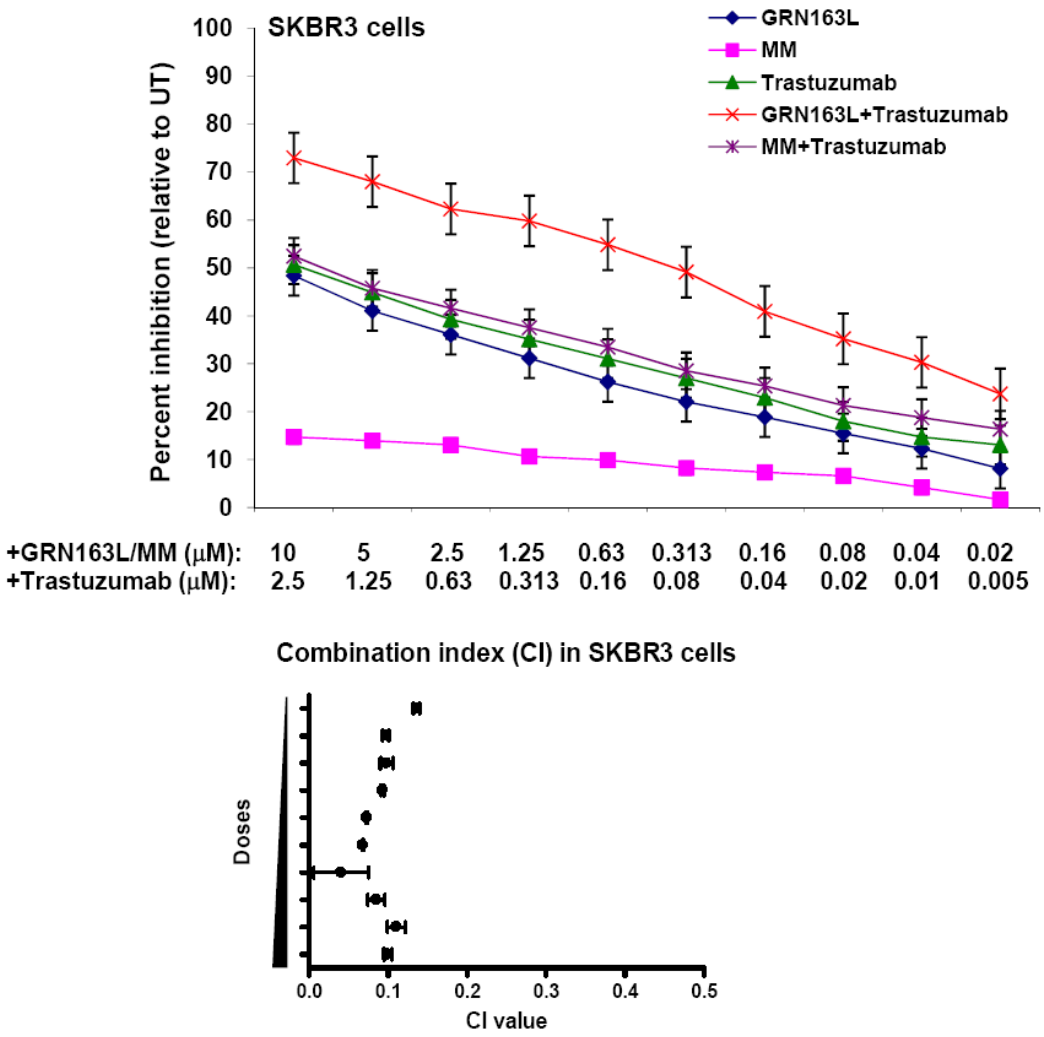


**Figure 4.4. GRN163L augments the effects of trastuzumab in HER2+ SKBR3 breast cancer cells.** SKBR3 cells were plated in 12-well dishes and treated with 1:2 serial dilutions of GRN163L or a mismatch oligonucleotide alone (MM), trastuzumab alone, or the combination of GRN163L/MM with trastuzumab at a 1:4 ratio (trastuzumab: GRN163L/MM). Cells were collected after five days to determine cell number and viability. Percent inhibition was plotted versus concentration for each treatment group (top panel). Calcusyn software (Biosoft, Cambridge UK) was used to ascertain the combination index (CI) value for each combination ratio (Dose), from highest to lowest (bottom to top) to determine whether combination treatments have an additive or synergistic effect (lower panel; ±SD). A CI value of CI <1.0 denotes synergistic interaction (more than additive), a CI of 1.0 demonstrates summation (additive), and a CI >1.0 indicates antagonistic interaction (less than additive).

**Table 4.1. Combination treatments with GRN163L and trastuzumab synergistically inhibit cell growth of SKBR3 breast cancer cells pre-treated with GRN163L to induce telomere shortening.**

| Sample: | Trastuzumab (μM) | GRN163L (μM) | Ratio | % Growth Inhibition | Fold increase (vs. Trastuzumab) | CI value |
|---|---|---|---|---|---|---|
| SKBR3+GRN163L (10-15 weeks) | 2.5 | 10 | 1:4 | 69.5 ± 4.1 | 1.77 | 0.111 |
| | 1.25 | 5 | 1:4 | 50.1 ± 2.5 | 1.34 | 0.297 |
| | 0.625 | 2.5 | 1:4 | 40.8 ± 10.6 | 1.35 | 0.323 |
| | 0.3125 | 1.25 | 1:4 | 33.91 ± 0.8 | 1.46 | 0.299 |
| | 0.156 | 0.625 | 1:4 | 30.33 ± 1.0 | 2.16 | 0.212 |

Cells were treated with a 1:4 combination (Ratio) of trastuzumab: GRN163L at the indicated concentrations. Percent growth inhibition of the combination treatments was calculated compared to untreated (media only) cells. The fold increase in percent growth inhibition of the combination treatment compared to trastuzumab alone was calculated (Fold increase). Calcusyn software (Biosoft, Cambridge UK) was used to ascertain the combination index to determine whether combination treatments have an additive or synergistic effect. A combination index (CI) value of CI <1.0 denotes synergistic interaction (more than additive), a CI of 1.0 demonstrates summation (additive), and a CI >1.0 indicates antagonistic interaction (less than additive).

**Table 4.2. Combination treatments with 5'oleic163 and trastuzumab synergistically inhibit breast cancer cell growth.**

| Cell line: | Trastuzumab (μM) | 5'oleic163 (μM) | Ratio | % Growth Inhibition | Fold increase (vs. Trastuzumab) | CI value |
|---|---|---|---|---|---|---|
| SKBR3 | 2.5 | 10 | 1:4 | 58.4 ± 1.2 | 1.25 | 0.527 |
| | 1.25 | 5 | 1:4 | 56.31 ± 0.03 | 1.35 | 0.307 |
| | 0.625 | 2.5 | 1:4 | 52.6 ± 1.0 | 1.36 | 0.209 |
| | 0.3125 | 1.25 | 1:4 | 48.3 ± 1.2 | 1.35 | 0.161 |
| | 0.156 | 0.625 | 1:4 | 45.2 ± 3.9 | 1.5 | 0.116 |
| SKBR3-R | 2.5 | 10 | 1:4 | 80.4 ± 3.5 | 11.14 | 0.162 |
| | 1.25 | 5 | 1:4 | 76.6 ± 3.1 | 12.38 | 0.132 |
| | 0.625 | 2.5 | 1:4 | 72.7 ± 4.2 | 20.21 | 0.103 |
| | 0.3125 | 1.25 | 1:4 | 68.3 ± 6.0 | 26.59 | 0.082 |
| | 0.156 | 0.625 | 1:4 | 65.5 ± 6.3 | 63.85 | 0.054 |

Cells were treated with a 1:4 combination (Ratio) of trastuzumab: 5'oleic163 at the indicated concentrations. Percent growth inhibition of the combination treatments was calculated compared to untreated (media only) cells. The fold increase in percent growth inhibition of the combination treatment compared to trastuzumab alone was calculated (Fold increase). Calcusyn software (Biosoft, Cambridge UK) was used to ascertain the combination index to determine whether combination treatments have an additive or synergistic effect. A combination index (CI) value of CI <1.0 denotes synergistic interaction (more than additive), a CI of 1.0 demonstrates summation (additive), and a CI >1.0 indicates antagonistic interaction (less than additive).

inhibition compared to untreated cells as well as the combination index. BT474 cells also showed a similar response to growth inhibition by trastuzumab and GRN163L as with the SKBR3 cells (Figure 4.5, upper panel). As with the SKBR3 cells, dual therapy of GRN163L and trastuzumab had synergistic effects on inhibiting cell growth of BT474 cells at all combination doses tested (Figure 4.5, lower panel). In addition, MCF7 cells overexpressing HER2 (MCF7/HER2) treated with GRN163L and trastuzumab also showed synergistic effects on cell proliferation. The combination of GRN163L and trastuzumab was synergistic (CI <1.0) for cell growth inhibition at all dose combinations tested for MCF7/HER2 cells. MCF7 control cells (MCF7pcDNA) did not respond to trastuzumab, no additional growth inhibitory effect was seen when combined with GRN163L or 5'oleic163 in these cells, and the combination index was greater than 1.0 (Table 4.3; data not shown).

*Telomerase template antagonists in combination with trastuzumab inhibit the growth of trastuzumab-resistant HER2+ breast cancer cells.*

To determine the effects of combination treatment on HER2+ breast cancer cells, the sensitivity to trastuzumab was first tested in both parental and trastuzumab-resistant SKBR3 cells. Parental SKBR3 cells had an 83% reduction in growth in response to 10 μg/ml trastuzumab ($p < 0.001$), whereas trastuzumab-resistant SKBR3-R cells had <10% reduction in growth in response to 10-20 μg/ml of trastuzumab (data not shown), which corroborated previously published reports (Nahta et al. 2004). Interestingly, the trastuzumab-resistant SKBR3-R cells were more sensitive to both telomerase inhibitors

