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Investigating the Efficacy of Tazemetosttat for In Vitro Treatment of Human Triple Negative Breast Cancer Cells

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Investigating the efficacy of tazemetostat for *in vitro* treatment of human triple-negative breast cancer cells

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I. Abstract

Cancer is a formidable, genetic disease that affects many people, either directly or indirectly. Breast cancer is the most commonly diagnosed cancer worldwide (31). Triple-negative breast cancer (TNBC) is a type of breast cancer that has a higher lethality compared to other breast cancers and has a poor prognosis due to its highly invasive nature and limited treatment options. Finding safe, effective, and accessible treatment for TNBC is integral to treating TNBC patients. Tazemetostat is an EZH2-inhibitor that has recently been approved for use in epithelioid sarcoma (23). EZH2 is an overexpressed protein in many cancers, including TNBC (11). However, there is limited literature on the applications of tazemetostat in TNBC. This project investigated the *in vitro* efficacy of tazemetostat in human TNBC cells. The results demonstrated that tazemetostat is effective at killing TNBC cell populations by activating cellular apoptotic pathways and decreasing cellular survivin expression suggesting its therapeutic potential as a monotherapy against TNBC. These findings could serve as a basis for *in vivo* studies evaluating the effects of tazemetostat on TNBC.

II. Background

A. Overview of Cancer and its Treatment

Cell theory states that all living organisms are composed of cells, cells are the basic units of life, and all cells come from preexisting cells. Cells have compartmentalized organelles that ensure the cell's sustenance and, by extension, the organism's survival. Cellular functions are carried out through cellular pathways. Proteins are the functional units of cells that can make up the receptors, signal transducers, and resulting products of a pathway. They are translated versions of genes. Genes can determine external features like eye color, hair type, and skin tone

or internal features such as metabolism, development, and immune system strength. When transcription and translation occur correctly, the proteins fulfill their roles and ensure the pathways are functional. When a mistake occurs in this process, the pathway can be disrupted.

Genetic ‘mistakes’ are called mutations. Mutations are changes in the sequence of a gene that can lead to the production of non-functional or malfunctioning proteins. When a mutation occurs, the cell either tries to correct the mutation, degrades the malformed protein, or commits to programmed cell death (apoptosis). Cells can correct genetic mutations through one of many available DNA repair systems. Cells are usually quite efficient at correcting mutations, but when they are unable to do so, the cells can activate backup processes that cover the disrupted pathway’s function. Cells undergo apoptosis if their DNA cannot be repaired and their pathways are severely compromised. Genetic mutations, DNA repair, and apoptosis are all normal cellular processes, among others like cell division, DNA replication, inter- and intracellular transport, immune defense, and pathway regulation. They are essential for the survival of the organism. Problems arise when these processes are disrupted, potentially leading to cancer.

Cancer is an umbrella term used to describe a group of genetic diseases that are caused by the uncontrolled growth of cells with mutated DNA. It affects a large range of organisms from clams to humans. In humans, cancer is classified into two major groups depending on the affected cell type: carcinoma (affects epithelial cells, or cells that are on the body’s outermost or innermost surfaces) and sarcoma (affects mesoderm cells, or cells that compose the body’s bones and soft tissues such as muscles, fat, and fibrous tissue). Other types of cancers include leukemias, lymphomas, germ cell tumors, neuroendocrine tumors, and brain and spinal cord tumors (32). Additionally, cancer is classified and named based on its site of origin and can have multiple subtypes. Everyone is susceptible to developing cancer at some point in his/her life, and

it is currently the second leading cause of death worldwide with a predicted 1,918,030 new cases in 2022 in the United States alone (3, 5). Despite centuries of cancer research, every 1 in 6 deaths globally is due to cancer (3). According to the American Cancer Society (ACS), males have a 1 in 2 and females have a 1 in 3 chance of developing an invasive cancer in the US; both males and females have a 1 in 5 chance of dying from cancer in the US (14). An understanding of the molecular biology of cancer development and progression can help explain the theory behind current cancer therapies.

Cancer development typically depends on cells accumulating specific mutations that affect proto-oncogene and tumor suppressor gene sequences. Proto-oncogenes are normal genes in the body that can promote cancer growth if mutated. These genes can, for example, regulate the rate at which cell growth occurs. When mutated, they can become oncogenes that produce oncoproteins with a malfunctioning growth regulation system resulting in uncontrolled growth. Tumor suppressor genes give rise to proteins that are responsible for identifying pre-cancerous activity in a cell and either repairing the problem or terminating the cell. Mutations in tumor suppressor genes can inactivate these proteins or block them from functioning properly. Therefore, cells typically need to over-activate oncogenes *and* deactivate tumor suppressor genes used in *multiple* cellular pathways to become cancerous. This process generally takes an extended period of time because cells are quite proficient at repairing DNA damage and not every mutation leads to cancer. If a cell does accumulate the necessary mutations, cancer may develop.

