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Effect of Telomerase Inhibitors on Malignant Breast Cancer Cells

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Abstract

Current therapies have nominal effect on the most intrusive-type of breast cancers (triple-negative) that have a higher tendency to metastasize or recur. Recent studies reveal an enzyme, telomerase, as key for unlimited cell growth (immortality) and replication. Therefore, our objectives were to assess (i) short- and (ii) long-term effects of a novel anti-telomerase agent (GV6) developed at our institute and compare it to a known analogue, BIBR1532 on MDA-MB 231 breast cancer cells. Cell viability was measured on days 5, 9, 14, 18 and 27 after treatment with GV6 (n=4), BIBR1532 (n=4) or Solvent alone (Control, n=3). The number of viable cells in GV6 and BIBR1532 treated flasks (T75) were about 40% (p<.05) of Control by day 14. It further dropped to 30% (p<.05) of Control by day 27 for both, GV6 and BIBR1532. Our results indicate the anti-proliferative effect of GV6 parallels that of BIBR1532 and should be investigated further.

Introduction

During 2013 alone, we estimate about 1,700,000 new cases of cancer to be diagnosed in the United States, leading to almost 1,600 deaths per day. Of the new cases, about 240,000 will be attributed to breast cancers diagnosed among women bringing with it a fatality rate of approximately 40,000 from this disease (1), making it one of the most deadly cancers. There are three key receptors that are commonly known to be triggers for breast cancer: the estrogen receptor, the progesterone receptor, and the human epidermal growth factor receptor. When a type of cancer has one or more of these receptors, it is considered to be “positive” for that specific receptor; while on the other hand, if the cancer does not have that receptor then it is considered to be “negative.” About 10-20% of all breast cancers diagnosed are a form of triple negative breast cancer (TNBC) because they lack these three pivotal hormone receptors, making TNBC the most aggressive type due to the high mortality rate. TNBCs exhibit a greater resistance towards the standard therapeutic procedures, requiring a more intrusive form of radio/chemo-therapies and even multiple surgeries to combat the higher metastatic and reoccurrence rates, confining the patient to a prolonged time for convalescence. Unfortunately the previously mentioned therapies are not capable of selectively targeting cancer cells specifically and
decimate both cancerous and healthy cells. The insurmountable side effects (hair loss, organ
damage, possible metastasis, secondary cancers etc.) of the current treatments have lead to an
increase in the collaborative efforts towards finding a new therapy to combat the disease through
the use of more precise agents.

Recent studies regard the over-expression of a particular reverse transcription (RT)
enzyme, Telomerase, as a pivotal indicator of the immortalization in 80%-90% of cancers (2).
Reverse transcriptase enzymes gain most of their notoriety for their crucial role in the
propagation of the HIV epidemic. Although these enzymes are similar and have the same
function, these RT enzymes are not the same. This dual part RT enzyme has a RNA template that
allows it to work in tandem with the catalytic part of the enzyme specific for humans, human
telomerase reverse transcriptase (hTERT), to elongate the protective end-caps (telomeres) of the
chromosomes through the addition of many repeats of the same base pair series TTAGGGG.
Telomeres will naturally reduce by approximately 30-120 base pairs with subsequent cycles of
cellular replication with no means of elongation in normal somatic cells. Healthy cells without an
active telomerase expect a set number of population doublings, known as the Hayflick limit,
before entering a state of replicative inactivity known as cellular senescence (3). Without this
growth arrest, a cell would perpetually divide and eventually damage genetic code crucial to the
survival of the cell, thus activating a DNA damage response apoptotic pathway. Therefore,
extension of the telomeres grants an unlimited cellular replication potential in somatic cells
through the evasion of expressing this senescence-like phenotype. Once a cell enters the state of
irreversible cellular senescence, its replicative potential becomes zero even though it is otherwise
a healthy and metabolically active cell. This ground breaking discovery catalyzed the production
of a novel strategy to combat TNBC through the use of novel molecules that effectively target
telomere/telomerase complex, and eventually hinder their proliferation rates (2).

