Research Article

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International Journal of Drug Development and Research

2020
Vol.12 No.1:146

DOI: 10.36648/0975-9344.12.1.146

Potent Suppression of Proliferation of Breast Carcinoma Cells by a Novel Anthranilic Acid Derivative

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Received date: Jan 10, 2020; **Accepted date:** Jan 21, 2020; **Published date:** Jan 28, 2020

Citation: Fisch A, Reiber C, Schroeder W, Smart R, Patel OV (2020) Potent Suppression of Proliferation of Breast Carcinoma Cells by a Novel Anthranilic Acid Derivative. Int J Drug Dev & Res. Vol. 12 No.1:1.

Abstract

Background/objective: Chemotherapy remains the mainstay therapy for the most intrusive-type of breast carcinoma, triple-negative breast cancer (TNBC) that has a higher tendency for visceral metastases, relapses, and poor prognoses. It is well recognized that the reactivation of a ribonucleoprotein enzyme, telomerase is among the key determinants of breast carcinogenesis, cellular immortalization and metastatic progression. Therefore, our objectives were to assess (i) short- and (ii) long-term effects of a novel anthranilic acid (GV6) developed at our institute and compare it to a known analogue, BIBR1532 on TNBC (MDA-MB 231) and non-TNBC (MCF-7) cells.

Methods: Seeded TNBC and non-TNBC flasks were supplemented with 25 µM of either BIBR 1532 or GV6 or solvent (DMSO) alone for 14 (Short-term) or 27 (Long-term) days. Trypan-blue dye exclusion test was utilized to determine viable cells, senescence-associated β-galactosidase activity was employed to detect senescent cells and qPCR was used to quantitate transcript abundance.

Results: Cell viability assay revealed that short- and long-term growth inhibitions of TNBC and non-TNBC cells were comparable between BIBR1532 and GV6. Cytochemical detection of β-Galactosidase demonstrated that GV6 was equally effective in inducing replicative senescence in treated TNBC and non-TNBC cells. Short- and long-term regimen of BIBR1532 and GV6 showed similar drug-induced downregulation of hTERT in TNBC and non-TNBC cells.

Conclusion: Results indicate that GV6 is an equally potent inhibitor of hTERT that induces growth impedance and triggers senescence in TNBC, as well as non-TNBC cells and merits further studies for improved treatment options.

Keywords: Anthranilic acid; Breast cancer; Cytochemistry; hTERT; Senescence

Introduction

Breast cancer remains the most common malignancy diagnosed in women worldwide [1,2]. Global estimates from the first decade of the 21st century indicate that close to 1.5 million new cases of breast cancer were diagnosed in women and about 500,000 individuals do succumb to the disease annually [1-3]. In the US, breast cancer remains the most diagnosed cancer in women with about 250,000 new cases per annum and accounting for approximately 40,000 deaths [4-6]. Alarmingly, the US has one of the highest prevalence rates of breast cancer in the world with one in eight women likely to be diagnosed during their lifetime [4-6]. Direct and indirect costs associated with treating breast cancer in the US are estimated to increase from about $15 billion in 2010 to over $25 billion by 2020 [7-9].

Generally, the molecular subtyping of breast cancers is based on the expression profiles of distinct hormonal and growth receptors [10-12]. Furthermore, the intrinsic molecular configuration of these breast tissue biomarkers is universally employed to dictate therapeutic approaches, as well as predict disease progression and prognosis [10,13,14]. Subsequently, targeted therapies for these hormone- and growth-receptor positive tumors have been developed and available to clinicians [12,15,16]. However, there is a unique molecular subtype of breast cancer characterized as triple-negative breast cancer (TNBC) that lacks the expression of these hormone- and growth-receptors [17,18]. Approximately, 20% of all breast cancers are identified as TNBC and the established targeted treatment modalities are of limited benefit [17,19]. In addition, a high percentage of patients with BRCA1 and BRCA2 mutants will likely develop TNBC [20,21]. Likewise, TNBC is occurring in higher rates in younger women and in African American women [17,19]. TNBC, in general, is a more aggressive tumor compared to other breast cancer subtypes and has a tendency to rapidly metastasize to visceral organs [10,12,18].

Relapse-free time is shortened with the disease returning within the first three years, and death occurring inside five years of initiating treatment [13,14]. Currently, the choice of treatment for TNBC remains the conventional cytotoxic chemotherapeutic agents and radiotherapy [22,23]. Routinely, a higher dose of chemotherapy and/or radiotherapy, or a combination of anti-cancer drugs are utilized to counteract the aggressive nature of TNBC [22,23]. However, major concerns with the current treatment paradigm are precipitation of chemoresistance, plus the myriad side-effects and triggering of secondary cancer in patients [10,15,22]. Therefore, absence of specific target, higher resistance towards the standard therapeutic approaches, and poor disease prognosis has led to a concerted effort to find a more robust and effective molecular target (s) to treat TNBC.