Cancer treatment is dependent on a variety of factors including cancer type, stage, size, patient medical history, and patient's cancer genetic makeup. Common treatment methods include surgery, chemotherapy, radiation, targeted therapy, and immunotherapy. Surgery involves

the physical removal of the tumor from the body and is usually employed in earlier-stage cancers. Chemotherapy is the use of a combination of drugs that generally enhance the effects of each other to diminish or eliminate the cancer cells' ability to grow and metastasize. Ionizing radiation works on the principle that a cell with too much damaged DNA undergoes apoptosis and, hence, intentionally causes genetic damage to cells. Because cancer cells depend on their mutated pathways to progress to later stages, targeted therapy tries to block those pathways or regulate their activity. Lastly, immunotherapy tries to weaponize the immune system against cancer cells so that it is better able to identify and eliminate cancer cells (25). The specific combination of treatment techniques used for each cancer is different because every cancer is unique. New drugs, drug combinations, or treatment techniques are constantly being evaluated in clinical trials in hope of establishing personalized medicine that can be tailored to the specific needs of each patient's cancer genetic profile. In spite of this, about 10 million people died of cancer in 2020 (3) which makes it imperative for research to continue that identifies new drugs, regimens, and treatment protocols.

B. Introduction to Breast Cancer

Breast cancer (BC) originates in the breast tissue and is the most common cancer diagnosed worldwide (31). The ACS estimates that in 2022 there will be 290,560 new cases of invasive breast cancer, 51,400 new cases of non-invasive breast cancer, and approximately 43,780 breast cancer deaths in the United States (5). Based on data collected from 2011-2017, the 5-year relative survival rate for women with breast cancer is 90% (30); this probability varies depending on the type and stage of breast cancer. For example, the 5-year relative survival rate is significantly lower for metastasized breast cancer: 29% (30). Approximately 30% of patients will have a recurrence of breast cancer after completing initial treatments (4). The type of recurrence

(local, regional, or distant) determines the survival rate of the patient. Treatment for BC also varies by type and stage. Common treatments include surgery, radiation, chemotherapy, hormone therapy (blocks hormone-receptor binding), targeted therapy, and immunotherapy (2).

Understanding normal breast functionality can be helpful to describe how breast cancer develops. In females, the breasts begin to grow in size after the onset of puberty. This increase is due to the elevated production of hormones (including estrogen and progesterone), growth factors, and growth regulators like human epidermal growth factor receptor 2 (HER2) (8). Through various processes, these hormones bind to their respective receptors and signal breast cells to start dividing and accumulating fat, continue growth and branching of the ductal system, and form secretory glands at the end of these milk ducts (18). The growth rate increases dramatically at certain phases during the menstrual cycle as the body prepares for a potential pregnancy. This is normal as long as it is regulated. Breast cancer arises when the regulatory systems are disrupted. Pre-BC cells exploit alterations of the regulatory pathways of estrogen, progesterone, HER2, BRCA1/2 (breast cancer type 1/2 susceptibility protein), and/or other genes to become cancerous. These cells can quickly accumulate other mutations that transform them into cancerous cells and compete with non-cancerous breast cells for resources. The type of breast cancer a person has can be classified by the type of receptors present on the cancerous breast cells.

C. Triple-Negative Breast Cancer and its Treatment

Triple-negative breast cancer (TNBC) is an aggressive type of breast cancer that does not express estrogen, progesterone, or HER2 receptors. It accounts for approximately 10-15% of breast cancer cases and is one of the more lethal types of breast cancer (30). Based on data collected by ACS from 2011-2017, the 5-year relative survival rate of patients with TNBC (all

stages combined) is 77%, with that of metastasized TNBC being 12% (30). Cells of this type of cancer tend to grow and spread at a faster rate than other breast cancers, which is one reason for the poor prognosis of TNBC patients. In addition, according to a 2019 study conducted by the American Association for Cancer Research (AACR), approximately 42% of patients with stage I-III TNBC experience rapid relapses after standard treatment with most occurring in the first five years after diagnosis (1, 28). However, the mechanisms by which TNBC develops are poorly understood.

Current targeted therapies against other types of breast cancer aim at the aforementioned hormones, their receptors, and/or other participants of their pathway and regulate their activity — thereby lengthening the lifespan of patients and enhancing their quality of life. The lack of these receptors in TNBC means the presently available targeted therapies are ineffective against these cells. A chemotherapy protocol that includes taxanes, doxorubicin, and/or platinum-based drugs is usually used to treat patients diagnosed with this cancer (15, 26, 27, 29). Oakman et al. believe these approaches have been determined retrospectively and there is a lack of substantial support for the use of these protocols in TNBC patients (15). These treatments have been chosen as they tend to show promising effects against many different types of cancer. In 2020, the Food and Drug Administration (FDA) approved the use of sacituzumab govitecan for the treatment of metastatic TNBC (20). Sacituzumab govitecan works by inhibiting the repair of damaged DNA in cancer cells which overexpress Trop-2. Trop-2 is a cell surface glycoprotein important for epithelial-to-mesenchymal transition (an essential step for metastasis) and is commonly overexpressed in most TNBCs (20).

