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Abstract

Breast cancer is the second leading cause of death among women in the United States. Among the different molecular sub-groups of breast cancer, the most invasive is Triple-Negative Breast Cancer (TNBC). TNBC has the worst prognosis, decreased overall survival rate and no targeted therapy available. On-going research is investigating new strategies and therapies for TNBC. Therefore, this study's objective was to compare and contrast the effects of continuous low-dose of BIBR 1532, a novel analogue of BIBR1532 (GV6), Paclitaxel and Doxorubicin on breast cancer (MDA-MB 231) cells. Culture flasks (T-25) were seeded with approximately $5.0x10^5$ cells/ml and supplemented with GV6 (n=4-8) or BIBR 1532 (n=4-8) or Doxorubicin (n=4-8) or Paclitaxel (n=4-8) or non-drug supplemented media (Control, n=4-8) for 21 days. Trypan Blue (Gibco) exclusion test was utilized to assess the viability of the cells. BIBR 1532, Doxorubicin and Paclitaxel reduced ($P<0.05$) proliferation of the cancer cells by approximately 20-35% by day 7 of treatment compared to the Control. By day 21 of low-dose GV6, BIBR1532, Doxorubicin and Paclitaxel supplementation, the cell counts dropped to about 25% ($P<0.05$), 55% ($P<0.05$), 75% ($P<0.05$) and 50% ($P<0.05$) of Control, respectively. Our results indicate that continuous low dose anti-telomerase and chemotherapeutic drugs do inhibit breast cancer cell proliferation and merits further investigation.

Introduction

In 2014, the United States will have an estimated 250,000 new cases of invasive breast cancer that accounts for 14% of all new cancer cases (1). Of the 250,000 new cases diagnosed in 2014, it is estimated that 40,000 will die due to the disease, which accounts for about 7% of all cancer-related deaths (1). In 2012 there were 1.7 million new cases of breast cancer worldwide that cost about $88 billion in health care (1). This number represented about 12% of all new cancer cases and 25% of all cancers in women (9).

Breast cancers are currently classified into subclasses based on their ultrastructural morphology. Among the most invasive, with high metastatic and recurrence rates, as well as one of the worst prognoses is triple negative breast cancer (TNBC). In the US, TNBC is occurring in higher rates in younger women

and in African American women. The currently available drugs targeting other subtypes that over express the estrogen receptor or progesterone receptor or human epidermal growth factor receptor two (HER2) are of limited benefit with TNBC. This type of cancer over time can become very aggressive and difficult to treat. Due to the aggressive nature of TNBC, a higher dose of chemotherapy and/or radiation is recommended to the patient. Studies have reported that patients show a variety of adverse side effects with higher doses ranging from: infertility, organ damage, secondary cancers, and fatality (1, 5, 6). For example, when high dose of cyclophosphamide (180 mg/kg) is administered, 28% of these patients start to show progressive signs of congestive heart failure, and 19% of these patients succumb due to cardiac failure (4).

Recent developments in life sciences are providing new understanding to the cellular and molecular flaws of cancer cells. One field of study, being pursued by cancer researchers is the regulation of cellular replication. The two important elements of cellular replication are; division of the cytoplasm and the duplication of DNA. The main focus of the scientist has been on DNA replication, as it is part of the cell that encodes for all processes within the cell, and is passed from one cell to another during cell division. Cancer emanates when there is a flaw within one of the phases associated with DNA duplication, therefore making the cancer cells divergent from normal cells. Cytoplasmic replication has a limited role to play in triggering cancer because it is mostly involved in transportation of intracellular components.

During DNA replication, the cell proceeds through an inherent systemic quality control process via checkpoints, which detect alterations within the DNA. The damage can be induced by the environment, chemicals or genetics. Damaged or mutated cells cannot pass these checkpoints and are directed towards senescence. These checkpoints are controlled by cyclins and if a cyclin is mutated, it will affect the overall quality control process. Consequently, cancer cells will be able to bypass these checkpoints with atypical cyclins without being eliminated. Once these cells evade the internal screening mechanism, they acquire unrestricted proliferative capacity leading to malignant cells (2). A number of drugs targeting enzymes associated with the DNA duplication cycle have been developed to halt progression of cancer cells. Among them is Doxorubicin (DOX) from the anthracycline family, which is routinely used as an anti-cancer drug. Doxorubicin inhibits enzymes, such as topoisomerase II that are associated with DNA duplication.