There are two main classes of telomerase inhibitors, each affecting a different site of
function for the telomerase enzyme. One is known as a nucleoside reverse transcriptase inhibitor
(NRTI) which is disguised as a nucleoside base used in transcription which will then disrupt the
replicative chain. The other class is known as non-nucleoside reverse transcriptase inhibitors
(NNRTI) which will non-competitively bind to the telomerase enzyme. One well-documented
synthetic agent known as BIBR1532 belongs to the class of NNRTIs and can effectively bind to
the enzyme, halting the enzymatic activity. The specific inhibitory mechanism of this NNRTI is not completely understood, though there are two main theories that have become generally accepted. The first states that BIBR1532 attaches to a binding site near enough to the binding site for replication that it blocks the replication binding site and thus hindering telomerase; the second, BIBR1532 causes a conformational change in the structure of the telomerase enzyme after binding to it which will then block the replication binding site from being active (4). Essentially both of these compounds directly impede upon the unlimited proliferative capacity of cancerous cells, slowing their propagation, and eventually stopping the disease’s progress.

A novel analogue of the NNRTI, BIBR1532, called GV6 was developed at Grand Valley State University and its potential as an anti-tumor/anti-telomerase agent is currently unknown. Therefore, our goals were to compare the short-term and the long-term anti-proliferative effects of GV6 on MDA-MB 231 TNBC cells to the previously investigated telomerase inhibitor BIBR1532, as well as to compare the effectiveness of the two inhibitors on the induction of a senescence-like phenotype in the cells. Our hypothesis is that the anti-proliferative and senescence inducing effects of GV6 will parallel that of BIBR1532.

Methods

Cell line

TNBC cells (MDA-MB231) were seeded into T-75 Ventilated Culture Flasks and cultured in RPMI (Life Technologies, NY) media 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) in an incubator set at 37 °C and 5% CO₂.

Treatment

Cells were seeded at a density of 1.00 x 10⁶ cells/ml and left undisturbed for 72 hours in RPMI control media to allow acclimation to the culture conditions. After the 72 hours had transpired, the media was aspirated and replaced with fresh RPMI media supplemented with either Solvent (5% DMSO) alone [control] (n=3), 25µm GV6 (n=4), or 25µm BIBR1532 (n=4).
Figure 1: The Chemical structures of BIBR1532 (R=COOH). The structure of GV6 is under intellectual property and therefore its structure has not been shown.

Viability Assessment

At days 5, 9, 14, 18, and 27 of culture in experimental treatments, relative cell densities were evaluated using a hemocytometer and the live to dead ratios were calculated using the Trypan Blue Exclusion Test (Life Technologies, NY). When a cell dies the cellular membrane becomes more permeable which allows the Trypan Blue Stain to be absorbed into the cell thus staining it blue. The Number of live/dead cells was estimated by counting and averaging the number of cells within the 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 days 14 and 27 to detect the cellular activity of β-galactosidase in the lysosomes at slightly acidic pH 6.0. The percentage of SA-βGal positive cells was estimated by counting the number of blue-stained cells in a micrograph obtained using an inverted microscope (Olympus, PA; 100X).

Figure 2: Micrograph of MDA-MB 231 Cells illustrating (i) cells exhibiting a Senescence-like phenotype (S) and Non-senescence (Replicatively Active) cells (A) [400X magnification], and (ii) viable and non-viable cells on one grid of a hemocytometer (Trypan Blue Exclusion Test).
**Statistics**

Statistical analysis was done using the computer program SPSS (Statistics Version 20, IBM Corporation NY). Difference of $P < 0.05$ was considered significant.

**Results**

![Effect of Solvent on Cell Growth Kinetics](image)

**Figure 3:** To determine the effect of the Solvent (DMSO 5%) on the MDA-MB 231 cells, flasks were seeded at a density of about $1.00 \times 10^6$ cells/ml in RPMI media supplemented with either Solvent (n=3) or Solvent-free (RPMI alone) (n=3).