Previously, the causation of breast cancer was poorly understood, but research over the decades has delineated some of the underlying causes of the disease based on its cellular and molecular signature [24-26]. The elucidation of critical differences between normal and cancerous cells has provided avenues to develop targeted therapy against the neoplasms. It is now recognized that cancer cells escape senescence and attain replicative immortality by activating telomerase [24-26]. Telomerase, an enzyme with reverse-transcriptase activity, elongates the protective ends (telomeres) of the DNA molecule and over time these ends progressively get shorter with successive cell division. Typically, telomerase is either barely active or is completely lost in differentiated human cells [26,27]. Unfortunately, the ability to extend the telomeres by telomerase allows the cell to proliferate with unlimited capacity by evading senescence [24,27]. Furthermore, majority of breast carcinomas are known to increase the activity and expression of this enzyme, including TNBC [28-31]. Therefore, targeting TNBC with telomerase inhibitors could potentially impede its replicative and metastatic propensity and reduce mortality rates associated with this cancer.

One of the first developed carboxylic acid molecule 2-[(E)-3-naphtalen-2-yl-but-2enoylamino]-benzoic acid (commonly referred to as BIBR1532), is known to impede telomerase activity by binding to the catalytic subunit of the enzyme [32]. BIBR1532 has been shown to inhibit cancer cell growth [33-40] including breast carcinomas [28,30,31]. However, a major issue with BIBR1532 is that there is a long time-lag between commencement of treatment and noticeable decrease in proliferation of tumor cells [33,38]. Additionally, BIBR1532 is only capable of suppressing cancerous cell growth rather than impeding their development indefinitely. Therefore, the present study was conducted to assess the anti-cancer properties of a novel carboxylic acid analogue with an aliphatic side-chain moiety, called GV6, employing TNBC and non-TNBC cells.

Materials and Methods

Synthesis of 4-chloro- (2-nonenoyl) anthranilic acid (GV6)

Starting material: Trans-2-nonenoic acid was purchased from Alfa Aesar (Haverhill MA) and used without additional purification. 2-amino-4-chlorobenzoic acid and dimethoxyethane were obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification.

Reaction steps/conditions: A mixture of trans-2-nonenoic acid (1.5 grams, 0.0096 moles), thionyl chloride (5 mL, 0.069 moles) and dimethylformamide (0.1 mL, 0.0013 moles) was stirred at room temperature for 15 minutes. The temperature of the reaction was increased 65°C for an additional 10 minutes.

Excess thionyl chloride was removed under reduced pressure on the Rotovap at 65°C. The resulting crude acid chloride was dissolved in dimethoxyethane (5 mL). This solution was added dropwise, with good stirring, to a solution of 2-amino-4-chlorobenzoic acid (1.0 grams, 0.0058 moles) and potassium carbonate (2.5 grams, 0.018 moles) dissolved in a mixture of water (15 mL) and dimethoxyethane (15 mL) at 5-10°C. After 15 minutes, the temperature was allowed to rise to room temperature and stirred for another 30 minutes. Addition of HCl (50 mL, 20%) gave a precipitate of the product (4-chloro- (2-nonenoyl) anthranilic acid) (**Figure 1**) which was collected, washed with water, dried in air and recrystallized from acetic acid water [yield 1.17 grams (65.1%)]. The identity and purity of the synthesized GV6 was confirmed by spectroscopy [^{1}HNMR, ^{13}C-NMR, JEOL 400 MHz (Peabody MA)], and elemental analysis.

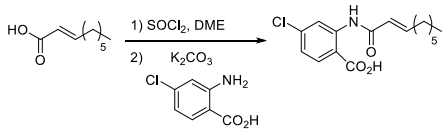

Figure 1: Reagents and synthesis pathway of 4-chloro- (2-nonenoyl) anthranilic acid (GV6).