In most human cells, an enzyme called telomerase helps cap the end of chromosomal DNA (telomere), this activity is low and barely detectable. As the DNA is replicated, telomere DNA becomes shorter with each cell division. Ultimately, when the telomere becomes too short, the chromosomal DNA is not protected and can be easily degraded (2). Interestingly now in cancer cells, we know the telomerase is reactivated with each cell cycle, which lengthens the

telomere to detectable lengths, causing the damaged DNA to stabilize and pass through the cell division checkpoints (2). Further understanding, of the telomere/telomerase complex has lead to the creation of a now common drug called Azidothymidine (AZT). AZT was used extensively to treat HIV and AIDS by targeting the replication of viral DNA. The knowledge of the telomere/telomerase complex has lead to a development of other synthetic anti-telomerase molecules, including BIBR1532 (3). BIBR 1532 blocks the limitless proliferative capacity of metastatic cancerous cells by impeding the progression of telomere/telomerase complex (2). A novel analogue of the BIBR1532, named GV-6, has been developed at Grand Valley State University.

To counteract the side effects of the high-dose of chemotherapy used to treat TNBC, a number of studies are investigating chronic low-dose therapies (5, 8, 9). Low dose therapies have been very effective at reducing the cytotoxic effect of the chemotherapeutic agents (5, 8, 9). Overall, studies show that in a low-dose therapy, the agent still has a significant effect on the cancerous cells, such as tumor size, while demonstrating limited toxicity (5, 8, 9). A recent study has shown that when a weekly low-dose was given, 36% of patients had a progressive decrease in tumor sizes, while having a limited number of side effects (5). The goal of this study was to evaluate the effect of chronic low-dose mono-therapies (BIBR1532 or GV6 or DOX) compared to combination (DOX+BIBR1532 or DOX+GV6) treatment on the proliferation of commercially available TNBC (MDA-MB 231) cells.

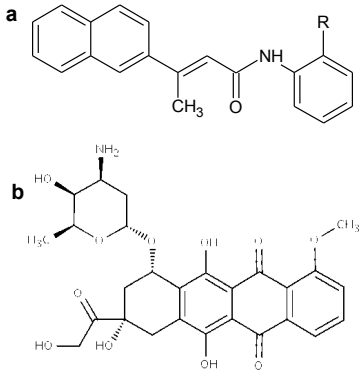

Figure 1: (**a**) Chemical structure of BIBR1532 (R=COOH) and GV6 (R=unknown). (**b**) Chemical structure of Doxorubicin

Materials & Methods

Cell line

Breast cancer cells (MDA-MB231) were cultured in RPMI (Life Technologies, NY) media supplemented with 10% fetal bovine serum (Innovative Research, MI) plus 100uL of 100X Anti-Anti (Life Technologies, NY) in an incubator set at 37 °C and 5% CO_2.

Treatment

Cells were seeded at a density of $0.50x10^6$ cells/ml (T25) and cultured for 72 hours in solvent-free RPMI media to allow cells to acclimatize to culture conditions. Thereafter, the media was switched to fresh RPMI media alone for Control (n=2-4) or media supplemented with 10μm GV6 (Fig 1), or 10μm BIBR1532 or 100nm DOX (Fig 1) or 100nm DOX+10μm GV6 or 100nm DOX+10μm BIBR1532 (n=4-6).