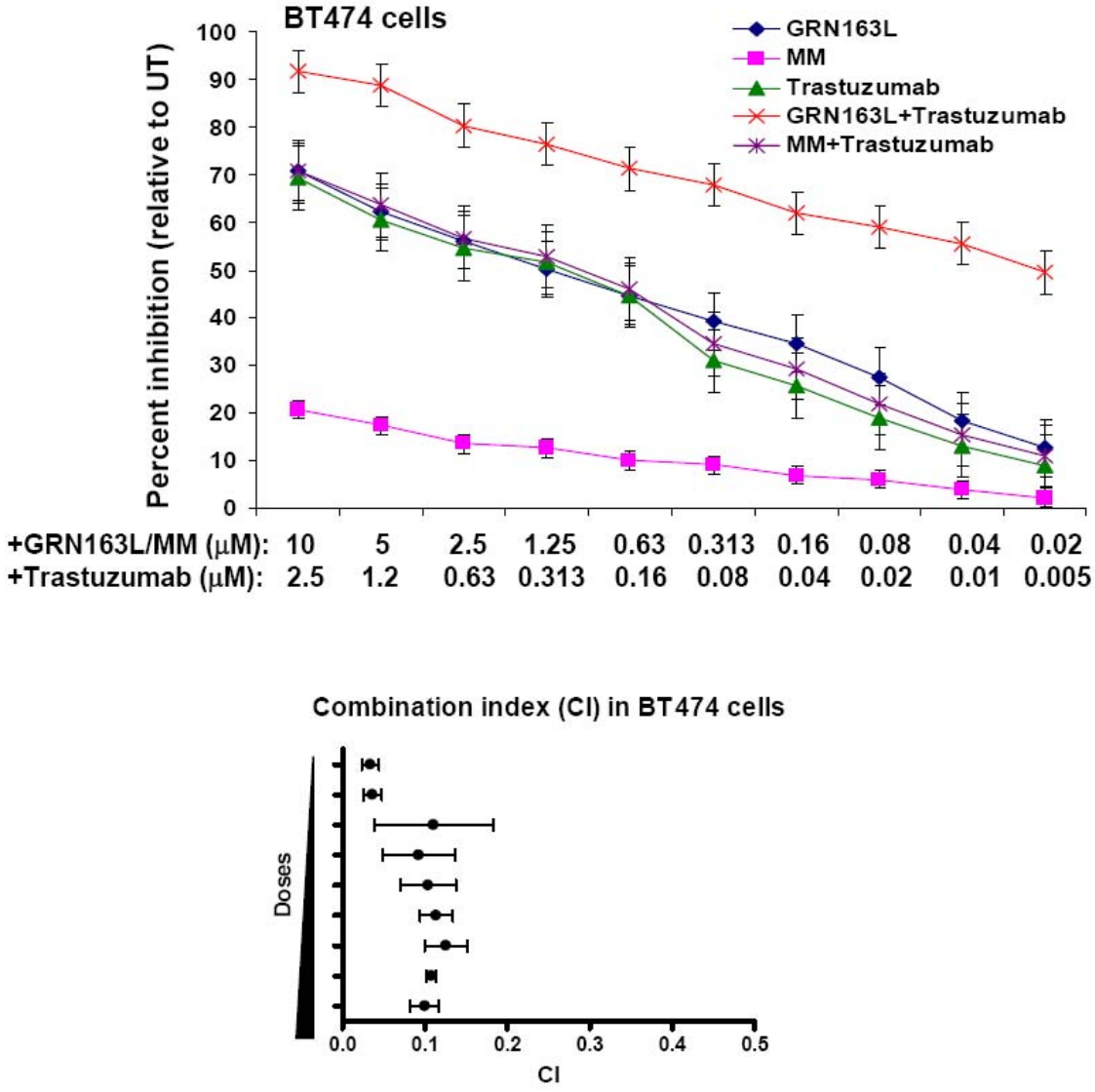


**Figure 4.5. GRN163L augments the effects of trastuzumab in HER2+ BT474 breast cancer cells.** BT474 cells were plated as in Figure 4.4. Cells were collected after five days to determine cell number (and viability). Percent growth inhibition was plotted versus concentration for each treatment group (top panel). Calcusyn software (Biosoft, Cambridge UK) was used to ascertain the combination index (CI) value for each combination ratio (Dose), from highest to lowest (bottom to top) to determine whether combination treatments have an additive or synergistic effect (lower panel; ±SD).

**Table 4.3. Combination treatments with GRN163L and trastuzumab synergistically inhibit cell growth of MCF7 breast cancer cells overexpressing HER2, but not MCF7pcDNA cells.**

| Cell line: | Trastuzumab (μM) | GRN163L (μM) | Ratio | CI value | Fold increase in growth inhibition vs. trastuzumab alone (MCF7/HER2 cells only) |
|---|---|---|---|---|---|
| MCF7pcDNA | 2.5 | 10 | 1:4 | 1.522 | |
| | 1.25 | 5 | 1:4 | 1.099 | |
| | 0.625 | 2.5 | 1:4 | 1.050 | |
| | 0.3125 | 1.25 | 1:4 | 1.026 | |
| | 0.156 | 0.625 | 1:4 | 1.133 | |
| MCF7/HER2 | 2.5 | 10 | 1:4 | 0.100 | 2.68 |
| | 1.25 | 5 | 1:4 | 0.137 | 2.93 |
| | 0.625 | 2.5 | 1:4 | 0.135 | 3.05 |
| | 0.3125 | 1.25 | 1:4 | 0.154 | 4.21 |
| | 0.156 | 0.625 | 1:4 | 0.146 | 4.71 |

Cells were treated with a 1:4 combination (Ratio) of trastuzumab: GRN163L at the indicated concentrations. Percent growth inhibition of the combination treatments was calculated compared to untreated (media only) cells. Calcusyn software (Biosoft, Cambridge UK) was used to ascertain the combination index to determine whether combination treatments have an additive or synergistic effect. A combination index (CI) value of CI <1.0 denotes synergistic interaction (more than additive), a CI of 1.0 demonstrates summation (additive), and a CI >1.0 indicates antagonistic interaction (less than additive). The fold increase in percent growth inhibition of the combination treatment compared to trastuzumab alone was calculated for MCF7/HER2 cells only which had a CI index of < 1.0.

compared to the parental SKBR3 cells. More importantly, telomerase inhibitors in combination with trastuzumab resulted in synergistic effects (CI < 1.0) on cell growth inhibition compared to either agent alone (Figure 4.6, bottom panel) at all combination doses tested. BT474 cells that had acquired resistance to trastuzumab (BT474-R), as previously described (Ritter et al. 2007), also showed enhanced sensitivity to the combination of GRN163L and trastuzumab compared to either agent alone (Figure 4.7). The combination index for BT474-R cells at all doses was also <1.0 (Figure 4.7, bottom panel).

*GRN163L and trastuzumab, alone or in combination, have no significant effect on cell growth of non-tumorigenic human mammary epithelial cells (HMECs) and MCF10A cells.*

To determine whether the effects of lipid-conjugated telomerase template antagonists and trastuzumab on cancer cell growth could also be seen in non-tumorigenic breast epithelial cells, HMECs from a patient with no history of breast cancer as well as the non-tumorigenic MCF10A cells were treated in the same manner as in the experiments with the SKBR3 and BT474 cells. These cells do not contain amplified levels of HER2 and are not expected to be sensitive to trastuzumab. As shown in Figure 4.8, GRN163L, a mismatch oligonucleotide control, and trastuzumab had no significant effect on the proliferation of HMECs and MCF10A cells, either alone or in combination. These results further support the suggestion of specificity of telomerase template antagonists towards cancer cells without limiting the proliferation and viability of normal cells.

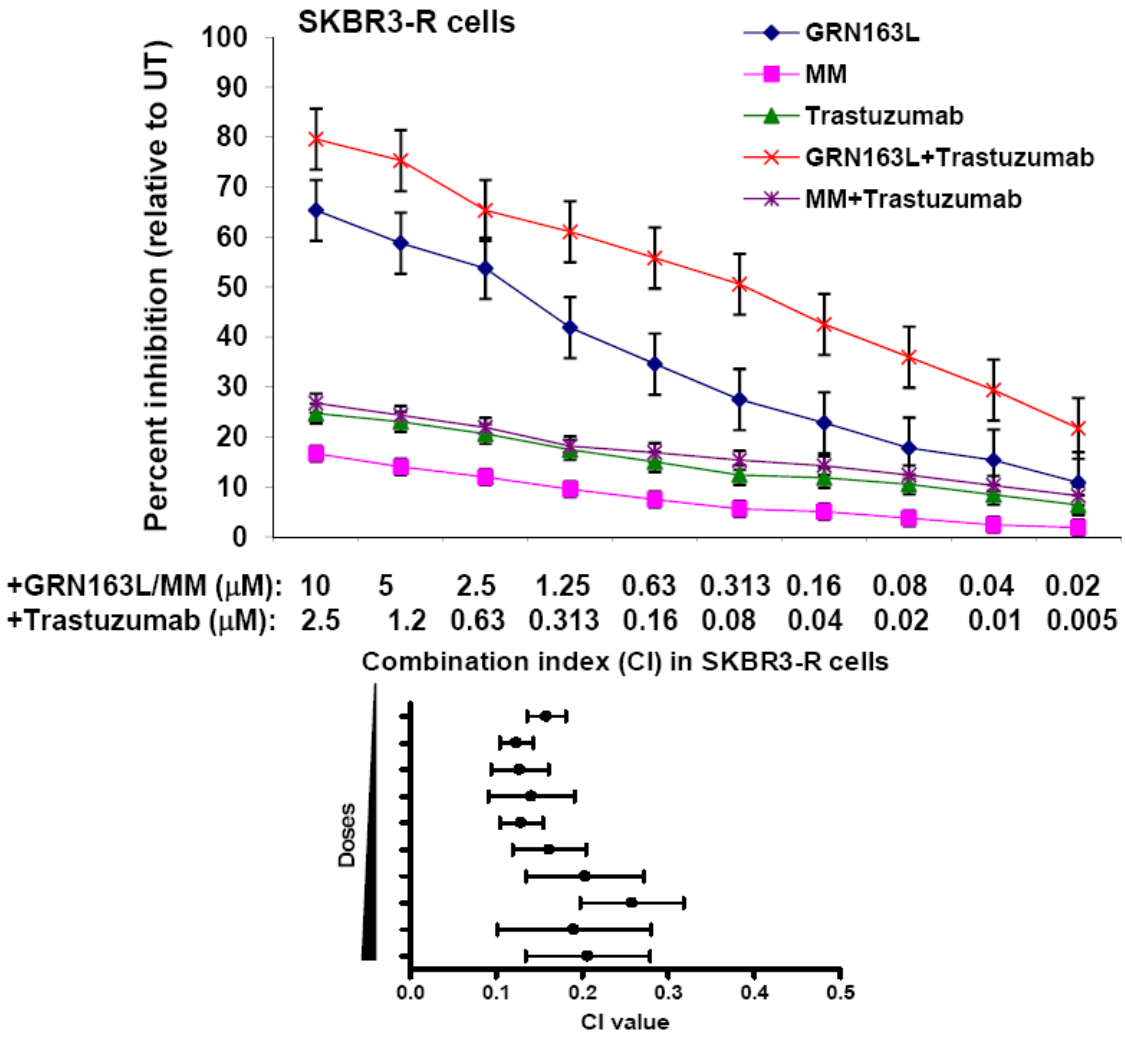


**Figure 4.6. GRN163L in combination with trastuzumab inhibits cell proliferation in trastuzumab-resistant SKBR3 breast cancer cells.** Trastuzumab-resistant SKBR3-R breast cancer cells were plated and treated as in Figure 4.4. Percent growth inhibition compared to untreated (media only) cells was plotted versus each versus concentration for each treatment group (top panel). Combination index value for each dose combination is shown on the bottom panel. Results are representative of multiple, independent experiments (± SD).

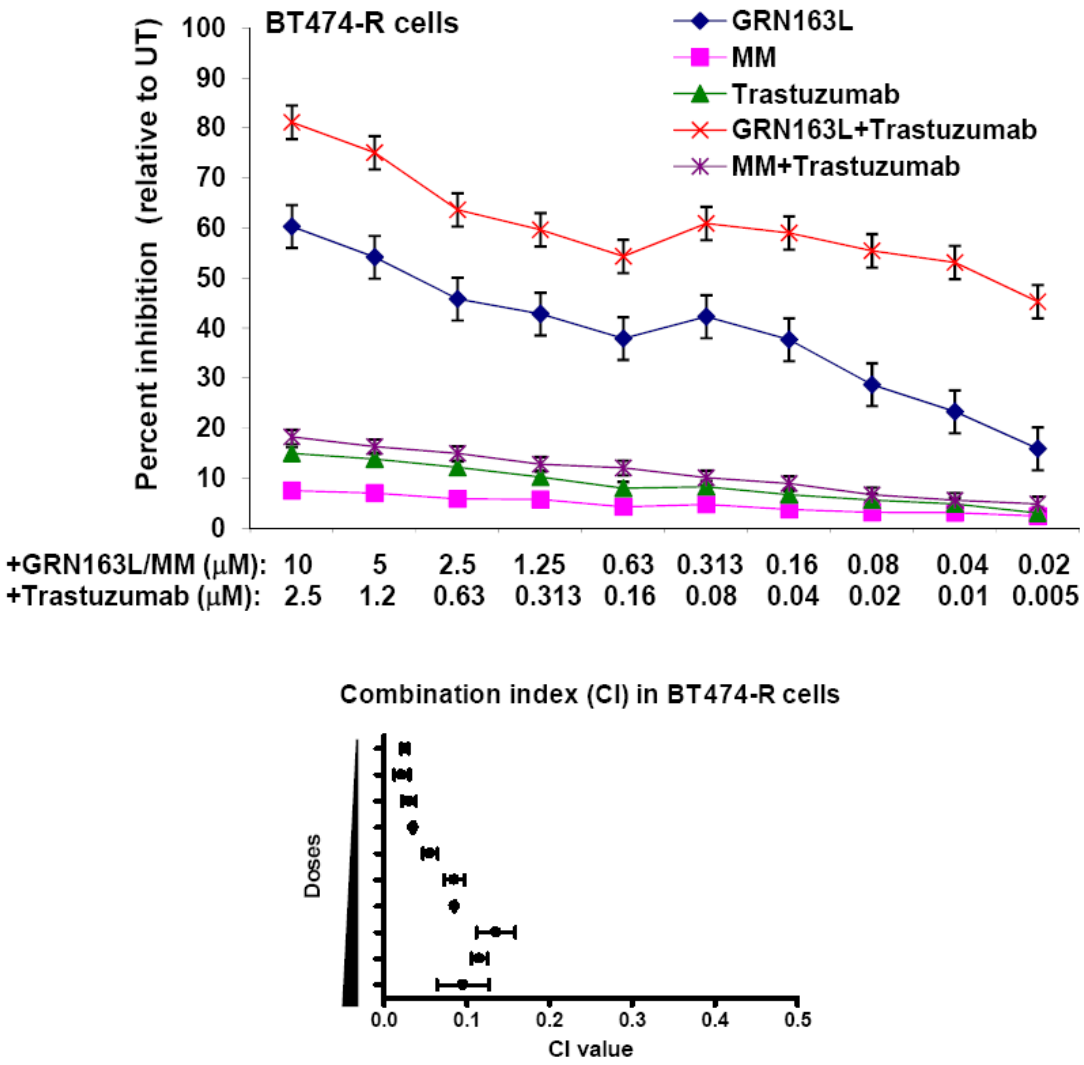


**Figure 4.7. GRN163L in combination with trastuzumab inhibits cell proliferation in trastuzumab-resistant BT474 breast cancer cells.** Trastuzumab-resistant BT474-R breast cancer cells were plated and treated as in Figure 4.4. Percent growth inhibition compared to untreated (media only) cells was plotted versus each versus concentration for each treatment group (top panel). Combination index value for each dose combination is shown on the bottom panel. Results are representative of multiple, independent experiments (± SD).

