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Effects of Adjuvant and Neoadjuvant Anti-telomerase with Anthracycline on Breast Cancer Cells

Effect of Adjuvant and Neoadjuvant Anti-telomerase with Anthracycline based Chemotherapy on Triple Negative Breast Cancer Cells

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Abstract

Breast cancer is the second leading cause of cancer related death in women in the US. In addition, 20% of all breast cancer cases in the U.S. are from the subtype known as Triple-Negative Breast Cancer (TNBC), which is the most aggressive and invasive form of the disease. This type of breast cancer has the worst prognosis, a decreased survival rate, and no targeted therapy. Over the decades, interest in pre- (Neoadjuvant) and post- (Adjuvant) chemotherapy treatments, in the management of TNBC has increased. Therefore, we evaluated the Adjuvant and Neoadjuvant effects of anti-telomerase (BIBR 1532 and GV6) with anthracycline-based (Doxorubicin) chemotherapy. In the initial (Neoadjuvant) experiment, MDA-MB-231 (TNBC) cells were supplemented with BIBR 1532 (n=4) or GV6 (n=4-6) for 14 days, then exposed to Doxorubicin (n=4) for 7 days. In the second (Adjuvant) experiment, cells were primed with Doxorubicin for 7 days (n=4) prior to 14 days of BIBR 1532 (n=4) or GV6 (n=4) therapy. The Trypan Blue (Gibco) exclusion test was used to assess the viability of the cells. After 14 days of Neoadjuvant treatment with BIBR1532, followed by 7 days of doxorubicin treatment, the cell density decreased to 59% of control (p<0.05). Adjuvant treatment with BIBR 1532 or GV6 had limited effect on the proliferation rate of MDA-MB-231 cells. A higher (p<0.05) percent of dead cells was observed in BIBR1532 adjuvant therapy with doxorubicin. These data indicates that neoadjuvant therapy with anti-telomerase in conjunction with anthracycline-based chemotherapy does have beneficial effects and warrants further investigation.
Introduction

Breast cancer remains a common cause of cancer-associated mortality among females in most countries (1). In 2015, approximately 250,000 new cases of invasive breast cancer will be diagnosed in the United States (US), with more than 40,000 cases being fatal, making this disease among the leading causes of cancer-related death in women in the US (2). Currently, breast cancers are classified into subclasses based on their ultrastructural morphology and receptor status (7). Cells that overexpress progesterone or estrogen receptors (PR or ER) are known as hormone receptor positive, and ones overexpressing the human epidermal growth factor 2 receptor (HER2) are known as HER2 positive (7). These receptors can be expressed individually or together on one cell. To treat these breast cancers, targeted therapies are used against the receptors to limit the proliferation of cancerous cells, such as, Trastuzumab, which is an antibody-based therapy that specifically targets the HER2 receptor on tumor cells (6). Breast cancer that does not overexpress any of these receptors is known as Triple-Negative Breast Cancer (TNBC), it accounts for about 15% of all breast cancer and is the most dangerous form, since it has the worst prognosis, a decreased survival rate, and no targeted therapies (1,9). In the US, TNBC is most common in women, who are younger, as well as women of African-American or Hispanic heritage, and specifically those who have mutations in the BRCA1 and 2 genes (9). Due to the lack of targeted therapy; chemo, radiation, and surgery are the only options available for women with this subtype of cancer. Due to its highly invasive and aggressive nature, as well as its’ ability to develop resistance, routinely a very high dose of chemo- and radiotherapy are utilized. Disappointingly, this approach precipitates a diverse array of side-effects, ranging from neutropenia to cardiomyopathies (13, 14).