Cell culture

MDA-MB 231 (TNBC) were maintained for the duration of the experiments in RPMI (Life Technologies, NY), while MCF-7 (non-TNBC) cells were grown in DMEM (Life Technologies, NY) media. Both mediums were supplemented with 10% fetal bovine serum (Innovative Research, MI) plus 100 unit/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL Amphotericin B (Life Technologies, NY). The TNBC and non-TNBC cells were seeded at a density of 1 × 10^{6} cells/mL (T-75 culture flasks, n=5-6 flasks per treatment) and allowed to acclimate for 72 hours before starting the treatments. The drugs (GV6 and BIBR1532) were prepared as 10 mM stock solutions in 0.5% DMSO. Thereafter, the acclimation-media was switched to media supplemented with either solvent

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alone (0.5% DMSO) [Control] or 25 µM BIBR1532 or 25 µM GV6 for fourteen (short-term experiment) or twenty-seven (long-term experiment) days. The short-term experiment was structured such that TNBC and non-TNBC cells were cultured continuously in the presence of the compounds for fourteen days and then cultured for an additional fourteen days minus the drugs (Control media only). Likewise, the longterm study was designed such that cells were first continuously exposed to the drugs for twenty-seven days, followed by twenty-seven days of growth in just Control media. The media in the drug-plus and drug-minus flasks was refreshed every third day for both experiments. The drug-plus and -minus flasks for short-term were passaged on days 5 and 9, while the long-term ones were passaged on days 9 and 18. All flasks were incubated at the same conditions (37°C and 5% CO_2).

Cell viability

Cell viability was assessed using the Trypan Blue Exclusion Test (Life Technologies, NY). The numbers of both live and dead cells were averaged using a set of four well-defined grids on the hemocytometer in tandem with an inverted microscope (Leica IL; 100x).

Cell senescence assay

Cellular senescence was determined as described in previous studies [41-45] employing a commercially available Senescence-Associated β-galactosidase (SA-βGal) Staining Kit (Cell Signaling Technology, MA). The average of SA-βGal positive cells was obtained by quantifying the number of stained cells in four randomly taken micrographs for short- and long-term treatments using an inverted microscope (Olympus, PA; 100X).

Isolation of total RNA and cDNA synthesis

Total RNA from cryo-aliquots of TNBC and non-TNBC cells was extracted using Trizol® Reagent (Thermo Fisher Scientific, Carlsbad, CA) according to manufacturer's instructions. Quantity and quality of the RNA was assessed using the Nanodrop® ND-1000 UVVis Spectrophotometer (Nanodrop Technologies, Wilmington, DE). Genomic DNA contamination of cDNA was removed with DNase I (Thermo Fisher Scientific) prior to reverse transcription (RT) reaction. Equivalent amounts of total RNA (1 µg) from each treatment was reverse transcribed into cDNA (Thermo Fisher Scientific) according to manufacturer's instructions. The qPCR analysis was performed as described earlier [46,47] using the Strategene MX3000P (Agilent, Santa Clara, CA) machine and TaqMan® Assays-on-DemandTM Gene Expression kits (Thermo Fisher Scientific, Waltham MA) specific for human Telomerase reverse-transcriptase (hTERT) and BCL2-associated X-protein (BAX). The qPCR data across the groups was normalized relative to the abundance of two (GAPDH, ACTB) recommended endogenous control genes for breast cancer [48]. The mean sample threshold cycle (CT) and mean endogenous control CT for each sample were calculated from triplicate wells. The relative amounts of target gene expression for each sample were then calculated using the formula $2^{-\Delta\Delta CT}$ [49].

Statistical analysis

Data are represented as Mean ± standard deviation (SD). Statistical analysis was done using the computer program SPSS (Statistics Version 20, IBM Corporation NY). Difference of $P < 0.05$ was considered significant. The dose-response data was analysed using the GraphPad Prism software (La Jolla, CA).

Results and Discussion

GV6 characterization (Spectrometry/elemental analyses) data

4-chloro- (2-nonenoyl) anthranilic acid was synthesized in a one pot reaction by first generating the acid chloride derivative of trans-2-nonenoic acid *in situ* followed by reaction with the amino functionality of 2-amino-4-chlorobenzoic acid under Schotten-Baumann reaction conditions. The product, 4-chloro- (2-nonenoyl) anthranilic acid, was obtained in 65% yield after recrystallization in a dilute acetic acid solution. The determined spectral and structural composition data of GV6 were:

^{1}**HNMR data:** (ppm): 11.33 (s, 1H), 8.67 (d, J=2Hz, 1H), 7.98 (d, J=8.8Hz, 1H), 7.20 (dd, J=8.8 and 2.4Hz, 1H), 6.82 (dt, J=15.2 and 7.2Hz, 1H), 6.05 (br d, J=15.2Hz, 1H), 2.49 (br s, 1H), 2.20 (dt, J=7.2 and 6.8Hz, 2H), 1.40 (m, 2H), 1.25 (m, 6H) and 0.84 (t, J=6.8Hz, 3H).

^{13}**C-NMR data:** (ppm):168.98, 163.87, 146.59, 142.11, 138.51, 132.87, 124.77, 122.54, 119.25, 115.02, 31.41, 31.09, 28.34, 27.71, 22.08, and 13.97.