Other drugs and therapeutic targets are being investigated to find more treatment options for TNBC. The EGFR pathway is one such target (15, 26, 27, 29). This is a growth factor

pathway that regulates cell growth, differentiation, and survival. Therapies that inhibit the pathway or block the EGFR receptor could potentially slow the growth of tumors. A second promising method is the usage of poly (ADP-ribose) polymerase (PARP) inhibitors (15, 26, 27). PARP is a key enzyme used in single-stranded DNA repair. Since many women with TNBC also have a BRCA1 mutation (key protein for double-stranded DNA repair), blocking the action of PARPs through PARP inhibitors would selectively target BRCA deficient cells (synthetic lethality) while sparing cells with preserved BRCA functionality (15). Other targets of interest include angiogenesis inhibitors and TRAIL (TNF-related apoptosis-inducing ligand), a molecule capable of inducing apoptosis (15).

D. Gene Regulation in Cancer

Gene regulation plays a key role in tumor development and progression. DNA methylation can regulate gene expression by the addition of methyl groups to the histone tails of nucleosomes thereby increasing the compaction and inaccessibility of gene segments in DNA. Polycomb group (PcG) genes are a family of epigenetic regulators that repress transcription. Polycomb repressive complexes 1 and 2 (PRC1 and PRC2) are PcG proteins that silence genes through chromatin modulation (10). Enhancer of zeste homolog 2 (EZH2) is an evolutionarily conserved catalytic subunit of PRC2 that catalyzes H3K27 (lysine-27 of histone H3) trimethylation in the cell nucleus. This modification enables PRC1 to attach to H3K27me3 and monoubiquitinates histone H2A at lysine 119, thereby promoting chromatin compaction and transcriptional silencing of downstream genes as shown in Figure 1 (10, 24).

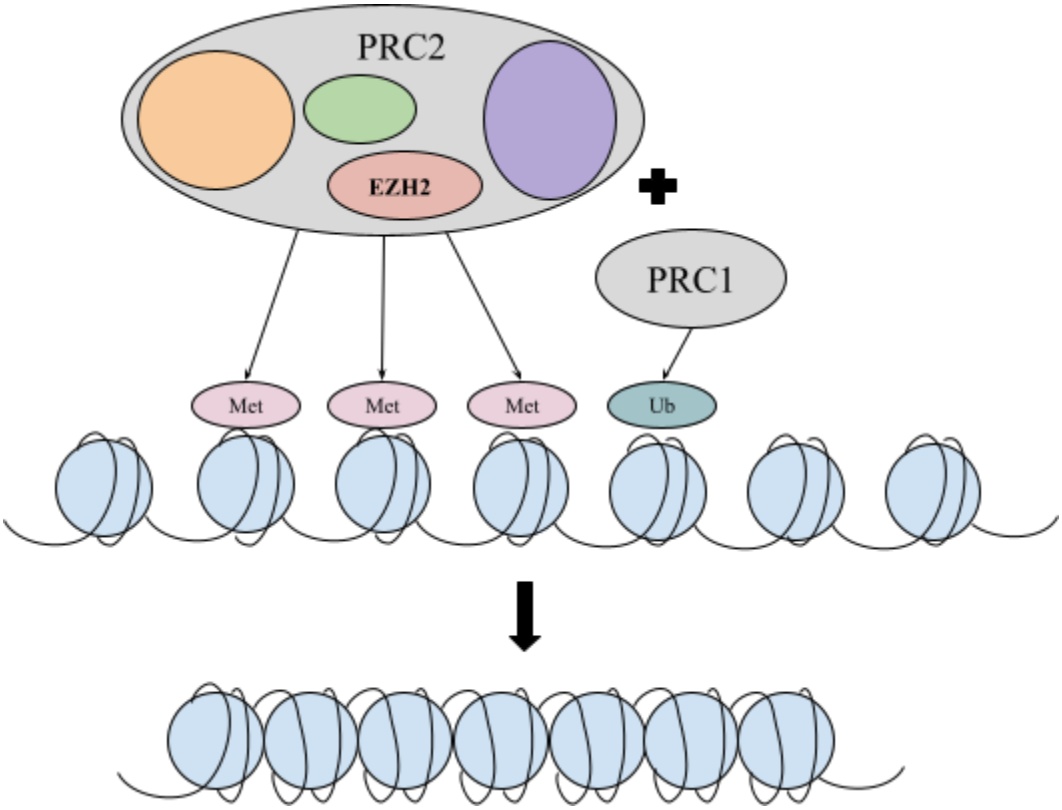

Figure 1: Schematic of PRC2-mediated gene regulation. Met = methyl groups, Ub = ubiquitin.