Among these chemotherapy drugs is Doxorubicin, which has been employed extensively in the treatment of almost every type of cancer, ranging from leukemia to breast cancer, and has shown great effect in its ability to reduce tumor size (15). Doxorubicin and other anthracyclines are drugs that specifically target DNA replication, and they do so by targeting an enzyme known as topoisomerase II, which is responsible for reducing torque on the DNA during replication, by making double strand breaks and religating the strands. To target this enzyme, anthracyclines use their aglycone ring structures to intercalate between the base pairs of DNA, where they stabilize the topoisomerase II-DNA complex, which results in double strand breaks and DNA damage (3, 13). Though this drug has high effectiveness in cancer therapy, one major drawback is the production of free radicals that can build up in and around the cardiac muscle and result in cardiomyopathies (13, 14). In order to reduce the risk of cardiomyopathies, many researchers have begun investigating using low-dose extended-period treatment regimens to reduce toxicity or combination treatment with other drugs in order to reduce the duration of doxorubicin exposure. A study done by Scheithauer et al., (15), showed that a weekly low-dose regime with doxorubicin did not elicit cardiomyopathies, and also had a relatively low generalized toxicity. This provides a significant improvement in the clinical outcome over the standard method of treatment with larger doses over a short period of time. Further studies have also shown that a reduced dose over a longer period does not diminish the efficacy of the Doxorubicin, but significantly reduces the associated risk of cardiomyopathies (14).

In recent years, interest in the biology of telomeres and the enzyme telomerase has increased in relation to cancer treatment. The telomeres cap and protect the ends of the chromosomes from end-to-end fusion, and are made up of a large number of repetitive sequences (TTAGGG in mammals) (10). In most human somatic cells, these telomeres gradually erode
away with each replication, since DNA polymerase I cannot replicate the ends (10). Once the
telomeres have eroded to a critical length, the cells go through cellular senescence and cease to
proliferate (10). To overcome this end-replication dilemma, some cells activate the enzyme
telomerase, to lengthen the telomeric sequence. Typically it is repressed in most somatic cells,
though now it is known to be activated in almost 90% of cancers (1,10), which allows these
tumor cell lines to elongate their telomeres and achieve immortalization. Recently, the drug
BIBR1532 (GV1) (4,5) and novel derivatives (GV6) (5) have shown promise in the inhibition of
the telomerase enzyme, which subsequently induces cellular senescence.

One drawback though of anti-telomerase therapies is that they require extended treatments in
order to have notable effects in vivo, since the cells only experience mitotic crisis when their
telomeres have shortened significantly, which occurs only after a large number of population
doublings (4,5). Because of this downside, many researchers have suggested using it in a
neoadjuvant or adjuvant setting, in order to either sensitize cancer cells to other therapies or
eliminate remaining cancer cells after the primary treatment (4,5,11). We thus investigated the
efficacy of neoadjuvant or adjuvant therapy with the anti-telomerases GV1 (BIBR1532) and
GV6 in potentiating the effects of anthracycline (Doxorubicin) based chemotherapy.
Materials and Methods

Cell line

Triple-Negative Breast cancer cells (MDA-MB-231) were cultured in RPMI (Life Technologies, NY) media supplemented with 10% fetal bovine serum (Innovative Research, MI) plus 500uL Antibiotic-Antifungal (Life Technologies, NY) in an incubator set at 37 °C and 5% CO₂.

Treatment

Cells were seeded at a density of 0.50x10⁶ cells/ml (T-25) and cultured for 72 hours in solvent-free RPMI media to allow cells to acclimatize to culture conditions. The media was then supplemented with 10μM GV6, or 10μM BIBR1532 for the neoadjuvant treatment or with 100nM Dox for adjuvant treatment. After 14 days of neoadjuvant treatment with GV6 or BIBR1532, cells were then supplemented with 100nM DOX for 7 days, or after 7 days of DOX for the adjuvant treatment, cells were then supplemented with 10μM GV6 or BIBR 1532 for 14 days.

Viability Assessment

At days 7, 14, and 21 of culture in treatment, relative cell densities were evaluated using a hemocytometer and the live/dead ratios were calculated using the Trypan Blue Exclusion Test (Life Technologies, NY). The number of live/dead cells was estimated by counting and averaging the number of cells within a set of four defined grids using an inverted microscope (Leica IL; 100X).

Senescence Test

A commercially available Senescence-Associated β-galactosidase (SA-βGal) Staining Kit (Cell Signaling Technology, MA) was used on day 21 to detect the cellular activity of β-galactosidase at an acidic pH. The average percentage of SA-βGal positive cells per treatment was estimated from three independently obtained micrographs using an inverted microscope (Olympus, PA; 100X).