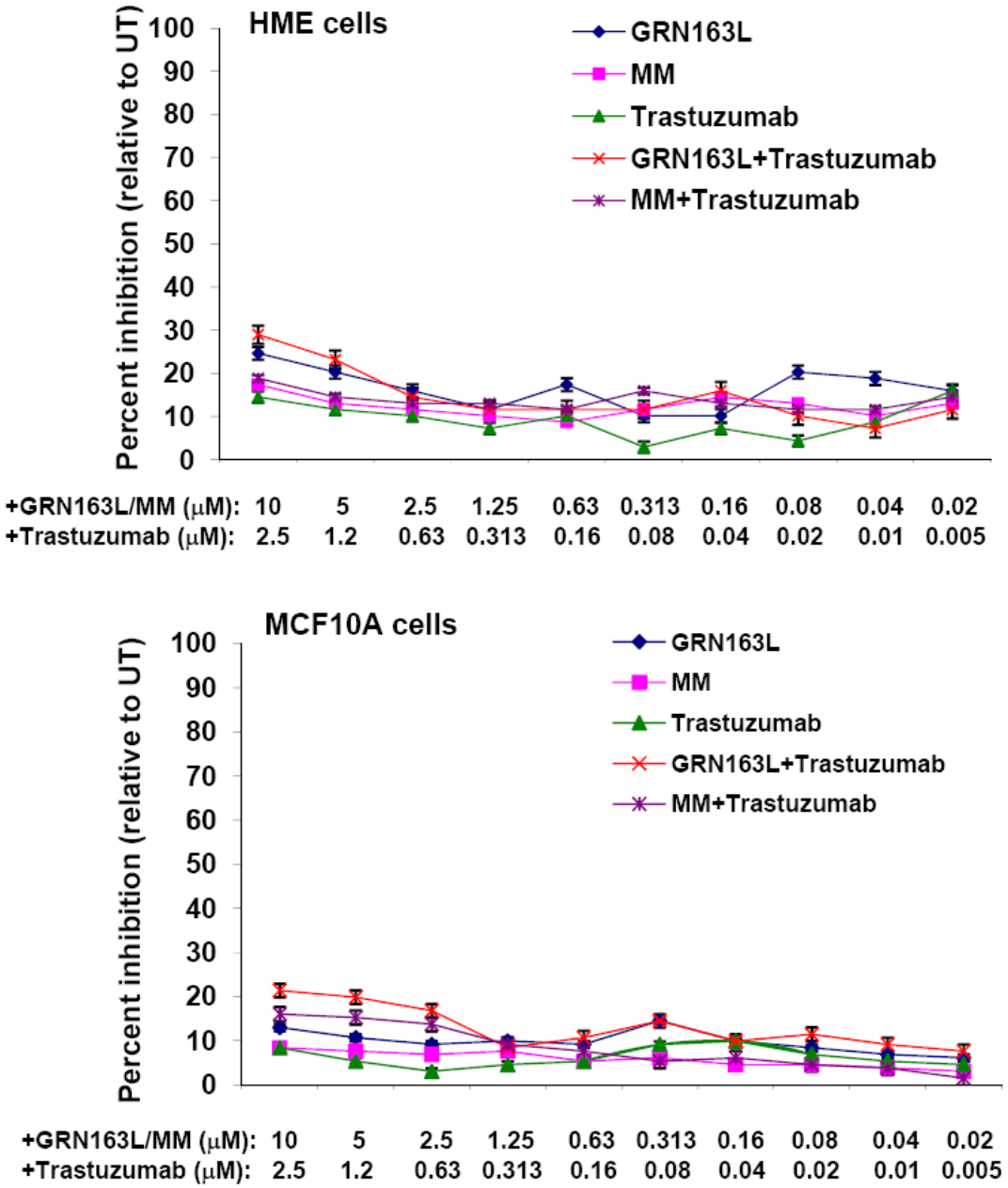


**Figure 4.8. GRN163L and trastuzumab have no affect on cell growth of nontumorigenic human mammary epithelial cells (HMECs) and MCF10A cells.** HMECs and MCF10A cells were plated, treated with GRN163L, it mismatch control, and trastuzumab, and analyzed for their effects on cell growth inhibition as described for Figure 4.4. GRN163L and trastuzumab treatment showed no significant effect on HMEC or MCF10A cell growth. Results are representative of multiple, independent experiments (± SD).

*Effect of telomerase inhibitors on HER2 expression and activity*

In an effort to understand the mechanism underlying the response to combination treatment with of GRN163L and trastuzumab, the level of HER2 and phosphorylated HER2 levels in SKBR3 and SKBR3-R cells was determined by Western blotting. 5'oleic163, GRN163 (non-lipidated), GRN163L (palmitoyl acid group), oleic acid, and mismatch oligonucleotide treated samples were used to determine whether the presence or type of lipid group was vital for the effect on HER2 expression. In addition, SKBR3 cells were treated with the $EC_{50}$ (for phosphorylated HER2 levels) concentration of trastuzumab (10 μg/ml) as a positive control. As expected, SKBR3-R cells did not exhibit a significant decrease in phosphorylated HER2 levels after treatment with HER2 inhibitors as expected. As an additional test for HER2 inhibition in resistant SKBR3 cells, the selective HER2 tyrosine kinase inhibitor AG825 was used. In our hands, AG825 did not decrease phosphorylated HER2 levels in the SKBR3-R cells which may reflect lost sensitivity to this kinase inhibitor in these cells. These results demonstrate that none of the compounds tested had a significant effect on the level of basal HER2 expression (Figure 4.9). Interestingly, GRN163L decreased the expression of phosphorylated HER2 by ~90% in both parental and trastuzumab-resistant SKBR3 cells (Figure 4.9). This decrease in HER2 phosphorylation may underlie the synergistic effect of combination therapy with GRN163L and trastuzumab on HER2+ breast cancer cell growth. The combination of GRN163L and trastuzumab (at the doses used in Figures 4.4-4.8) also displayed reduced levels of phosphorylated HER2 (data not shown). Similarly, the level of phosphorylated HER2 in the MCF7/HER2 cells was decreased by 50-70% upon treatment with GRN163L or GRN163, indicating that the lipid group was not a key

factor (Figure 4.10). Additionally, MCF7/HER2 cells were treated with 20 μg/ml trastuzumab as a postitive control. Treatment with trastuzumab resulted in approximately 90% inhibition of phosphorylated HER2. In all cell lines tested, a mismatch control oligonucleotide did not significantly alter HER2 levels. However, phosphorylated HER2 levels were altered upon oligonucleotide treatment after 48 hours in the SKBR3 and SKBR3-R cells (Figure 4.9) inhibition with trastuzumab after 5 days (Figures 4.4 and 4.5). This may reflect a temporal change in phosphorylated HER2 levels as mismatch oligonucleotide treatment of the MCF7/HER2 cells did not result in changes in phosphorylated HER2 levels (Figure 4.10). It has been reported that in trastuzumab-resistant BT474 cells, the level of EGFR and phosphorylated EGFR was significantly increased, and that inhibition of that phosphorylation via lapatinib (a dual tyrosine kinase inhibitor for EGFR and HER2) could inhibit cell growth (Ritter et al. 2007).To determine whether the combination of GRN163L and trastuzumab was acting in a similar fashion, the level of phosphorylated EGFR was measured via ELISA in the BT474 parental and resistant cells at the same time point as performed for Figures 4.4-4.8 (5 days). Treatment with GRN163L did not, however, result in a significant decrease in the level of EGFR phosphorylation in response to GRN163L (data not shown). These results indicate that the growth inhibition induced by GRN163L in combination with trastuzumab may be complex and require further investigation.

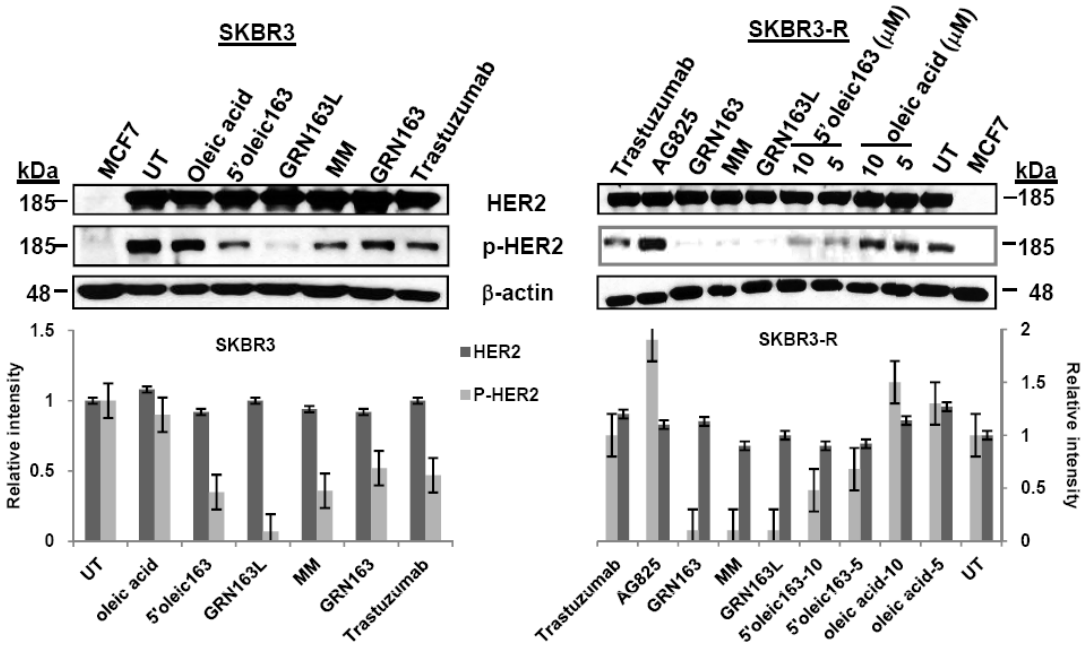


**Figure 4.9. Effects of telomerase template antagonists on HER2 expression and signaling in SKBR3 cells.** Western immunoblotting was performed to examine HER2 and phosphorylated HER2 (P-HER2) protein expression in treated cells. Both trastuzumab sensitive (SKBR3) or trastuzumab-resistant (SKBR3-R) breast cancer cells were treated with 10 μM oleic acid, 5-10 μM 5'oleic163, GRN163L, GRN163, or mismatch (MM) oligonucleotides for 48 hours following a 24 hour serum starvation. The selective HER2 tyrosine kinase inhibitor AG825 (5 μM) and/or trastuzumab (10 μg/mL; $EC_{50}$) were used as positive controls for HER2 inhibition. Untreated (media only) MCF7 cells were used as a negative control, as this cell line does not have HER2 amplification. Densitometry levels for each blot was determined using β-actin as a loading control; relative intensity levels for each sample were plotted and normalized to untreated (UT) samples. Results are representative of at least two experiments.