Elemental analysis (MidWest Micro Lab, Indianapolis, IN): For $C_{16}H_{20}NO_3Cl$

C-62.03; H-6.51;N-4.52;Cl-11.44. Found with 2 assays.

C-61.79; 61.86; H-6.44;6.51; N-4.52;4.50; Cl-11.89;11.92.

Dose-response relationship of BIBR1532 and GV6

A preliminary study was initially conducted to assess the effect of DMSO on growth kinetics of TNBC as well as non-TNBC cells and our results indicate that there was no difference in the proliferation profiles of cells grown in presence of 0.5% DMSO and that of cells grown in DMSO-free media (data not shown). Thereafter, we proceeded to evaluate the dose-response effects of BIBR1532 and GV6 using these breast carcinoma cells. The cells were exposed to concentrations ranging from 1 nM to 1 mM for 96 hours and the half maximal concentration (IC_{50}) to reduce proliferation by 50% for BIBR1532 and GV6 with TNBC was 21.6 µm and 15.9 µm, respectively. The IC_{50} values for BIBR1532 and GV6 with non-TNBC cells was 13.2 µm and 35.6 µm, respectively. From these results, the 25 µm dose was utilized to further

evaluate the anticancer properties of the synthesized 4-chloro-(2-nonenoyl) anthranilic acid.

Short-term effect of BIBR1532 and GV6

The short-term drug-plus and drug-free proliferative rates of TNBC and non-TNBC cells are depicted in **Figure 2.** The number of viable TNBC cells decreased by about 35% and 20% in BIBR1532 and GV6 supplemented flasks by day 5 (P<0.05) relative to control, respectively **(Figure 2a).** The cell densities in the drug-treated flasks continued to drop and by day 14 were approximately 50% of the control for both treatments (P<0.01). Like the effects noted with TNBC cells, treatment with BIBR1532 and GV6 reduced the growth of non-TNBC cells by ~30% at day 5 (P<0.05) and an additional ~20% by day 14 (P<0.01) in comparison to the control **(Figure 2b).**

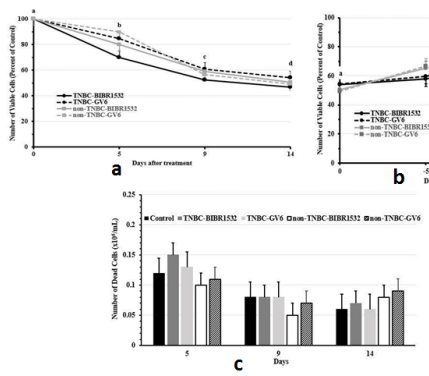

Figure 2: Short-term BIBR1532 and GV6 effects on breast carcinoma cells. (a) The TNBC and non-TNBC were treated with 25 µm of BIBR1532 and GV6 for fourteen days, followed by (b) additional fourteen days minus the drugs (Control media only). The number of dead cells was similar between of BIBR1532 and GV6 for the short-term regimen of drug exposure (c). Cell counts in 2a and 2b are expressed as a percent relative to the control (data is presented as Mean ± SD). ^{ab}Means, ^{ac}Means and ^{ad}Means are significantly different (p<0.05), (p<0.01) and (p<0.001), respectively.

A 10% increase in the number of viable TNBC cells was detected after five days of switching the culture media to drug-free media. Thereafter, a steep (~40%) increase in cell density was observed following fortnight's (P<0.01) culture in the drug-free media **(Figure 2b).** A similar growth pattern was evident with non-TNBC cells in BIBR1532 and GV6 drug-free culture experiment were it also gradually (~10%) increased for the first five days, followed by a surge to ~98% of control-cells growth by day 14 (P<0.01) of drug-minus culture **(Figure 2b).** There was no significant variation in number of dead cells between the control and the BIBR1532 and GV6 treated groups of TNBC and non-TNBC cells **(Figure 2c).**

Long-term effect of BIBR1532 and GV6

In long-term trials, the number of viable cells by day 9 (P<0.01) drop by ~50% and 40% in BIBR1532 and GV6 treated TNBC cells relative to solvent-control, respectively **(Figure 3a).** A further decrease of about 10% (P<0.01) in viability of TNBC cells was observed for both drugs by day 18. By day 27, the counts of viable cells in the drug-treated cells had diminished to about ~20% (P<0.001) of the control treatment. Likewise, the growth of non-TNBC cells was inhibited by approximately 40% and 35% by day 9 (P<0.01) in BIBR1532 and GV6 supplemented flasks, respectively **(Figure 3a).** A further 30% reduction in the number of viable non-TNBC cells was found by day 27 (P<0.001) for both drugs.