Statistical

Statistical analyses were performed using the Student paired t-test. P<0.05 were considered significant.
Results

Effects of Neoadjuvant and Adjuvant Anti-Telomerase with Anthracycline on MDA-MB-231 Proliferation

Exposure to fourteen days of GV1 prior to seven days of doxorubicin treatment, significantly (P<0.05) decreased (59%) MDA-MB-231 cell counts in relation to control (Fig 3). On the other hand, Neoadjuvant use of GV6 with the doxorubicin had no significant effect on proliferation rates in relation to control. A significant (P<0.05) drop in cell counts was observed between days 14 and 21 in neoadjuvant GV1 treatment (doxorubicin exposure), whereas GV6 neoadjuvant showed no decrease within the same timeframe.

![Figure 3: Number of viable MDA-MB-231 cells in media supplemented for 14 days with GV1 (BIBR1532) or GV6, then supplemented with Doxorubicin for 7 days, at days 7, 14, and 21, as determined by Trypan-Blue Exclusion test (n=4). Cells are expressed relative to control. Dashed line indicates DOX treatment. Data are shown as Mean ± SD; ac are significantly different (p<0.05).](image-url)
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In the adjuvant treatment (Fig 4) approach of seven days of doxorubicin followed by continuous fourteen days of GV1/GV6 treatments, there was a significant drop (p<0.05) in cell numbers by day 14 of trial (61% with GV1 and 47% with GV6 compared to control). However, after an additional exposure of seven days (day 21 of trial) to GV1, a significant (p<0.05) increase in MDA-MB-231 cell counts in relation to control was noted. GV6 trend paralleled that of GV1 (Fig 4).

Figure 4: Number of viable MDA-MB-231 cells in media supplemented with Doxorubicin for 7 days, then GV1 (BIBR 1532) for 14 days, at days 7, 14, and 21 as determined by Trypan-Blue exclusion test (n=4). Cells counts are expressed relative to Control. Dashed line indicates DOX treatment. Data are shown as Mean ± SD; ac are significantly different (p<0.05).
Percentage of Dead MDA-MB-231 Cells in Neoadjuvant and Adjuvant Anti-telomerase Treatment with Doxorubicin

In figure 5, significant increases in dead cell counts were only observed between days 7 to 14 in the adjuvant GV1 treatment group.

**Figure 5:** Dead MDA-MB 231 cells as a percentage of the total cells supplemented with Neoadjuvant BIBR1532 and GV6, Adjuvant BIBR1532 and GV6, or Control media, at days 7, 14, and 21. ab are significantly different (P<0.05)
Evaluation of Senescence and Proliferation in MDA-MB-231 Cells

In figure 6A, it is apparent that both adjuvant and neoadjuvant GV1/6 significantly decreased (p<0.05) the number of replicatively active cells in relation to control, with no notable differences between the adjuvant and neoadjuvant groups. In contrast, Figure 6B demonstrates that though both adjuvant and neoadjuvant GV1/6 significantly increased the number of cells expressing the senescent phenotype, but with neoadjuvant GV1/6 having a significantly higher fold change of senescent cells (p<0.05) than in the adjuvant GV1/6 group.

Figure 6: Number of (A) non-senescent (replicatively active) cells, and (B) senescent cell/treatment in comparison to Control on day 21 of treatment. Data are shown as Mean ± SD. ab, ac, ad, and ae are significantly different (p<0.05) in each panel.
Discussion

TNBC has the worst prognosis, a decreased survival rate, and no targeted therapies, due to its lack of the key receptors (ER/PR/HER2) that drive most breast cancer cell proliferation (7,9). Without these receptors, there is no method of targeted therapy to counteract it, and thus higher doses of chemo- and radiation therapies are necessary. Higher doses of these therapies, specifically chemotherapy, can have adverse side effects, including cardiomyopathies and neutropenia, which can be fatal (3,13,14). Because of these effects, as well as the lack of targeted therapies for TNBC, many researchers have begun exploring new and novel pathways to treat TNBC, including targeting of the telomere/telomerase complex, which is responsible for cell line immortalization (10). Drugs targeting the telomeres have notable drawbacks, and thus researchers have suggested that they be used in neoadjuvant or adjuvant settings with chemotherapy drugs in order to sensitize the cancer cells to the chemo, so smaller doses can be used that are less detrimental to patient health (4). BIBR1532 (GV1) and GV6 are novel anti-telomerase drugs, and Doxorubicin is a universally employed chemotherapy drug (3,4,13).