After culture for five and nine days, assessment of the live/dead ratio was performed using the Trypan-Blue exclusion test and data are presented as Mean ± SD. At day 5, both trial flasks exhibited a corresponding cell density of roughly $1.50 \times 10^6$ cells/ml. By day 9 the cells density peaked in both treatments at approximately $2.00 \times 10^6$ cells/ml. These results indicate that the presence of 5% DMSO in the media has no effect on growth kinetics of the MDA-MB 231 cells. Therefore, the difference noted in cell kinetics during our experimental trials can be justifiably attributed to the effects of the GV6 and BIBR1532, and not to the presence of 5% DMSO.
Figure 4: The number of viable MDA-MB 231 cells in media supplemented with GV6 and BIBR1532 at days 5, 9, 14, 18, and 27 had been determined using the Trypan-Blue exclusion test (n=3). The cell counts are expressed as percents relative to Solvent-Control and data are shown as Mean ± SD. The anti-proliferative effects of the compounds are shown to be time dependent. The \( a, b, c, d, e, \) and \( f \) means within each of the treatment are significantly different (\( p<0.05 \)) and means between the treatments at each point \( a, b, c, d, e, \) and \( f \) are not statistically different (\( p>0.05 \)).

During the short-term treatment trial, the decrease in the number of viable cells in BIBR1532 (40%) supplemented media was almost double of GV6 (20%) by day 5. However, by day 14, corresponding growth deficits were noted under both treatments. Long-term treatment trials depicted a comparable drop in number of viable cells at days 9 (~50%), 18 (~45%) and 27 (~30%), relative to Solvent-control, respectively. Both, the short-term and long-term trials, indicate that GV6 has an anti-proliferative effect that mirrors the overall effect of BIBR1532 on the TNBC cells. These results parallel the current published literature on small molecule inhibitor that indicate the effectiveness of BIBR1532 as a telomerase inhibitor, as well as demonstrate a similar negative effect on the growth kinetics of the MDA-MB 231 cells, dropping to nearly 25% of the proliferation rates of the control cells at concentration of 25\( \mu \)M. (2) The next step would be to compare and contrast the dose-dependent curves of BIBR1532 and GV6 and validate whether it corresponds to published results.
**Figure 5:** Number of dead MDA-MB 231 cells in media supplemented with GV6 and BIBR1532 at days 5, 9, 14, 18, and 27 in comparison to Solvent-Control as determined by Trypan-Blue exclusion test (n=3-5). Data are shown as Mean ±SD.

The amount of dead cells was approximately 5% of the normal live-cell rate of Solvent-control (2.00 x 10⁶ cells/ml) and fluctuated only a negligible amount throughout the trials. This means that GV6 and BIBR1532 do not cause an increase in the rate of cell death but, instead, only slow the proliferation rate of the cells.
Figure 6: Number of (A) senescent cells/treatment in comparison to Control (Solvent), and (B) non-senescent (replicatively active) cells on days 14 and 27 of treatment. Data are shown as Mean ± SD. ab, ac, ad, and ae, means within each panel are significantly different (p<0.05).

The time dependent increase in the number of cells expressing a senescence-like phenotype is equivalent for GV6 and BIBR1532. By day 14, GV6 and BIBR1532 supplemented flasks have approximately double the number of senescent cells in comparison to Solvent-control and the number of replicatively active cells corresponds between the two treatments. A three-fold increase in the number of senescent cells and a nearly 70% reduction in the number of active cells was observed for both treatments by day 27.

Conclusion and Future Projects

This study illustrates that the anti-proliferation effect of GV6 is as potent as BIBR1532 in inhibiting rates of triple-negative breast cancer cells both in the short- and long-term setting. The ability of GV6 to effectively induce a senescence-like phenotype in treated cells has also been observed. Further studies include using real time PCR to determine the level of relative gene expression in treatment supplemented cells as opposed to solvent supplemented cells and quantifying the levels of telomerase expression in the cells.

References