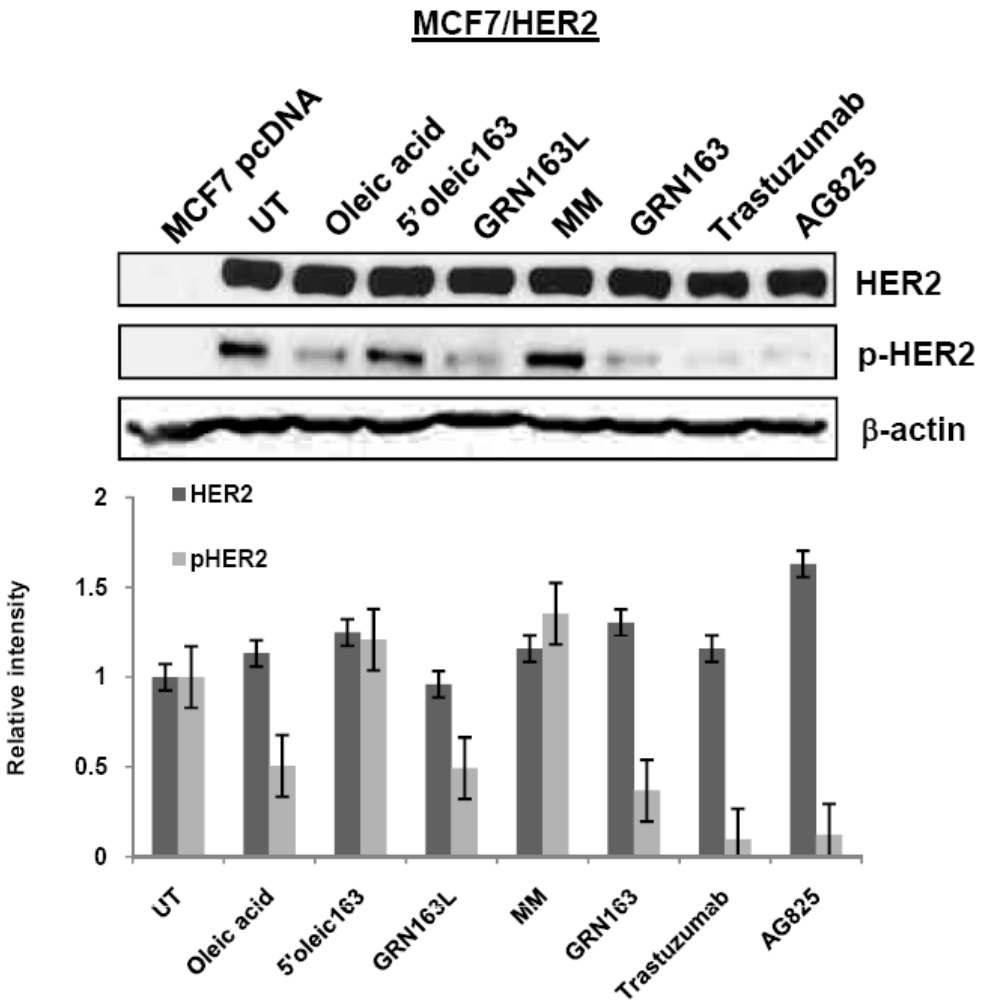


**Figure 4.10. Effects of telomerase template antagonists on HER2 expression and signaling in MCF7/HER2 cells.** MCF7 cells overexpressing HER2 (MCF7/HER2) were treated under estrogen-free conditions with 10 μM oleic acid, 5'oleic163, GRN163L, GRN163, or mismatch (MM) oligonucleotides for 48 hours following a 24 hour serum starvation. Western immunoblotting for HER2 and phosphorylated HER2 (P-HER2) was performed. AG825 (5 μM) and trastuzumab (20 μg/mL) were used as positive controls for P-HER2 inhibition. Untreated (media only) MCF7pcDNA (empty vector control) cells were used as a negative control, as this cell line does not have HER2 amplification. Densitometry levels were determined by comparing intensity of P-HER2 and HER2 to β-actin levels and relative intensity levels for each sample were plotted and normalized to untreated (UT) samples.

**Discussion**

Trastuzumab administration in breast cancer therapeutic regimens has had a major impact on the treatment of HER2+ metastatic breast cancer. Adding trastuzumab to therapy regimens has been associated with a longer time to disease progression, higher rate of treatment response, longer survival time, and lower death rates (Ross et al. 2003). However, trastuzumab can also fail to prevent breast cancer relapse due to acquired resistance (Nicolini et al. 2006, Sledge 2004). Therefore, experimental therapeutic studies, such as those presented herein, allow for the continued development of improved combination therapy for breast cancer using multiple molecular targets. Combining chemotherapy drugs or irradiation (IR) with trastuzumab enhances the effects of these anti-cancer therapies (Pegram et al. 2004, Liang et al. 2003). However, the negative side effects of trastuzumab can be exacerbated by the effects of chemotherapy drugs or IR. The effect of telomerase inhibition in sensitizing cancer cells to chemotherapy drugs and IR is well documented, but has been shown to require telomere shortening prior to detecting sensitization, indicating a long lag time (Cerone et al. 2006a, Djojosubroto et al. 2005, Gomez-Millan et al. 2007, Ward and Autexier 2005). The data presented here shows that telomerase template antagonists can significantly increase the sensitivity of HER2-positive breast cancers to trastuzumab within five days, including cells which have developed resistance to HER2 inhibition. These results have significant implications for the treatment of breast cancers that have acquired resistance to anti-HER2 therapies. As shown by the efficient uptake and inhibition of telomerase activity (Figure 4.1, page 94), the oligonucleotide telomerase inhibitors used in this study may therefore be effective in limiting relapse and delaying recurrence in HER2 breast cancer cells. This may be a

novel use for a telomerase template antagonist that does not depend on telomeres becoming critically short prior to inducing growth arrest or cell death. However, we cannot rule out a bystander effect of a few dysfunctional telomeres within a few population doublings that may play a role in the synergistic effect of telomerase inhibitors and targeted therapy. Therefore, treatment with telomerase template antagonists in therapeutic regimens could have beneficial outcome not only on primary tumor growth due to the effects of telomere shortening, but also in therapeutic regimens for sensitization to therapy and prevention of recurrence.

Dietary fatty acids have been shown to regulate signal-transduction pathways and may be associated with the risk of developing many diseases including cancer. This relationship between fatty acids and regulation of cell signaling in cancer remains unclear (Ip 1997, Hardman 2004, Hwang and Rhee 1999). Previous reports have shown that oleic acid, the main monounsaturated fat in olive oil, can regulate HER2 expression and activity (Menendez et al. 2004, Menendez et al. 2005, Menendez et al. 2007). This regulation can in turn inhibit cell proliferation, induce apoptosis, and alter downstream targets of HER2 signaling in breast cancer cells that have HER2 overexpression. The reports also showed that oleic acid worked synergistically with trastuzumab to enhance the effects of either drug alone (Menendez et al. 2004, Menendez et al. 2007). We therefore included within this study an oleic-conjugated telomerase template thio-phosphoramidate antagonist to test whether the oleic group is important for combination effects with trastuzumab on HER2 signaling. The 5'oleic163 thio-phosphoramidate is an effective telomerase inhibitor, reducing telomerase activity in a variety of cancer cell lines at low nM concentrations without exerting immediate cytotoxicity. However, the

oleic-conjugated thio-phosphoramidate displayed similar results as GRN163L in combination with trastuzumab, suggesting the type of lipid group was not a key factor in the synergism.

HER2 amplification is also associated with increased hTERT expression and telomerase activity (Goueli et al. 2004). How telomerase is regulated within a cell is still not completely understood, although numerous reports have shown that telomerase can be upregulated through the MAPK pathway (Goueli et al. 2004, Maida et al. 2002, Ge et al. 2006). The activation of hTERT can be inhibited by MAPK pathway inhibitors, which further supports the role of HER2 signaling in the induction of telomerase activity. In this study, however, there was no change in the basal level of HER2 expression after treatment with GRN163L, but instead resulted in lower levels of phosphorylated HER2 in the breast cancer cell lines tested in this study. Ritter et al. showed that breast cancer cells selected for resistance to trastuzumab *in vivo* overexpressed epidermal growth factor receptor (EGFR) and phosphorylated EGFR. Furthermore, this group showed that these cells, which contained ErbB ligands, remained dependent on ErbB receptor signaling and were sensitive to EGFR tyrosine kinase inhibitors, including the dual EGFR/HER2 inhibitor lapatinib (Ritter et al. 2007, Nahta et al. 2007b). Ectopic expression of hTERT in human mammary epithelial cells is associated with upregulation of EGFR compared to untransduced human mammary epithelial cells (Smith et al. 2003). Targeting telomerase via siRNA and antisense oligonucleotides (AS-ODNs) in bladder cancer cells resulted in the down-regulation of EGFR (Kraemer et al. 2006). We did not, however, observe a significant decrease in EGFR or phosphorylated EGFR associated with telomerase inhibitor treatment. However, it is likely that the combination of GRN163L and lapatinib

would be effective in inhibiting breast cancer cell growth, as trastuzumab-resistant cells remain sensitive to both compounds (Ritter et al. 2007, Nahta et al. 2007b). This appears to be regulated through insulin-like growth factor I (IGF-I). The involvement of another major signaling pathway in trastuzumab-resistant cells indicates that the exact mechanisms of GRN163L effects on cell growth inhibition via the HER2/EGFR or IGF-I pathway may be complex and warrant further attention.

In summary, a lipid-conjugated thio-phosphoramidate targeting the RNA template region of telomerase (GRN163L) is an effective telomerase inhibitor for HER2+ breast cancer cells, as shown by the dose-dependent decrease in telomerase activity and progressive telomere shortening with each population doubling. GRN163L, a telomerase inhibitor that is currently in clinical trials for cancer therapy, was synergistic with trastuzumab to inhibit breast cancer cell growth. More importantly, telomerase template antagonists restored sensitivity to trastuzumab in cells that had acquired resistance. These results further support the use of telomerase inhibitors in combination with other molecular targeted therapies for the treatment of breast cancer, metastasis, and recurrence. Published reports demonstrated synergy between HER2 inhibition via trastuzumab and a variety of cytotoxic chemotherapeutic drugs (Pegram et al. 1997, Pegram et al. 1999, Liang et al. 2003, Pegram et al. 2004). Despite the benefits of combination therapy, many patients have still developed resistance and progressed despite therapy, demonstrating the need for research into the mechanisms underlying resistance to trastuzumab and how resistance can be reversed. The research presented here supports published reports that trastuzumab sensitivity can be restored through combination therapy with trastuzumab. This restoration of sensitivity has been studied

using IGF-1 receptor signaling inhibitors, and AKT/ PI3K pathway inhibitors. Additionally, sensitivity to trastuzumab has been shown to be restored through dual therapy with lapatinib and pertuzumab, which inhibits HER2 from binding to EGFR or HER3 and initiating downstream signaling pathways (Nahta and Esteva 2006, Nahta and Esteva 2007, Suzuki and Toi 2007, Park and Davidson 2007, Berns et al. 2007).

## Materials and Methods

### *Cell culture*

Trastuzumab-resistant and parental SKBR3 cells, a generous gift from Dr. Francisco Esteva (UT MD Anderson Cancer Center), were cultured in DMEM/F12 media containing 10% fetal bovine serum; resistant cells were cultured with the addition of 4 μg/ml trastuzumab (Nahta et al. 2004). Trastuzumab-resistant and parental BT-474 cells, a generous gift from Dr. Carlos Arteaga (Vanderbilt University School of Medicine), were cultured in IMEM (Invitrogen) containing 10% fetal bovine serum; resistant cells were cultured with the addition of 10 μg/ml trastuzumab (Ritter et al. 2007). MCF7pcDNA and MCF7/HER2 breast cancer cells were kindly provided by Dr. George Sledge of the IU Simon Cancer Center (and originally from Dr. Dennis Slamon of UCLA). MCF7pcDNA and MCF7/HER2 cells were grown in 1X MEM media containing 10% fetal bovine serum, 1X non-essential amino acids, and 0.6 mg/ml G418 to maintain selection. Experiments with MCF7 cell lines were performed in phenol red-free media containing 2% charcoal stripped fetal bovine serum. All cells were cultured at 37°C and 5% $CO_2$ and routinely checked for mycoplasma contamination.