The percent of viable TNBC cells tripled (P<0.01) post nine days of culture in drug-free media [~20% at day 0 to ~60% by day 9] **(Figure 3b).** A further eighteen days of drug-free culture increased the number of TNBC cells to approximately 90% of the control. On the other hand, the cell density of non-TNBC cells only doubled (P<0.01) following first nine days of culture in treatment-free media [~30% at day 0 to ~63% by day 9] **(Figure 3b).** However, the growth rate of non-TNBC cells was comparable to that of TNBC cells by day 27 of drug-free culture. No difference was observed in the number of dead cells between the BIBR1532 and GV6 treated groups and relative controls of TNBC and non-TNBC cells **(Figure 3c).**

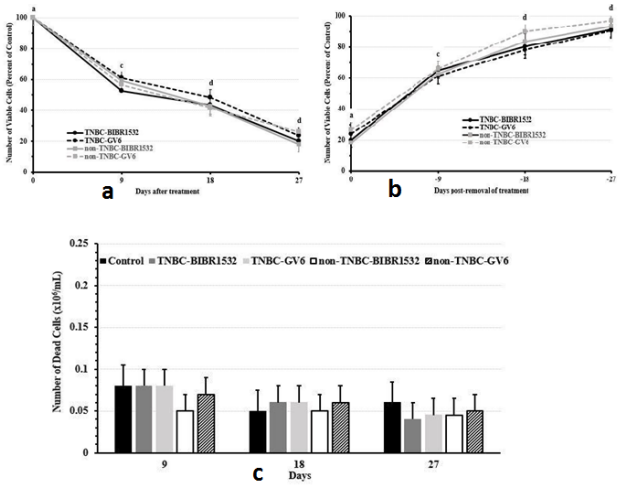

Figure 3: Long-term BIBR1532 and GV6 effects on breast carcinoma cells. (a) The TNBC and non-TNBC were treated with 25 µm of BIBR1532 and GV6 for twenty-seven days, followed by (b) additional twenty-seven minus the drugs (Control media only). The number of dead cells was similar between of BIBR1532 and GV6 for the long-term regimen of drug exposure (c). Cell counts in 3a and 3b are expressed as a percent relative to the control (data is presented as Mean ± SD). ^{ac}Means and ^{ad}Means are significantly different (p<0.01) and (p<0.001), respectively.

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Short- and long-term effects of BIBR1532 and GV6 on induction of cell-senescence

The TNBC and non-TNBC drug-treated flasks had about 50% (P<0.05) more senescence associated β-Gal positive cells in comparison to respective controls with short-term treatment **(Figure 4a).** Doubling the duration of treatment from fourteen to twenty-seven days almost doubled (P<0.001) the number [~150% to 250%] of senescent cells for both TNBC and non-TNBC cells in comparison to their relative controls. Additionally, there was no difference in the number of senescent cells between BIBR1532 and GV6 treated TNBC cells in the long-term regime. While with the non-TNBC cells, BIBR1532 had an increased (P<0.05) number of senescent cells compared to GV6 with long-term drug exposure **(Figure 4b).**

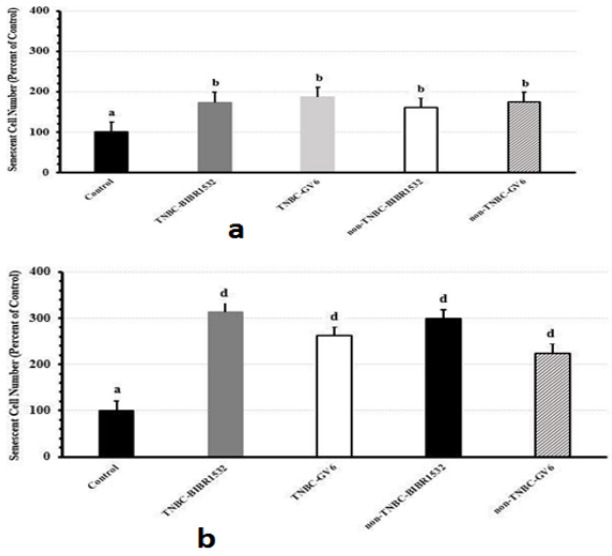

Figure 4: Effect of BIBR1532 and GV6 on induction of senescence in treated breast carcinoma cells. (a) Fourteen (short-term) days of treatment with 25 µm of BIBR1532, and (b) twentyseven (long-term) days of treatment with 25 µm of BIBR1532 (data is presented as Mean ± SD). [ab]Means and [ad]Means are significantly different (p<0.05) and (p<0.001), respectively.