Viability Assessment

At days 7, 14, and 21 of culture with or without treatments, relative cell densities were evaluated by 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)

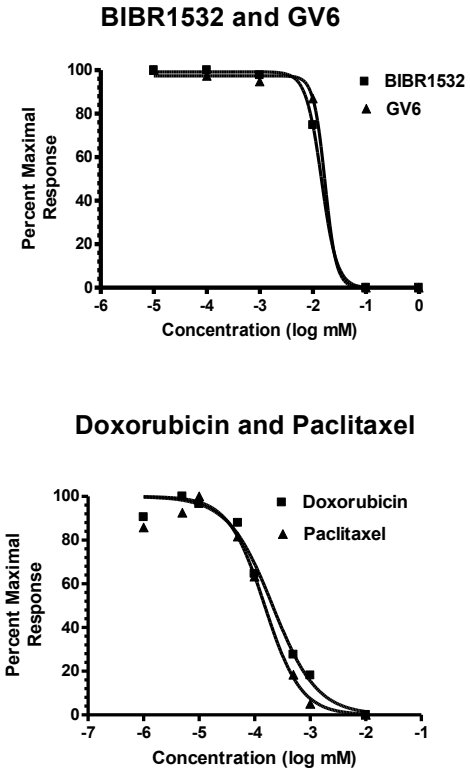

Figure 2: The Dose-Response curves of DOX, Paclitaxel, BIBR1532 and GV6. Figure 3b DOX and paclitaxel dose curve is made up of concentrations from 10μM to 1nM. Figure 3a BIBR1532 and GV6 dose curve is made up of concentrations form 1mM to 1nM. The EC_{50} for DOX, PAC, BIBR1532 and GV6 is: 201nM, 152nM, 15μM and 17μM, respectively. (R^2 =0.98 to 0.99)

Statistical

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

Results

Dose Curve

The dose response curves of BIBR 1532, GV6, doxorubicin and paclitaxel are shown in Figures 2a and b. There was not a large difference in the EC_{50} concentrations of BIBR1532 and GV6 (201nM vs 152nM, respectively). Similarly, the EC_{50} for doxorubicin was comparable to that of paclitaxel (15μM vs 17μM).The correlation coefficient (R^2) values for BIBR 1532, GV6, and doxorubicin, paclitaxel dose-response curves were 0.98 and 0.99, respectively.

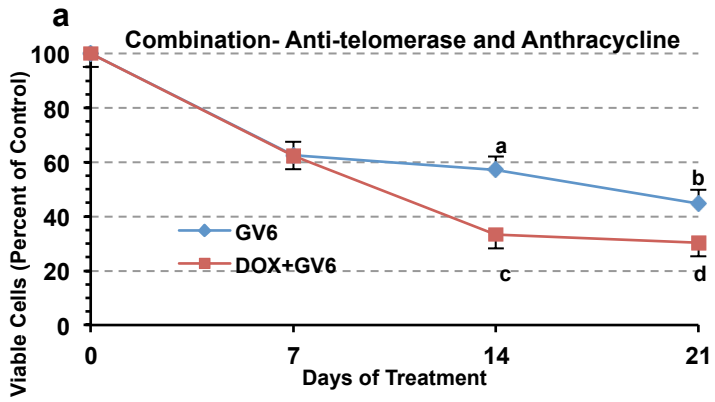

Figure 3: Number of viable MDA-MB 231 cells in media supplemented with GV6 and DOX+GV6 at days 7, 14, and 21 as determined by Trypan-Blue exclusion test (n=4-6). Cells counts are expressed relative to Control. Data is shown as Mean ± SD; [ac, and bd] means are significantly different ($p<0.001$).