*Reagents*

The telomerase template antagonists used in this study are complementary to the telomerase RNA component (hTR) template region for telomerase (5'-CUAACCCUAAC-3'). The lipid-modified N3'→P5' thio-phosphoramidate oligonucleotide (GRN163L, 5'-Palm-TAGGGTTAGACAA-NH2-3') , the non-lipidated parental compound (GRN163), the 5'-palmitoyl mismatch control oligonucleotide (5'-Palm-TAGGTGTAAGCAA-NH2-3'), and the oleic acid conjugated oligonucleotide (5'oleic163; 5'-Oleic-TAGGGTTAGACAA-3') were prepared as previously described (Herbert et al. 2005). 5'oleic163 was prepared with the substitution of an oleic acid for the palmitoyl modification of the GRN163L oligonucleotide. Trastuzumab was kindly provided by the Indiana University Cancer Center Pharmacy. The selective HER2 inhibitor AG825 was purchased from AG Scientific (San Diego, CA) and was dissolved as a 10 mM stock in DMSO. Oleic acid (18:1n-9) was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved as a 100 mM stock in ethanol.

*Cell Growth and Viability Assays*

In order to determine how treatment with telomerase template antagonists or trastuzumab affect cell growth and viability, $5x10^4$ cells were plated with 2.5 μM GRN163L or mismatch oligonucleotides in 12-well dishes. Cells were collected weekly and counted via Beckman Coulter Counter to determine cell growth. Triplicate samples were combined prior to replating to avoid selection of resistant cells. Student's t-test was performed using Microsoft Excel.

*Telomere Repeat Amplification Protocol (TRAP) and Telomere Restriction Fragment Assay (TRF)*

The TRAP assay was performed using a Cy5 fluorescently labeled TS primer and established protocols (Chapter Two; Hochreiter et al. 2006, Herbert et al. 2006). Relative telomerase activity (RTA) was calculated as the ratio of the 6-bp telomerase-specific ladder to the 36-bp internal standard. The $EC_{50}$ for telomerase inhibition by the telomerase template antagonists was determined using GraphPad Prism (GraphPad Software, Inc, San Diego, CA). Measurements of telomere lengths were done as described previously (Hochreiter et al. 2006). Average telomere lengths in kb were calculated using TELORUN© as previously described (Chapter Two; Herbert et al. 2003) and plotted versus population doubling (PD) level.

*Cell Growth and Viability Assays to Determine the Combination Index*

In order to determine how treatment with telomerase template antagonists or trastuzumab affect cell growth and viability, $5x10^4$ cells were plated in 12-well dishes in triplicate and were treated with 1:2 serial dilutions of GRN163L (0.0195-10 μM) or trastuzumab (0.005-2.5 μM) in culture media. Cells were then collected after five days to obtain cell number and viability as measured by the Trypan blue exclusion assay. Percent inhibition of cell growth compared to untreated cells was determined and plotted. In order to determine the effects of combination therapy on breast cancer cells, parallel studies were done in which cells were treated with 1:2 serial dilutions of GRN163L (0.0195-10 μM) in combination with trastuzumab (0.005-2.5 μM), in which the two agents were at a 1:4 ratio of each other (trastuzumab: GRN163L). A mismatch palmitoyl

thio-phosphoramidate oligonucleotide (MM) was used as an oligonucleotide control. CalcuSyn software (Biosoft, Cambridge, United Kingdom) was used to determine the combined effect of multiple drug treatment via the Chou and Talalay method (Chou and Talalay 1984). This program analyzes the effect of combining drugs with different mechanisms of action in order to determine whether single drug therapy is improved upon the addition of a second compound. A combination index (CI) value of <1.0 denotes synergistic interaction (more than additive), a CI of 1.0 demonstrates summation (additive), and a CI >1.0 indicates antagonistic interaction (less than additive).

*Western immunoblotting*

Cells were harvested after treatment and lysed in buffer containing 5% 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. 10-50 μg protein lysate was subjected to SDS-PAGE followed by a two hour transfer to PVDF 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: anti-c-ErbB2 antibody (Ab-3; Oncogene Research Products, San Diego, CA), anti-phospho-HER2 (Cell Signaling Technology; Beverly, MD). Densitometry levels for each blot was determined using β-actin as a loading control; relative intensity levels for each sample were plotted and normalized to untreated (UT) samples. Results are representative of at least two experiments.

# Chapter Five

## Conclusions and Future Directions

The overall goal of this research project was to gain insight into the role of telomerase in breast cancer cell survival and the mechanisms of how the telomerase template antagonist GRN163L effectively inhibits the growth of breast cancer cells. *In vivo* studies from our laboratory have shown that telomerase inhibition not only inhibits the growth of human breast cancer cells in the mammary fat pad of mice, but also inhibits breast cancer metastases to the lung (Hochreiter et al. 2006). Despite individual efficacy, telomerase inhibitors have been proposed for use in combination with current therapeutic regimens (Shay and Wright 2002). Using combination therapies is likely to improve treatment regimens, as targeting multiple pathways involved in cancer progression can increase patient survival over single drug therapy. The research presented herein demonstrates that in addition to the known telomere dysfunction effects from continuous telomerase inhibition, GRN163L induces a rapid inhibitory effect on cell growth and invasion. This rapid response synergistically inhibits the growth of breast cancer cells *in vitro* and *in vivo* with paclitaxel and trastuzumab, supporting the use of this compound in the clinical setting. The combination treatments proposed in this study could therefore effectively shorten treatment time, thus reducing the negative side effects of chemotherapeutic drugs and mortality due to metastatic disease.

GRN163L has been shown to be effective in a wide variety of cancer cell lines Djojosubroto et al. 2004, Dikmen et al. 2005, Hochreiter et al. 2006, Gellert et al. 2006, Gomez-Millan et al. 2007). The combination of telomerase inhibition via GRN163L and

irradiation (IR) was shown to significantly enhance the effects of radiation on breast cancer cell growth *in vitro* and primary tumor growth *in vivo* (Chapter Two; Gomez-Millan et al. 2007). This effect was shown to be due to the induction of telomere dysfunction, suggesting a relationship between extended telomerase inhibition, telomere length, and impairment of DNA damage-induced repair. The combination of telomerase inhibitors and IR may allow for the use of lower doses of IR, a reduction in the overall time of treatment, and a decrease in secondary effects on normal cells. Although treatment with GRN163L for 9-20 days did not show any synergism with IR, the decrease in proliferation observed during this time period could have a potential clinical effect when combined with IR (Gomez-Millan et al. 2007). The *in vivo* results with MDA-MB-231 xenografts support GRN163L as a promising adjuvant cancer treatment in combination with radiation therapy (Chapter Two; Gomez-Millan et al. 2007). With GRN163L currently in clinical trials, it will be of interest to further study whether other tumor models respond to GRN163L in combination with radiation therapy.

Published reports have demonstrated that inhibiting telomerase activity alone or in combination with current therapeutic techniques can inhibit tumor cell growth *in vitro* through telomere dysfunction following protracted telomerase inhibitor treatment (Ward and Autexier 2005, Cerone et al. 2006a, Gomez-Millan et al. 2007). The data presented in Chapter Three shows that telomerase template antagonists can significantly increase the sensitivity of breast cancer cells to paclitaxel at five days, without a significant effect on non-tumorigenic cells. This rapid response may be a novel use for a telomerase template antagonist that does not depend on the average bulk of telomeres to be shortened prior to inducing growth arrest or cell death. These results indicate that the reduction in

metastasis observed previously in response to GRN163L (Hochreiter et al. 2006, Jackson et al. 2007) could be due to a loss of proliferative potential in breast cancer cells, as demonstrated by a reduction in growth rates and DNA synthesis (Chapter Three; Hochreiter et al. 2006, Gomez-Millan et al. 2007). Additionally, GRN163L treatment in combination with the mitotic inhibitor paclitaxel resulted in reduced or delayed MDA-MB-231 tumor growth *in vivo* and invasive potential *in vitro*. The combination of GRN163L and paclitaxel may, therefore, be effective in the treatment of both primary and metastatic breast cancer. Furthermore, the data presented in Chapter Four shows that telomerase template antagonists can significantly increase the sensitivity of HER2-positive breast cancers to trastuzumab at five days, including cells which have developed resistance to HER2 inhibition.

Telomerase inhibition results in the lack of maintenance of telomeric DNA and eventually leads to apoptosis or senescence (Herbert et al. 2005, Gellert et al. 2006 for review, Shay 2003 for review). As discussed previously, the effects of telomerase inhibition are predicted to follow a lag phase in which telomeres have reached a critically short length due to the end replication problem (i.e., telomeres progressively shorten with each cell division). The studies presented in Chapters Two and Three, however, demonstrate that there is a rapid response to the second generation, potent telomerase template antagonist, GRN163L, which does not rely on the bulk of telomeres to reach a critically short state. Published reports have demonstrated that a small number of short telomeres are responsible for cell viability rather than the average telomere length of the all the chromosomal ends (Zou et al. 2004, Ouellette et al. 2000, Hermann et al. 2001). These critically short telomeres may not be accurately measured if present in small

numbers, and may trigger growth arrest or cell death without affecting average telomere size. The effects of GRN163L on breast cancer cell growth within five days could be due to the loss of a sentinel, critically short telomere(s). It would therefore be valuable to measure telomere lengths at earlier time points, using sensitive techniques such as fluorescence in situ hybridization (FISH) or single telomere length analysis protocol (STELA), to determine whether there is a rapid reduction in the size of a small number of telomeres, resulting in the shorter response time shown in these studies.

The balance between cell growth and death is tightly regulated. Resistance to cell death, particularly apoptosis, is an important aspect of cancer development and can result in resistance to therapy. There are several forms of cell death; apoptosis, autophagy, senescence, and mitotic catastrophe (Okada and Mak 2004). Apoptosis is a form of programmed cell death, or cellular suicide, which is essential during development and tissue homeostasis (Bröker et al. 2005, Okada and Mak 2004). Genomic instability or metabolic abnormalities can also trigger apoptosis. This form of cell death is associated with chromatin condensation and cellular disintegration, resulting in the elimination of a cell that has the potential to become tumorigenic. Senescence is a permanent cell-cycle arrest, or terminal growth arrest, after which cells can no longer replicate. This form of cell death can be induced by telomere shortening, or through DNA damage. While senescent cells remain quiescent, they maintain their viability and metabolic functions. Senescent cells are capable of secreting factors that affect the growth of surrounding cells, and can have a significant effect on the growth and survival of tumor cells (Roninson 2003).

Autophagy is a form of cell death seen in the absence of apoptosis. Autophagy is associated with the destruction of unwanted or unnecessary proteins and is often activated during nutrient starvation or cellular damage. The exact function of autophagy in humans is not yet understood, although it is thought to be required for cellular remodeling during differentiation, stress, or exposure to cytotoxins (Hait et al. 2006, Okada and Mak 2004). Dysregulation of autophagy may result in diseases such as cancer, as some oncogenes and tumor suppressor genes play a role in the autophagic process. Additionally, at least one of the major proteins involved in autophagy has been found to be located to a human tumor-susceptibility locus and is deleted in a high percentage of human epithelial cancers, including breast cancer (Okada and Mak 2004, Hait et al. 2006). Furthermore, tumor cells that have been treated with anticancer drugs (chemotherapy and molecular targeted therapy) can activate the autophagy pathway, although whether this is a protective response or a death promoting activity is still unclear (Okada and Mak 2004, Hait et al. 2006). Autophagy can be measured by the appearance of cytoplasmic vesicles (autophagosomes) that engulf non-essential cellular components such as mitochondria and endoplasmic reticulum (Kroemer and Jäättelä 2005, Gozuacik and Kimchi 2004). The formation of autophagosomes is visible using electron microscopy. The microtubule-associated protein-1 light chain-3 (LC3) can be physically associated with forming autophagic vesicles and is a well-characterized marker for autophagosome formation. LC3 is redistributed from the cytoplasm to the autophagosomes, which is associated with the conversion of LC3 from the LC3-I isoform to the LC3-II isoform (Lum et al. 2005a, Lum et al. 2005b). The conversion of LC3-I to LC3-II can also be measured via western immunoblotting.