Effect of short- and long-term BIBR1532 and GV6 exposure on expression of hTERT and BAX mRNA levels

There was a time-dependent decrease in the relative abundance of hTERT mRNA, such that it was about 40% (P<0.01) and 70% (P<0.001) less in BIBR1532 treated TNBC and non-TNBC cells at days 14 and 27, respectively. In the GV6-exposed TNBC and non-TNBC cells, the relative expression respective to control decreased approximately by 25% (P<0.05) and 60% (P<0.001) by days 14 and 27, respectively **(Figure 5a).** There was no significant difference in the relative abundance of BAX mRNA at days 14 and 27 for both TNBC and non-TNBC cells relative to their controls **(Figure 5b).**

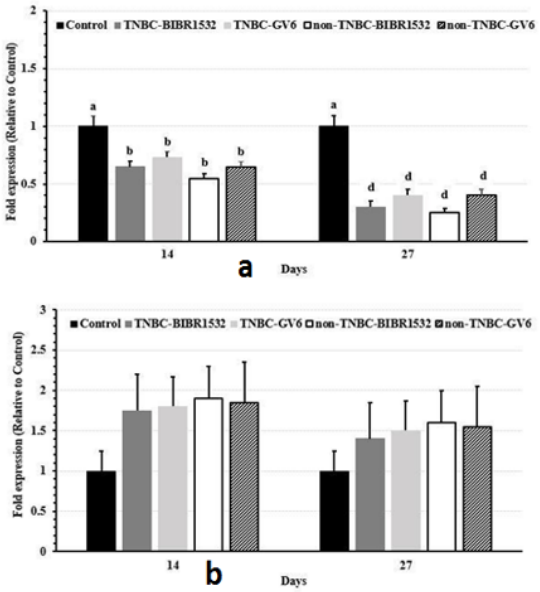

Figure 5: Effect of BIBR1532 and GV6 on the steady-state mRNA expression of (a) apoptosisassociated BAX gene and, (b) gene encoding for the catalytic unit of telomerase at days 14 and 27 of treatment. Transcript abundance in 5a and 5b are expressed relative to the control (data is presented as Mean ± SD). [ab]Means and [ad]Means are significantly different (p<0.05) and (p<0.001), respectively.

Discussion

The activation of telomerase in the molecular pathogenesis of human cancers is well documented [24-26]. Telomerase is detected in the vast majority (~90%) of tumors and its abundance is correlated with a tumor's metastatic potential [50,51], clinical outcome [52-54], relapse-free duration and overall prognosis [55,56]. Therefore, an array of approaches that target the telomerase-telomere complex have been developed as potential therapeutic options, including the highly selective inhibitor of telomerase, BIBR1532 [57-59]. On the other hand, it is wellknown that the pharmacodynamics of several surrogate carboxylic acid-based drugs marketed globally has been significantly improved over the years through bioisosteric modification [60,61]. The novel GV6 has a nucleophilic aliphatic substitution of the acyl group in comparison to the highly conjugated 3- (2-Naphthyl)-2-butenoyl attachment in BIBR1532.

Dose-dependent studies indicate differential sensitivities to BIBR1532 based on anatomical origin of the tumors. *In-vitro* studies employing cancer cells originating from the pulmonary and gynecological tracts demonstrated an IC_{50} concentration of less than 1 µm [33,38]. While studies with cells of hematopoietic origin revealed an IC_{50} concentration of >20 µm [35,39]. Neoplasia ' s emanating from germ-cells and connective tissues show efficacy of BIBR1532 nearer to 100 µm [36,37]. In the present study, both molecular-subtypes of

breast cancer showed cytotoxicity in dose-dependent manner, but the levels of response varied depending on the molecular subtype. The IC_{50} values of the TNBC subtype indicate that it was more sensitive to GV6 compared to non-TNBC. Additionally, the TNBC cells had a lower threshold dose relative to the non-TNBC cells. Shi et al., [40] evaluating BIBR1532 reported an IC_{50} values of approximately 55 µm and 10 µm with MDA-MB-231 and MCF-7 cells, respectively. Whereas, a recent study [34] associated with MCF-7 found an IC_{50} value of 35 µm using BIBR1532. These IC_{50} differences between studies could be attributable to seeding densities, dosing metrics, duration of exposure, and disparate techniques employed to validate cell viability [62,63]. However, our results together with these two current studies [34,40] do demonstrate that breast carcinoma cells require a significantly higher therapeutic dose of anthranilic acid than the 100 nm reported in the pioneering studies with cancers of the nonmammary origin [33,38]. In this study, aliphatic chain substitution rendered TNBC cells more sensitive to the anthranilic acid compared to non-TNBC cells. Taken together, these findings do show that bioisosteric modification of anthranilic acid does improve potency of these carboxylic acid derivatives.