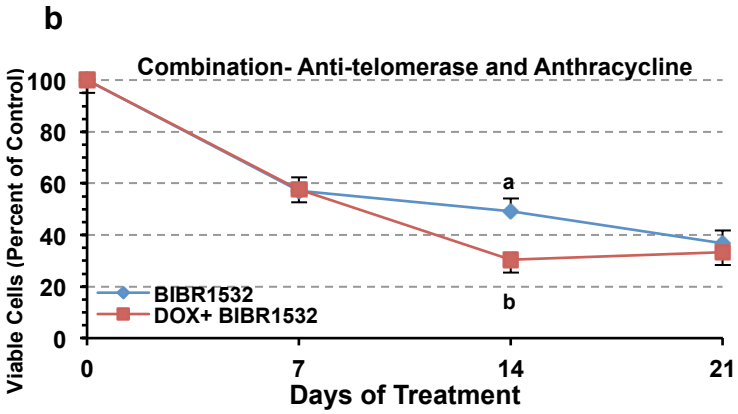

Figure 4: Number of viable MDA-MB 231 cells in media supplemented with BIBR1532 and DOX+BIBR1532 at days 7, 14, and 21 as determined by Trypan-Blue exclusion test (n=4-6). Cells counts are expressed relative to Control. Data is shown as Mean ± SD; [ab] means are significantly different ($p<0.001$)

Effects of Single and Combination Anti-telomerase and Anthracycline therapy on MDA-MB 231 cells

The Figures 3 and 4 show the effects of single and combination of anti-telomerase (GV6 or BIBR1532) and anthracycline (doxorubicin) therapy on viable MDA-MB 231 cells for 7, 14 and 21 days compared to Control.

The effects of the combination of doxorubicin with either BIBR1532 or GV6 revealed a significant effect ($P<0.001$) compared to single treatments. Both GV6 and BIBR1532 depicted similar potencies when compared to control. The efficacy of the combination treatment increased with duration of culture ($p<0.001$).

Senescence Test

No difference was noted between the number of active cells between single and combination treatments (Figure 5a). The number of senescent cells was more in single and combination treatments when compared to control (Figure 5b). The amount of dead cells did not change much between treatments (Figure 6).

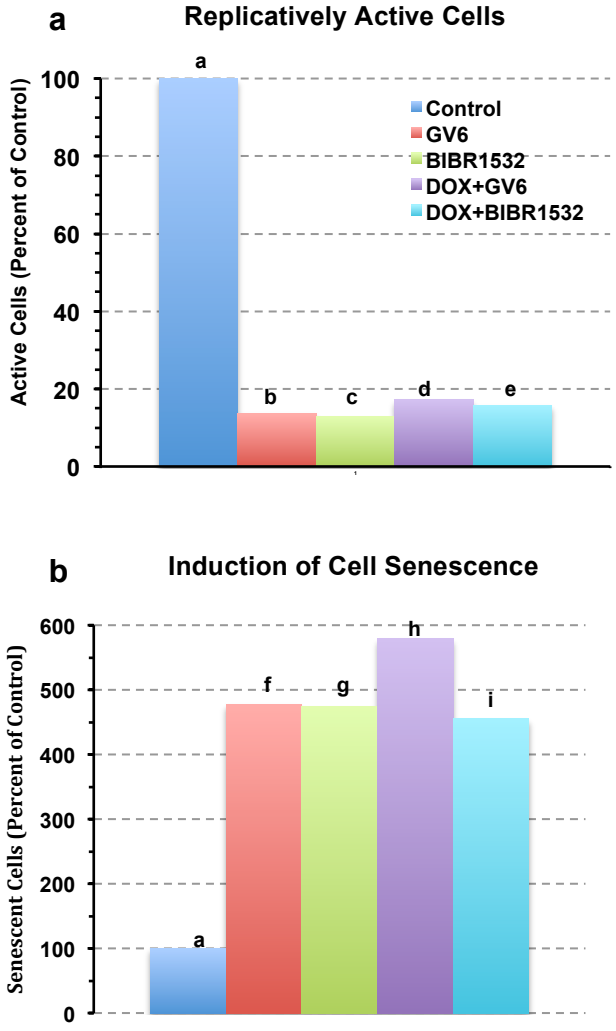

Figure 5: Number of (A) non-senescent (replicatively active) cells, and (B) senescent cell/treatment in comparison to Control on day 21 of treatment. Data is shown as Mean SD. $^{ab, ad, af, ag, ah, and\ ai}$ means within each panel are significantly different ($p<0.05$)