The response to GRN163L does not induce rapid cell death via apoptosis or senescence (Chapter Three; Hochreiter et al. 2006), or decreased cell viability over the first two weeks (by three weeks, an increase of senescence and apoptosis can be seen with a significant decrease in the average telomere length). This does not, however, rule out the fact that loss of a sentinel, critically short telomere(s) may trigger alternate forms of growth arrest or cell death, such as autophagy as shown by the use of telomere-targeting oligonucleotides (Aoki et al. 2007). As shown in Figure 5.1, there is preliminary evidence supporting the hypothesis of GRN163L-triggered autophagy by a decrease in the level of LC3-I and an associated increase in LC3-II over 72 hours after treatment with GRN163L.

Mutations in the PTEN tumor suppressor gene as well as increased expression of PI3-kinase and Akt oncogenes are frequent events in human tumors. Interestingly, PTEN has been shown to have a role in inducing autophagy (Gozuacik and Kimchi 2004, Hait et al. 2006). Along with the increase in LC3-II, an increase in phosphorylated PTEN after treatment with GRN163L was observed (Figure 5.1). Taken together, the upregulation of LC3-II and PTEN suggest a potential induction of autophagy in response to GRN163L. However, PTEN has also been shown to have a role in FAK regulation as well, desphosphorylating FAK and inhibiting cell migration (Figure 5.2, Li et al. 2005). The upregulation of PTEN, therefore, could have multiple roles in the response to GRN163L. Although the basal level of FAK was measured, it is the phosphorylation pattern that regulates protein activity. It is possible that FAK phosphorylation is inhibited by GRN163L, resulting in the upregulation of a protein that has similar function in cell proliferation and migration.

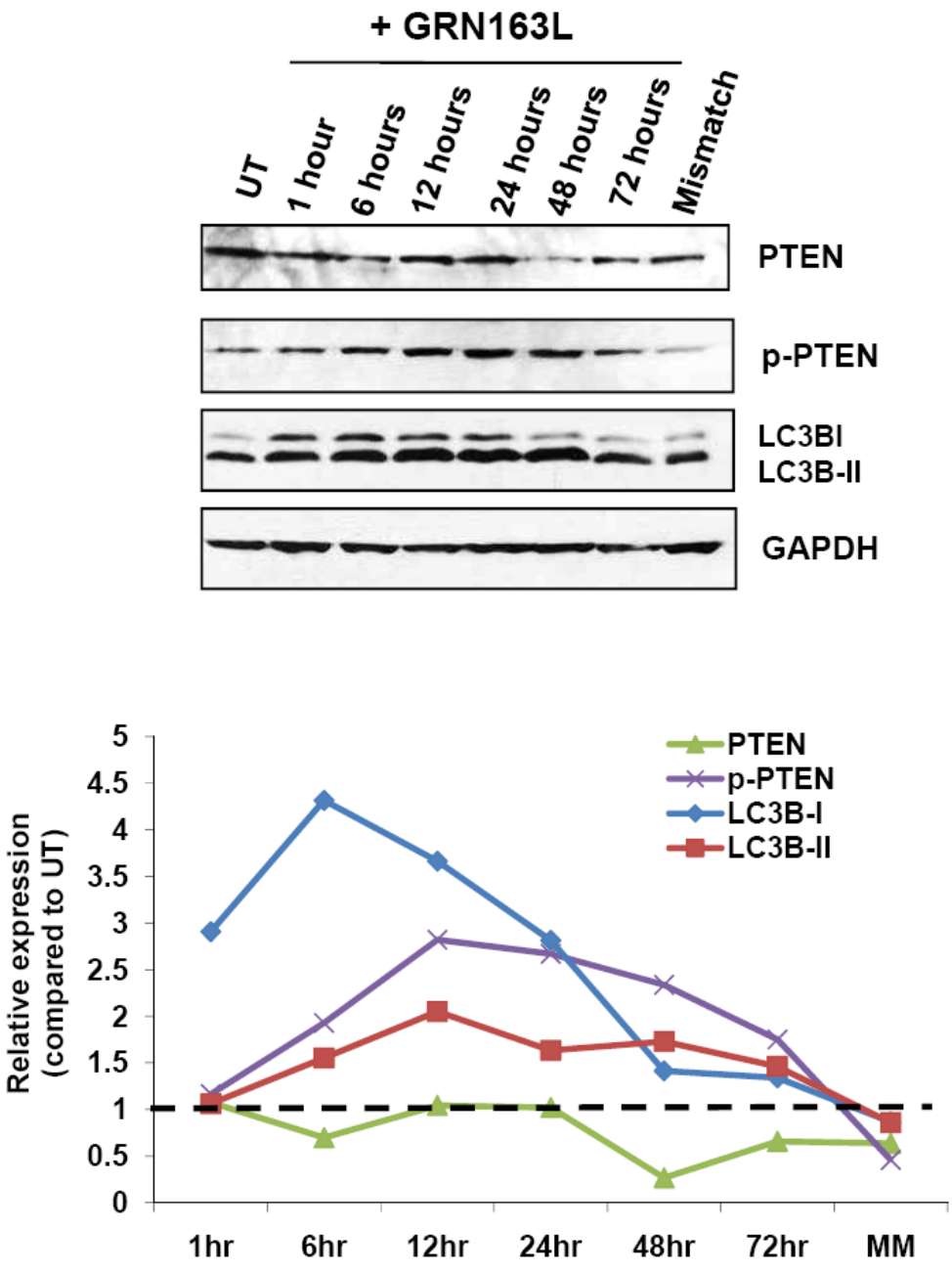


**Figure 5.1. GRN163L induces the conversion of LC3B-I to LC3B-II and the phosphorylation of PTEN.** MDA-MB-231 cells were treated with 2.5 μM GRN163L or mismatch oligonucleotides for the depicted times. Western immunoblotting for the indicated adhesion and morphology proteins was performed. Densitometry levels were determined by comparing intensity of LC3B-I, LC3B-II, PTEN and phopsho-PTEN to GAPDH and relative intensity levels for each sample were plotted and normalized to untreated (UT) samples.

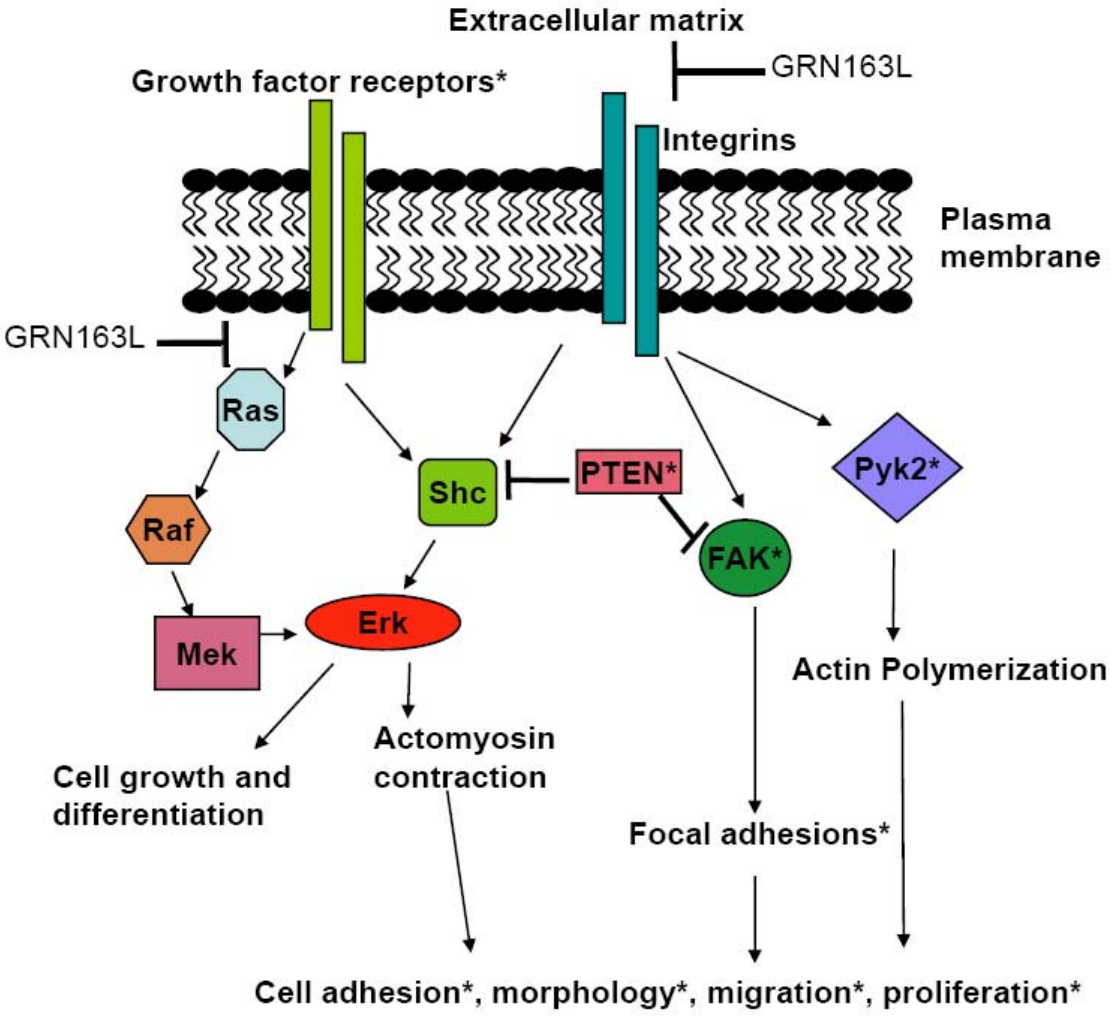


**Figure 5.2. GRN163L disrupts cell structure and function.** GRN163L has been shown to have an effect on cell adhesion, growth, migration, and proliferation. ⊢ = inhibition (direct or indirect). The processes specifically investigated within this study are highlighted by an asterisk (*). Adapted from Li et al., *Annu Rev Biomed Eng* 2005.

As shown in Chapter Three, the reduction in breast cancer cell growth by GRN163L may be explained by the alterations in actin filament organization and decreased attachment to extracellular matrix ECM proteins, resulting in changes in proteins involved in maintaining cell shape, and regulating cell proliferation and migration. To support this hypothesis, antisense oligonucleotides have previously been reported to induce reversible, non-toxic effects on cells that are not related to protein inhibition. One of the more common events observed in response to antisense oligonucleotides is an inhibition of adhesion to the ECM, most likely due to interaction with membrane associated receptors (Figure 5.2). The structure of the oligonucleotides seems to play a role in this phenomenon, with oligonucleotides containing triplet-G and G-quartet sequences influencing cell morphology (Anselmet et al. 2002, Jackson et al. 2007). The effects of GRN163L on cell adhesion and architecture is reversible with the addition of ECM proteins to cell culture dishes (Goldblatt et al. unpublished observations; Jackson et al. 2007). This reversibility indicates that GRN163L may be interfering with the ability of breast cancer cells to make or secrete ECM proteins, or the binding of integrins to the ECM (Figure 5.2).

As an alternative mechanism underlying the response to GRN163L in cancer cells, based on the data derived from these studies, it is hypothesized that there is an induction of mitotic catastrophe. Mitotic catastrophe is cell death due to aberrant mitosis, often due to defective cell cycle checkpoints, DNA damage, abnormal spindle formation, and the subsequent generation of aneuploid cells (Eom et al. 2005, Castedo et al. 2004). This can often be due to premature mitosis prior to the completion of DNA replication and repair, or induced due to microtubule damage (Eom et al. 2005, Okada and Mak

2004, Bröker et al. 2005). Additionally, shortening of telomeric repeats beyond a critically short length leads to genomic instability and the formation of structural rearrangements, which can lead to the induction of mitotic catastrophe (Gisselsson 2005). There has been evidence that mitotic catastrophe can be induced by IR and chemotherapy (Gerwitz et al. 2008). Additionally, telomerase inhibition and subsequent telomere shortening has been shown to result in mitotic cell death, with evidence of senescing cells undergoing mitotic catastrophe (Gerwitz et al. 2008).