In the original study, Damm et al., [33] employed a standard concentration of 10 µm BIBR1532 against several cell-lines originating from diverse tumors and observed that there was a protracted lag-period (>120 population-doublings) before growth inhibition was evident. On the other hand, El-Daly et al., [35] found that increasing the dose above 20 µm induced growth arrest within a significantly shorter time-frame compared to the above original study. Additionally, they [35] showed that the cell viability precipitously declined to near-zero following exposure to doses in excess of 50 µm for a week. Another study revealed ~95% decrease in cell viability when cancer cells were treated continuously with 30 µm of BIBR1532 for about a fortnight [64]. Taking into consideration these earlier findings [35,64] as well as our own dose-response data, we resolved to use 25 µm of BIBR1532 & modified derivative (GV6) to evaluate the biological effects in our experimental strategy of continuous short- and long-term exposure of breast carcinoma cells. Relatedly, majority of the previous studies evaluating the dose- and time-dependent effects of BIBR1532 utilized the 96-well plate setup [33,34,38,40,65-67]. The 96-well format provides a simple and cost-effective method for drug screening. However, the limited geometric surface (<0.5 cm^2) and amount of media (100-200 µl) per well requires frequent passaging and media changes [68]. The frequency of passaging, dissociation and trituration steps associated with subculturing are known to affect the growth kinetics of cells [68,69]. Likewise, the underlying edge-effect phenomenon associated 96-well plate design and dissimilar evaporation rates across the plate results in uneven growth of cells [68,69]. Accordingly, we opted to use large (75 cm^2) vented culture-vessels in present study to mitigate the edge-effect evaporation and exploit the larger surface area for longer periods of uninterrupted growth of cells. Utilizing the T-75 flask substantially reduces the frequency of physical handling of cells compared to the 96-well setup. To the best of our knowledge, this is the first study to address short- and long-term exposure of cancer cells to these anthranilic acid derivatives without repeatedly perturbing growth cycles by incorporating a larger culture vessel.

The goal of this study was to avoid the doses already reported as cytotoxic in previous studies [35,64] and select a larger vessel to facilitate extended period of cell growth that permits a longer dosing regimen. We observed a time-dependent effect on proliferation of TNBC and non-TNBC cells with both BIBR1532 and GV6. Although TNBC cells were more sensitive to BIBR1532 compared to GV6 during the initial phases of treatment, the response was almost parallel between the two drugs for both short- and long-term dosing schedules. Additionally, there was no difference observed in the growth-inhibitory rates of short- and long-term treatments in non-TNBC cells between BIBR1532 and GV6. Interestingly, the ~20% drop in viability relative to control detected in TNBC cells with GV6 at day 5 is comparable to that observed in hematopoietic cancer cells treated with BIBR1532 [64]. On the other hand, increasing the dosage of BIBR1532 by five units to 30 µm led to 50% reduction in proliferation rate by day 9 [35,64] instead of fourteen as seen in the present study. However, a higher number of apoptotic cells were found with increasing the dose to 30 µm [35,64]. On the contrary, glioma cells viability rapidly declined to 50% of control within 72 hours of exposure to 25 µm of BIBR1532 [66]. Surprisingly, in the same study increasing the BIBR1532 concentration eightfold (25 to 200 µm) only reduced viability of the glioma cells by further 20% within the same time-frame of treatment [66]. Similar findings of limited decrease in viability with exponential increase in dosage were noted elsewhere [35,64]. This suggests that telomere attrition with carboxylic acid derivatives is gradual but continuous and the shortening of telomeres needs to surpass the critical threshold to halt proliferation and that the attrition of telomeres cannot be precipitously accelerated by increasing the dose of the anthranilic acid drug. Doubling the drug exposure time from 14 to 28 days in the present study led to the growth rates of TNBC and non-TNBC cells dropping from ~50% to ~25% of the control for both anthranilic acid analogues, respectively. Other studies have shown that either a sustained prolonged exposure (>120 population doublings) or a substantially higher dose is needed to totally halt proliferation [33,35,64,67]. However, a major drawback of elevating doses it that there is dose-dependent increase in apoptosis [34,35,40,64]. Our selection of the 28-day duration was based on the premise that a low-dose chemotherapy treatment-cycle on an average last between three- to fourweeks.