In this study, MDA-MB-231 breast cancer cells were either treated with neoadjuvant GV1 and GV6 then exposed to doxorubicin, or were treated with adjuvant GV1 and GV6 after exposure to doxorubicin. Under the neoadjuvant approach, GV1/6 revealed (Figure 3) the most notable changes between days 0-7, and days 14-21. The drop between days 0-7 correlates with initial sensitization of the cells to GV1/6 and this effect is sustained up to day 14. This is likely due to the varying lengths of the telomeres within the cell lines, as noted by Damm et. al. (4), which causes the cells to enter replicative senescence in a sequential fashion, thus the initial count reduction would be from cells with originally short telomeres entering senescence. Our data indicates that introduction of doxorubicin between days 14 to 21 leads to further drop of 15% in proliferation rates in the group with GV1 as a neoadjuvant treatment. However, in our earlier continuous combination (GV1+doxorubicin) treatment studies (data not shown), a decrease of approximately 46% in proliferation rate was observed after 21 days of exposure, indicating that the drugs are more potent when given synergistically rather than in a neoadjuvant manner for sensitization, though the exact mechanism of this synergism is not known. Within this same timeframe, cell counts are not significantly affected by doxorubicin with neoadjuvant GV6, indicating that GV6 is not as effective as GV1. Though when interpreting GV6+doxorubicin synergism data (not shown), it is noted that counts drop to 44% of control by day 21, indicating that GV6 and DOX do have a synergistic effect.

For these trials, dead cell percentages were also collected for each treatment day, and senescence data was collected on day 21. No increase in dead cell percentages was noted compared to control, denoting that the concentrations utilized were not cytotoxic to the cells (Figure 5). The senescence data (Figures 6A and B) shows that both, adjuvant and neoadjuvant anti-telomerase therapy are significantly effective at decreasing the number of replicatively active cells compared to control. However, there were significantly more senescent cells in the neoadjuvant group compared to adjuvant (p<0.05); implying that a combination of reduction in number of actively replicating cells plus increased rate of senescence leads to decreased proliferation of tumor cells in all of the experimental groups.

The data collected here indicates that GV1 is more effective as a neoadjuvant treatment with doxorubicin on MDA-MB-231 breast cancer cells, based on its decrease of cell counts on day 21 by 40%, which is significantly more than that of GV6 which only reduced cell counts by 24% by day 21. In contrast, GV6 appears to be more effective than GV1 in the adjuvant model,
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since it reduced cell counts more significantly by days 14 and 21 (40% vs 25% and 51% vs 40% of control, respectively). This indicates that GV6 becomes significantly more effective in inhibiting MDA-MB-231 cancer cell growth when cells are exposed to anthracycline therapy prior to GV6 exposure, while GV1 is more effective as pretreatment for cells before they are exposed to chemotherapy, indicating possible separate mechanisms of sensitization within the cells to the different treatments. Furthermore, our data suggests that induction of senescence rather than apoptosis leads to the reduction in proliferation of MDA-MB-231 breast cancer cells. This data is consistent with that collected by Legha et al., (19), who observed significantly decreased cardiotoxicity when patients were exposed to continuous low dose doxorubicin treatment over a period of 48 or 96 hours, but did not observe reduced antitumor efficacy, supporting the idea that continuous low dose anthracycline can retain its toxicity to cancer cells. Further, it agrees with Damm et al., (4) who noted BIBR1532’s (GV1) ability to induce the senescence phenotype in MDA-MB-231 cells (4,19).

This study indicates that adjuvant and neoadjuvant anti-telomerase drugs with anthracyclines are effective in inhibiting proliferation of TNBC cells, as well as inducing the senescence phenotype within these cells. These results contradict Mauri et al., (16), who found that adjuvant and neoadjuvant treatment types were for the most part, similar in their effect on invasive breast cancers. Though, they based their assumption on data collected at the patient level rather than the in-vitro cell based model utilized in this study. In spite of this, further studies are needed to elucidate the mechanisms for the anti-proliferative properties of adjuvant and neoadjuvant anti-telomerase approach, as well as verify and expand upon these findings.
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References

1) World Cancer Research Fund International 2013

2) American Cancer Society, Inc. 2015