The structural alterations induced by GRN163L (Chapter Three) characterized by disruption of actin filaments indicates that the response to GRN163L may, at least in part, be due to inability of cells to properly divide, further supporting the hypothesis that there may be an induction of mitotic catastrophe (Eom et al. 2005, Castedo et al. 2004). Telomere dysfunction induced by GRN163L could also lead to an induction of mitotic catastrophe (Chapter One), which would also help explain the reduction in growth rates seen in cancer cells *in vitro*, as well as tumor growth *in vivo*. To further support this idea, telomerase template mutations have been shown to block chromosome separation in anaphase (Kirk et al. 1997). Mutant hTR transfected into cells resulted in delayed doubling times, altered cell morphology, and cell death. These results indicate an important role for hTR in proper cell division (Kirk et al. 1997). In order to further understand the role of mitotic catastrophe in the response to GRN163L, the morphology of mitotic cells would be examined for abnormalities including multipolar anaphases, uneven chromosome distribution and cell cycle arrest. Additionally, the expression of genes that control mitosis can be measured via Western immunoblotting or real-time RT-PCR techniques (Chang et al. 2000).

The relationship between telomerase and growth factor receptors in breast cancer is complex. The regulation of telomerase is not completely understood, although numerous reports have shown that telomerase can be upregulated through the MAPK pathway (Goueli et al. 2004, Maida et al. 2002, Ge et al. 2006). One published report demonstrated that HER2 amplification is associated with increased hTERT expression and telomerase activity (Goueli et al. 2004). Additionally, there is a link between telomerase activity and EGFR, with an upregulation of EGFR in telomerase-positive cells versus telomerase-negative cells. Furthermore, EGFR inhibition reduced the proliferative advantage of telomerase-positive cells (Smith et al. 2003). The activation of hTERT has been shown to be inhibited by MAPK pathway inhibitors, which supports the role of growth factor signaling in the induction of telomerase activity. Furthermore, targeting telomerase via siRNA and antisense oligonucleotides (AS-ODNs) in bladder cancer cells resulted in the down-regulation of EGFR (Kraemer et al. 2006).

Ritter et al. showed that breast cancer cells selected for resistance to trastuzumab *in vivo* overexpressed epidermal growth factor receptor (EGFR) and phosphorylated EGFR. Furthermore, these cells remained dependent on ErbB receptor signaling and were sensitive to EGFR tyrosine kinase inhibitors, all of which may be regulated through insulin-like growth factor I (Ritter et al. 2007, Nahta et al. 2007a, Nahta et al. 2007b). Recent studies have demonstrated that HER2+ breast cancer cells have increased TGFβ signaling, resulting in increased survival and migration, as well as resistance to trastuzumab (Wang et al. 2008). As discussed in Chapter Four, GRN163L did not induce a change in the basal level of HER2 or EGFR activity (Figures 4.9-4.10 and data not shown). There was, however, a reduction in phosphorylated HER2 in the breast cancer

cell lines, indicating that GRN163L may be able to affect the expression and/or activity of growth factor receptors (Figure 5.2). This decrease in HER2 phosphorylation may underlie the synergistic effect of combination therapy with GRN163L and trastuzumab on HER2+ breast cancer cell growth. The involvement of major signaling pathways in trastuzumab-resistant cells indicates that the exact mechanisms of GRN163L effects on cell growth inhibition via the HER2/EGFR or IGF-I pathway may be complex and warrant further attention. Future implications of the work presented in Chapter Four include the design of clinical trials for GRN163L in combination with trastuzumab.

One concern with the use of telomerase inhibitors in the clinical setting is the potential effect on stem and progenitor cells found in highly replicative tissues, such as bone marrow, B and T cells, and gastrointestinal crypt cells. These cells, while largely quiescent, transiently express high levels of telomerase during proliferation (Chiu et al. 1996, Broccoli et al. 1995, Counter et al. 1995, Hiyama et al. 2001, Bodnar et al. 1996, Hodes et al. 2002). They have also been found to have longer telomeres than most cancer cells. During the time that cells are quiescent, telomere shortening does not occur and telomerase activity is negligible (Chiu et al. 1996). It is therefore reasonable to expect that quiescent stem cells will not be significantly affected by short courses of telomerase inhibition therapy (Wright et al. 1996, Forsyth et al. 2002, Shay and Wright. 2002). Indeed, such effects have not been observed in either clinical trials or in preclinical toxicology studies of GRN163L. Importantly, Phase I trials have demonstrated that GRN163L is tolerated by patients with no dose-limiting toxicities or serious adverse side effects yet reported. More importantly, there has been a beneficial effect of treatment in

multiple patients with CLL, although results are still pending for the other clinical trials discussed in Chapter One.

A major limitation of cancer immunotherapy in general is that for most cancers no foreign gene or protein is crucially involved in causing or maintaining tumor growth. The catalytic component of telomerase, hTERT, appears to be one such antigen due to near universal expression and crucial function in most tumors (Harley 2008). The use of anti-telomerase immunotherapy is also being studied in non-small cell lung cancer, acute myeloid leukemia, chronic myeloid leukemia, gastrointestinal tumors, and central nervous system/brain tumors, pancreatic cancer, prostate cancer, and breast cancer. Clinical trials have revealed that patients treated with an hTERT vaccine have induced hTERT-specific cytotoxic T lymphocyte production without serious side effects (Vonderheide 2008, Brunsvig et al. 2006, Su et al. 2005, Vonderheide et al. 2004, Domchek et al. 2007). While there are many benefits to using anti-telomerase immunotherapy, there are also several drawbacks. Because many cancer cells have a low number of telomerase molecules, it is possible that tumor cells might present low levels of the hTERT epitope as well, which might be a disadvantage for triggering killing by hTERT-specific immune cells. Additionally, many antigen presenting cells are primed *ex vivo*, introducing the risk of immune response against these cells. Furthermore, many patients have a weak immune system, thus hindering the effectiveness of vaccines (Harley 2008). Importantly for the continued use of telomerase vaccines, there have been no serious adverse side effects reported (Shay and Wright 2006).

Oncolytic viruses as gene therapy that utilize the hTERT promoter to drive expression of apoptosis genes have also shown potential to kill cancer cells (Shay and

Wright 2006, Gellert et al. 2005, Zimmerman and Martens 2007, Gu et al. 2000, Koga et al. 2000). This method of targeting telomerase-postive cells drives the expression of toxic genes, or "suicide genes" using the hTERT promoter to trigger cell death (Shay and Wright 2002, Shay and Wright 2006). The expression of these suicide genes results in the rapid killing of telomerase-positive cells based on the increased expression of either hTR or TERT promoters in cancer versus normal cells. This method has limitations, however, with potential toxic effects of normal cells expressing the hTERT promoter. Additionally, effective delivery of gene therapy to cancer cells throughout the body is challenging, and vector systems have a propensity to induce immunological responses (Harley 2008, Shay and Wright 2002, Shay and Wright 2006). Additionally, gene therapy has been shown to result in "leaky" expression of the target gene, which could result in inappropriate expression of suicide genes in normal cells.

As discussed throughout this thesis, one potential problem with using telomerase in cancer therapy is the theoretically predicted lag time associated with effective treatment (Hahn et al. 1999, Zhang et al. 1999, Herbert et al. 1999, Kelland 2005, Zimmermann and Martens 2007, Saretzki 2003). While this means that telomerase inhibitors may not be used as solo therapy, there have been numerous reports that telomerase inhibition can sensitize cells to chemotherapy or irradiation (Masutomi et al. 2005, Nakamura et al. 2005, Saretzki 2003, Kim et al. 2001, Goldkorn and Blackburn 2006, Cerone et al. 2006a, Cerone et al. 2006b, Djojosubroto et al. 2005, Gomez-Millan et al. 2007, Ward and Autexier 2005). Furthermore, recent studies have shown that GRN163L has potent anti-metastatic potential *in vivo* (Dikmen et al. 2005, Hochreiter et al. 2006, Jackson et al. 2007). In addition to the known telomere dysfunction effects from

continuous telomerase inhibition, GRN163L has been shown to induce a rapid inhibitory effect on cell growth and invasion (Chapters Three and Four; Jackson et al. 2007). This rapid response to GRN163L synergistically inhibited the growth of breast cancer cells *in vitro* and *in vivo* with paclitaxel and trastuzumab, supporting the use of this compound in the clinical setting.

Two of the biggest problems associated with current therapeutic regimens are recurrence and the development of drug resistance in residual cells remaining after initial treatment. As discussed in this dissertation, GRN163L is readily taken up by cancer cells, inhibits telomerase activity, and reduces cell growth. Additionally, as discussed in Chapter Four, GRN163L is effective in drug resistant cells, and augments the effects of trastuzumab in these cells. One explanation underlying recurrence and drug resistance is the existence of cancer stem cells (CSC). Cancer stem cells are immortal, pluripotent cells with the capability for self-renewal and the capacity to give rise to multiple different lines of cancer cells (Shay and Wright 2006). As most therapeutics rely on rapid cell division, quiescent cancer stem cells are likely to be resistant to treatment have the ability to survive an initial tumor kill and generate tumor transit cells, which might allow the tumor to re-grow once the therapeutic regimen has ended (Smalley and Ashworth 2003). However, elimination of CSCs from a tumor could effectively eliminate recurrence. Importantly, CSCs have been reported to express telomerase activity, thus indicating the potential use of telomerase inhibition in targeting CSCs. The implications of this research demonstrating that GRN163L can sensitize drug-resistant cells can lead to future research into the effects of GRN163L on cancer stem cells.

Based on the pre-clinical results from our laboratory, GRN163L entered Phase I/II trials for breast cancer in August 2008 in combination with paclitaxel and the anti-angiogenic agent, bevacizumab (ClinicalTrials.gov Identifier: NCT00732056). The primary objectives of this study are to determine the maximum tolerated dose (MTD) of GRN163L in combination with paclitaxel/ bevacizumab in patients with locally recurrent or metastatic breast cancer, as well as the rate and duration of response to combination therapy. There are several known risks associated with phosphorothioate (PS) oligonucleotides that have been observed in certain pre-clinical studies evaluating the toxicity of GRN63L. These risks include prolonged clotting times, thrombocytopenia and anemia. All of these outcomes have been observed after treatment with GRN163L in the current clinical studies; however the effects of GRN163L are transient. In this clinical study, patients are treated with GRN163L and paclitaxel once weekly for three weeks, followed by one week of rest. The starting dose and schedule utilized in this study is based upon the results from the phase I single agent studies in patients with solid tumors and CLL.

To measure the effectiveness of GRN163L in breast cancer patients, blood samples are being collected by our laboratory and analyzed for circulating tumor cells (CTCs). CTCs express telomerase activity, making them useful for screening and early cancer detection, but also for detection of residual disease after standard therapy (Mabruk and O'Flatharta 2005, Herbert et al. 2001, Soria et al. 1999). In addition, the level of telomerase activity in CTCs may have prognostic value, since cancers with high telomerase activity generally have poorer outcomes as compared to cancers with low telomerase levels. In addition, counting circulating tumor cells will determine whether

therapy with GRN163L is reducing the absolute number of CTCs or just inhibiting telomerase activity. However, cell death induced by standard therapeutics may decrease the number of CTCs and level of telomerase activity seen in blood samples, which could limit the knowledge of the exact contribution of GRN163L to standard therapeutic regimens. The telomerase activity assay described throughout this dissertation therefore has important applications as a prognostic tool (Herbert et al. 2006).