Investigators employing reverse genetics studies were first to establish that inhibition of hTERT led to attrition of telomeres and subsequently growth arrest [70]. Shortly thereafter, other groups demonstrated that a synthetic molecule, BIBR1532, could induce similar replicativearrest by inhibiting telomerase [33,38]. These investigators also showed that the BIBR1532 specifically targeted the overexpressed telomerase in cancerous cells and had no effect on normal hematopoietic cells [33,38]. A recent study identified a more probable molecular mechanism of inhibition of hTERT by

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BIBR1532 that paves way for developing more efficacious inhibitors of telomerase [32]. Our findings of decreased expression of hTERT mRNA with short-term exposure of TNBC and non-TNBC cells to BIBR1532 and GV6 is comparable to earlier studies with cancers of diverse origin [35,64,67]. Similarly, the ~75% reduction in abundance of hTERT mRNA following the long-term treatment with both drugs in our study parallels the findings of both, fifteen-day treatment of leukemia cells [64] and 48-hour exposure of endometrial carcinoma and glioma cells to >50 µm BIBR1532 dose [65,66]. However, with increasing doses of BIBR1532, these studies reported greater cytotoxic effects [64-66]. In our study, utilizing a lower dose than the previous studies showed no significant difference in the number of dead cells between short- or long-term BIBR1532 and GV6 exposure of TNBC and non-TNBC cells.

Replicative-senescence is a complex biological phenomenon that limits the indefinite proliferation capability of mammalian cells [27]. It is well-documented that cells that evade senescence do acquire replicative immortality, which is a pivotal catalyst in malignancy progression [24-26]. On the other hand, telomere shortening has been demonstrably linked to senescence and previous studies have long-established that BIBR1532 progressively truncates telomeres, culminating in replicative-senescence [32,33,35,38,67]. Among the important hallmarks of senescent cells is an elevation of intracellular β-galactosidase activity that can be identified by utilizing cytochemical approaches [41-45]. Majority of the previous studies tracked telomere lengths as a marker of senescence [33,35,38,64,67]. As far as we know, this study is first in relation to BIBR1532 studies to report on population of senescent cells using levels of β-galactosidase as a biomarker. Regardless of using a different approach to track senescence in the present study, our data of increasing number of senescent cells with longer durations of exposure parallels with earlier findings [33,35,38,67]. Additionally, modifying the carboxylic acid structure with an aliphatic chain did not significantly increase the percent of senescent cells in TNBC and non-TNBC populations for both short- and long-term drug exposures. In our experiments, doubling the exposure time to the drugs did not induce 100% senescence in the treated flasks. Conversely, Bashash et al. [64] demonstrated that the proliferation-rate of hematological cancers cells dropped close to zero following a fortnight's exposure to 30 µm of BIBR1532. This difference between the two studies could be attributable to disparate sensitivity of solid tumors like breast cancer to the carboxylic amides compared to tumors of hematological origin. Our findings of rapid growth-recovery of previously drug-exposed cells agree with earlier studies [33]. In the original study, the cells exhibited a growth pattern comparable to the control within ~72 hours of withdrawal of BIBR1532, even though the cells had been exposed to the drug for 220 days [33]. Ward et al., [67] found that the growth-recovery post-withdrawal of drug was progressive and significantly influenced by dose and duration of exposure. In our study, we noted a more accelerated recovery in both carcinoma cell types within the first nine days of withdrawal of BIBR1532 and GV6 treatments in the long-term model compared to the short term. Overall, the post-treatment proliferative capacity of TNBC and non-TNBC for short- and long-term treatments were almost identical, matching their control cohorts following 14 and 27 days of drug-free culture, respectively. We only cultured the carcinoma cells for the same duration in drug and drug-free experiments. However, it is a known phenomenon that cancer cells do develop drug resistance with progressive exposures to cytotoxic drugs [71-73], but this is not known whether this applies to carboxylic acid derivatives, too. Additionally, we made no attempt in this study to segregate and identify cells resistant to BIBR1532 and GV6 and monitor their growth patterns separately. Drug-resistant cells are renowned for evading treatment and growing more aggressively compared to other cells originating from the tumor [71-73]. These drug-resistant cells are notorious for initiating relapses of tumors despite multiple rounds of therapy. Cytotoxicity resistance is becoming a common issue with conventional chemotherapeutics and it is imperative to develop novel approaches as knowledge on molecular mechanism of carcinogenesis, cellular immortality and metastases expands.

Conclusion

To conclude, our study demonstrates that the anti-proliferative effects of GV6 are as potent as BIBR1532 for both TNBC and non-TNBC cell types. Similarly, GV6 induced a comparable rate of senescence and downregulation of steady-state levels of hTERT mRNA within the breast carcinoma cells. Further research is needed to confirm these findings and extend investigations on exploring benefits of utilizing this aliphatic-chain substituted anthranilic acid in either a combinatorial, adjuvant or neoadjuvant chemotherapeutic setting in comparison to BIBR1532

Acknowledgments

We indebted to Rebecca Uzarski (Central Michigan University) and John Risinger (Michigan State University) for the kind gift of ATCC-acquired MDA-MB 231 (TNBC) and MCF-7 (non-TNBC), respectively. We also acknowledge the Grand Valley State University for providing financial support for our undergraduate student's (AF, CR) to participate in summer (Student Summer Scholars Program) research projects.

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