Discussion

TNBC has one the worst prognosis because it is missing the key factors that typical breast cancer has (1). These key factors are the reason why TNBC has one of the poorest prognoses. With these factors missing TNBC has a more aggressive nature and is therefore harder to treat. There is no specified treatment for TNBC. The only current treatment for TNBC is either a high-dose of chemotherapy or radiation. The high dose of these agents has adverse side effects that can lead to organ failure or even death (1,4,5,6). Without a specific target to use against TNBC, scientists have been looking for other factors which can be targeted to fight TNBC. A few of the factors they have found that can be targeted in TNBC cells are, the amount of telomere DNA and the cell checkpoints within the cell replication stages. One of the most common anthracycline is doxorubicin and the most common anti-telomerase agents is BIBR1532 and the novel GV6 (2,3).

Doxorubicin is noted to be very effective against TNBC (5,6,7,8). A recent study has shown that it reduces tumor size significantly but is cytotoxic and cells do develop resistance (5,6,7,8). On other hand, BIBR1532 is also shown to be very effective against TNBC. A recent study showed that BIBR1532 worked very well against TNCB cells when glucose levels were increased (13). The single and combination of effects of chronic low dose of these chemotherapy drugs on MDA-MB 231 cells is the main target of this study.

To our knowledge, this is the first study that demonstrates effectiveness of BIBR1532 or GV6 in combination with DOX against TNBC in a chronic low-dose regime. The steady decrease in the number of viable MBA-MB 231 cells

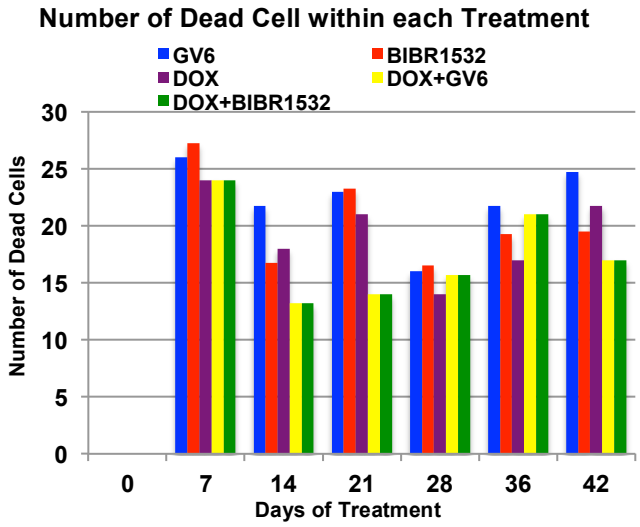

Figure 6: Number of dead cells per treatment over a 42 day period.

indicates that the treatments of BIBR1532 or GV6 in combination with DOX are efficacious (figure 3 and 4). The results also show that the number of senescent cells in the combination trails have not increased when compared to the single treatments (figure 5). The amount of dead cells within the combination trails also has deceased slightly form the single treatment trails. The results of this experiment have shown that there is a significant difference between single and combination treatments.

The combination of the BIBR1532 or GV6 with DOX shows similar results (figure 3 and 4). Recent studies have shown that the combination of different drugs with DOX when used against TNBC has been more effective then single treatments with less cytotoxic effects (5,8,9,10). A recent study revealed that the combination of doxorubicin or paclitaxel with another anti-cancer or anti-telomerase drug is very effective against many different cell lines (12). These results are very comparable to the results shown here, presenting that both treatments works better in combination versus single treatment.

The dose curve for this trial showed an EC-50 of about 201nM for doxorubicin. Another study has shown an EC-50 of 130 nM for doxorubicin (11). This difference is plausibly from using two different cells lines and having slightly different culture conditions between the two studies (11). Additionally, our study found that the number of dead cells were generally higher in the single treatments versus the combination treatments.

This preliminary study illustrates that the combination of anti-telomerase with an anthracycline agents is more effective in inhibiting proliferation of TNBC when compared to both single-drug anti-telomerase mono-therapies. Further studies are necessary to verify and expand upon these current findings by next using patient derived cells.

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