In summary, a lipid-conjugated thio-phosphoramidate targeting the RNA template region of telomerase (GRN163L) is an effective telomerase inhibitor, and can synergize with radiation, paclitaxel, and trastuzumab therapy. These results further support the importance of telomerase in cell growth and survival and the dependency of cancer cells to telomerase. The findings presented in this thesis suggest several mechanisms underlying reduction in cell proliferation, tumor growth, and metastasis by GRN163L (Figure 5.3). The pathways underlying cell migration, death, and resistance to therapy are closely intertwined. GRN163L could be targeting multiple pathways in breast cancer cell growth and progression (Figure 5.2 and 5.3, pages 128-129). Importantly, there is an overarching theme for the mechanism underlying augmentation of the effect of all three forms of breast cancer therapy. Irradiation, paclitaxel, and trastuzumab treatment all invoke responses involving the DNA damage repair pathway, either through induction of irreparable damage, or through inhibition of damage repair (Bernier et al. 2004, Le et al. 2005, Ramanathan et al. 2005). There is also a link between telomeres and DNA damage, as components of DNA damage response pathways have been found at the telomeres, and telomerase inhibition interferes with DNA repair (Nakamura et al. 2005, Gomez-Millan et al. 2007). Taken together with previous reports, the studies presented herein support

the use of telomerase inhibitors in combination with other therapeutic regimens for the treatment of breast cancer, metastasis, drug resistance, and recurrence. Treatment with telomerase template antagonists in therapeutic regimens could have beneficial outcome not only on primary tumor growth due to the effects of telomere shortening and dysfunction, but also in therapeutic regimens for sensitization to other forms of therapy.

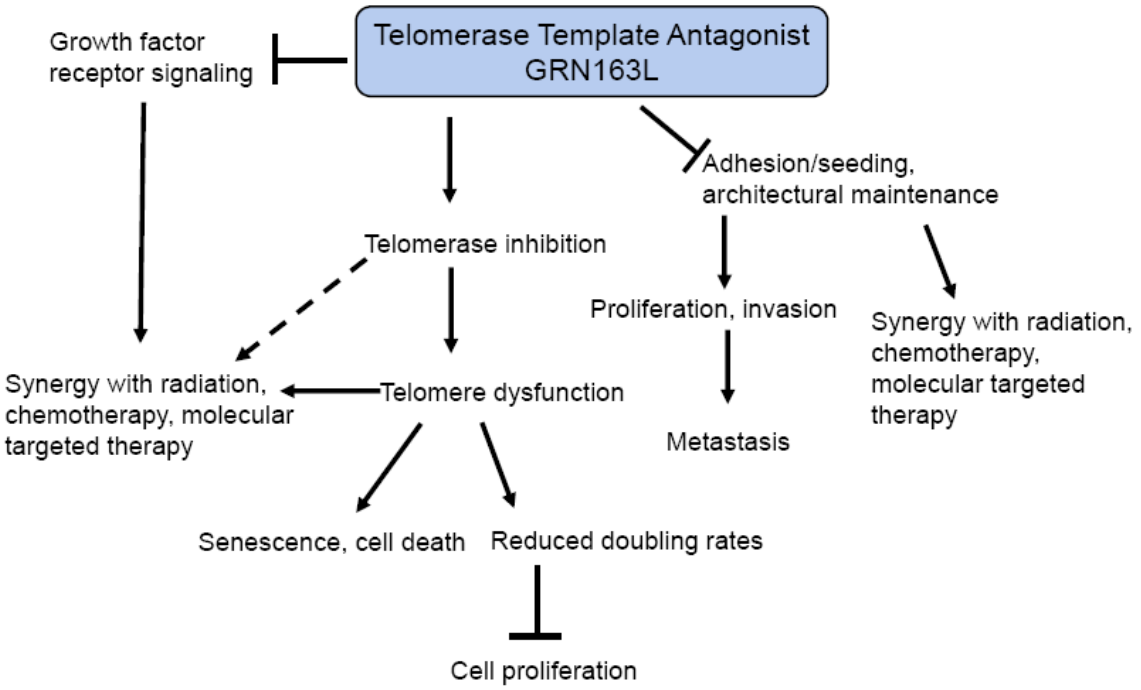


**Figure 5.3. Proposed mechanisms underlying response to GRN163L.** The effects of GRN163L result in synergy with three types of breast cancer therapy through inhibition of multiple cellular functions. Telomerase inhibition is known to result in telomere dysfunction, reduced cell growth, and the induction of senescence or cell death. The research presented in this dissertation demonstrates that telomerase inhibition via GRN13L also results in inhibition of cellular adhesion, proliferation, structural maintenance, and migration.

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CURRICULUM VITAE

Erin M. Goldblatt

**Education:**

Indiana University, Indianapolis, IN
Ph.D., Medical and Molecular Genetics, Minor in Cancer Biology
December 2008

University of Cincinnati, Cincinnati, OH
B.S. Biology with Honors
June 2004

**Honors and Awards:**

| | |
|---|---|
| 2007 | Best Poster Award- graduate student category, IU Melvin and Bren Simon Cancer Center Cancer Research Day |
| 2004-2005 | Indiana University Purdue University of Indianapolis Fellowship |
| 2003, 2004 | The National Dean's List |
| 2003 | American Heart Association Summer Research Fellowship |
| 2001-2004 | University of Cincinnati Dean's List |
| 2000-2004 | Cincinnatus Scholarship from the University of Cincinnati |
| 2000-2002 | Mary Rowe Scholarship from the University of Cincinnati |

**Fellowships:**

Graduate Fellowship in Translational Research 08/01/2006-07/31/2007
Indiana University School of Medicine
Title: Telomere Independent Effects of Telomerase Antagonist Treatment: Implications for Augmenting Chemosensitivity in Breast Cancer

Aging Fellowship 08/01/2006-07/31/2007
Indiana University School of Medicine graduate minor in Aging
Title: Role of the Longevity Gene SIRT1 (hSIR2) in Human Replicative Aging and Cancer
*Declined in lieu of the IUSM Translational Research Fellowship*

**Research and Training experience:**

| | |
|---|---|
| 2004-2008 | Indiana University School of Medicine, Dept of Medical and Molecular Genetics (Brittney-Shea Herbert, Ph.D., advisor and chair of graduate thesis committee)<br>My project is to understand the use of telomerase template antagonists in combination with other forms of cancer therapy in breast cancer cells, and how this can be translated into the clinic. The ultimate goal is to develop treatment regimens that are more specific to killing cancer cells while being less toxic to normal cells. |
| 2005 | Indiana University School of Medicine, Dept of Medical and Molecular Genetics (Kenneth Cornetta, M.D., research rotation mentor)<br>My project was to design a lentiviral vector that contained suicide genes under the control of the hTERT promoter so that expression of introduced apoptotic genes would only be active in cancer cells with telomerase reactivation. |
| 2002- 2004 | University of Cincinnati, Dept of Pathology (David Hui, Ph.D., mentor)<br>My project was to help identify a heparin sulfate proteoglycan that was involved in the anti-atherosclerotic effects of apolipoprotein-E, and to understand the mechanism underlying this effect. |
| 2001 | Children's Medical Center, Dayton, OH (Leslie Willis, supervisor)<br>My project was to test PCR efficiency of different Taq enzymes for genetic testing. |

**Skills:**

Cell culture- cell proliferation, viability, colony formation, DNA synthesis, flow cytometry
Western and Southern Blotting
Reverse transcriptase and real time PCR
Small animal handling
Subcutaneous and mammary fat pad *in vivo* tumor models
Immunofluorescence- cells and tissue
Radiation proficiency
Computer Software- Microsoft Office, GraphPad Prism, Adobe Photoshop, Primer Express, CalcuSyn

**Publications:**

1. Hochreiter AE, Xiao H, Goldblatt EM, Gryaznov SM, Miller K, Badve S, Sledge G, and Herbert B-S. The telomerase template antagonist GRN163L disrupts telomere maintenance, tumor growth and metastasis of breast cancer. *Clin Cancer Res* 2006;12: 3184-3192.

2. Gomez-Milan J, Goldblatt EM, Gryaznov SM, Mendonca MS, and Herbert B-S. Specific Telomere Dysfunction Induced by GRN163L Increases Radiation Sensitivity in Breast Cancer Cells. *Int J Radiat Oncol Bio. Phy*. 2007; 67: 897-905.

3. Herbert B-S and Goldblatt EM. Therapeutic Targets and Drugs 1: Telomerase and Telomerase Inhibitors. In: *Cancer Drug Discovery and Development: Telomeres and Telomerase in Cancer*. New York: Humana Press, Springer Science and Business Media; 2008.

4. Goldblatt EM, Erickson PA, Gentry ER, Gryaznov SM, and Herbert B-S. Lipid-conjugated telomerase template antagonists sensitize resistant HER2-positive breast cancer cells to trastuzumab. In press; *Breast Cancer Research and Treatment* 2008. DOI 10.1007/s10549-008-0201-4.

5. Herbert B-S, Goldblatt EM, Gentry ER, Smith D, Vance G, Kaur K, White MW, Shay JW. Tumorigenic conversion of Li-Fraumeni syndrome human breast epithelial cells in the absence of viral oncoproteins. In preparation.

6. Goldblatt EM, Gentry ER, Gryaznov S, and Herbert B-S. The Telomerase Template Antagonist GRN163L Alters MDA-MB-231 Breast Cancer Cell Growth and Structure, and Augments the Effects of Paclitaxel. In preparation.

**Abstracts and Presentations:**

1. Goldblatt EM, Erickson PA, Gentry ER, Gryaznov SM, and Herbert B-S. The telomerase template antagonist GRN163L sensitizes resistant HER2-positive breast cancer cells to trastuzumab. AACR Annual Meeting 2008; IU Cancer Center Scientific Poster Session 2008; Sigma Xi Research Society Graduate Research Competition 2008.

2. Goldblatt E, Ositelu O, Gryaznov S, Tressler R, and Herbert B-S. The effects of GRN163L and sensitization of breast cancer cells to paclitaxel. AACR special conference; The Role of Telomeres and Telomerase in Cancer Research 2007.

3. Goldblatt E, Hochreiter A, Stanton K, Clare S, Miller K, and Herbert B-S. Anti-tumorigenic Effects of the Novel Telomerase Inhibitor GRN163L in Breast Cancer Cells. IU Cancer Center Scientific Poster Session 2007.

4. Erin Goldblatt. “An extra virgin approach to breast cancer therapy: Targeting HER2 and TERT using olive oil” oral presentation at the IUSM Department of Medical and Molecular Genetics Research Club. May 2007.

5. Ositelu O, Goldblatt E, and Herbert B-S. The Anti-Adhesive Effects of GRN163L Sensitize Breast Cancer Cells to Paclitaxel. Summer Research Program Poster Session 2007, Medical and Molecular Genetics Retreat 2007.

6. Goldblatt E, Hochreiter A, Xiao H, and Herbert B-S. Effects of Telomerase Inhibition in MDA-MB-231 Breast Cancer Cells. Amelia Project poster session 2006; IU Cancer Center Scientific Poster Session 2006.

7. Gomez-Millan J, Goldblatt EM, Gryaznov SM, Mendonca MS, and Herbert B-S. Specific Telomere Dysfunction Induced by GRN163L Increases Radiation Sensitivity of Breast Cancer Cells. 8$^{th}$ Annual Midwest DNA Repair Symposium 2006; 48th Annual ASTRO Meeting 2006; Translational research in Radiation Oncology symposium 2006.

8. Goldblatt EM, Hochreiter AE, and Herbert B-S. Anti-tumorigenic Effects of the Novel Telomerase Inhibitor GRN163L in Breast Cancer Cells. 14th ACS Great Lakes Cancer Symposium 2006.

9. Hochreiter A, Xiao H, Goldblatt E, Gryaznov S, and Herbert B-S. Effects of the Potent Telomerase Template Antagonist GRN163L in Breast Cancer. Amelia Project poster session 2005.

**Teaching experience:**

| | |
|---|---|
| 2007 | Teaching Assistant, Q580 Basic Human Genetics lecture: “Recombinant DNA technology” (supervisor: Terry Reed, Ph.D.) |
| 2007 | IUCC Summer Research Program mentor for Oluwaranti Ositelu |
| 9/03-12/03 | Teaching Assistant, Honors course: “Science and Society,” University of Cincinnati (supervisor: Charles Sidman, Ph.D.) |

**Professional Activities:**

| | |
|---|---|
| 2007-present | AACR Associate Member |
| 2006-2007 | AAAS/Science Program for Excellence in Science |
| 2003-2004 | Golden Key International Honor Society |
| 2002-2004 | Alpha Epsilon Delta Pre-Medical Honor Society |
| 2000-2001 | Caducea Pre-medical Society |