EZH2 is overexpressed in many cancers, including TNBC, with multiple studies having shown a correlation between increased EZH2 expression and more aggressive forms of cancer along with a poorer prognosis (12, 19, 24). EZH2 functions in the regulation of the cell cycle, tumor suppressor gene expression, DNA damage repair, and immune system surveillance/detection among others (10, 24). For a cancer cell, many, if not all, of these mechanisms need to be disrupted for survival. This makes EZH2 aberration an ideal step choice for cancer progression and a novel target for cancer therapy.

E. Tazemetostat

Tazemetostat (Figure 2) is an oral, small-molecule inhibitor of EZH2 developed by Epizyme and Eisai and approved by the Federal Drug Administration (FDA) in January 2020 for the treatment of locally advanced epithelioid sarcoma (22, 23). It works by inhibiting the trimethylation of H3K27. As of March 2020, there were two completed and ten ongoing clinical trials using tazemetostat either individually or in combination with other chemotherapeutic drugs (23). None of the trials was for breast cancer. Commonly reported side effects are pain, fatigue, decreased appetite, vomiting, and nausea (23).

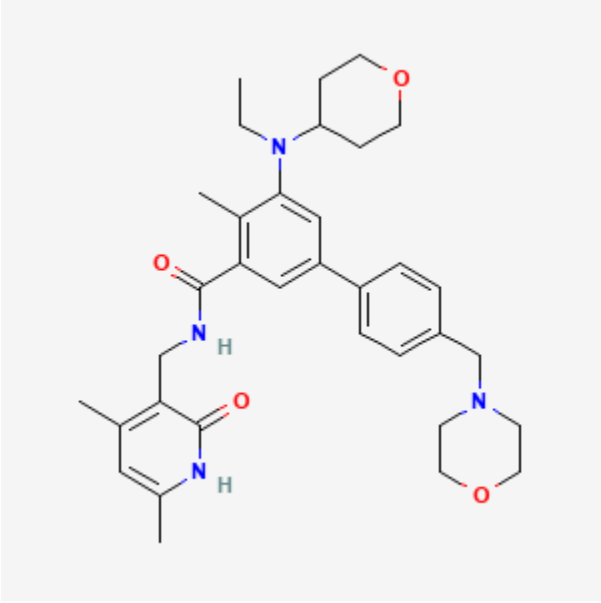

Figure 2: Structure of tazemetostat.

As of March 2022, there is limited published material on the use of tazemetostat in BC despite the fact that it has been established that EZH2 plays a key role in BC progression. Zhang reported that tazemetostat reversed EZH2-mediated chemotherapy-induced drug resistance in BC (7). More studies have been reported on TNBC. Multiple reports have shown that using tazemetostat or a similar EZH2 inhibitor impaired the metastatic ability of TNBC cells and

sometimes improved the sensitivity of these cells to other therapies (12, 13, 24). Wang et al. observed that EZH2 inhibitors sensitized TNBC cells to PARP inhibitors and thereby made them susceptible to synthetic lethality approaches (9). These authors synthesized a dual PARP and EZH2 inhibitor, based on the structures of olaparib and tazemetostat, which showed synergistic effects. In this paper, the dual inhibitor, referred to as compound 5a, was presented as a potential anticancer drug candidate for TNBC. Many sources suggested tazemetostat be used for TNBC in combination with miR33a-EZH2-signaling targeted drugs, histone deacetylase inhibitors, PARP inhibitors, or specific drugs like paclitaxel (9, 11, 16, 17). However, data supporting the use of tazemetostat as a monotherapy in the treatment of TNBC are lacking. This study investigates the effect of tazemetostat alone on the viability of BT549 human TNBC cells.

III. Experimental Design

A. Cell Culture

The BT549 human TNBC cell line was purchased from American Type Culture Collection (ATCC) and was used for all experiments. Cells were grown in RPMI-1640 media from ATCC, containing 10% fetal bovine serum (Hyclone) and 1% antibiotic-antimycotic (Grand Island Biological Company). BT549 cells were washed with 5 ml phosphate-buffered saline (PBS), then 1 ml trypsin (Sigma LifeSciences), and subcultured twice every week. They were grown in a ThermoScientific Forma Series II Water Jacket CO_2 incubator at 37°C with 5% CO_2.

B. Cell Viability Assays

BT549 cells were plated in a 96-well plate in nine columns of triplicates with the first column having no treatment and alternating columns thereafter having vehicle or drug treatments. Tazemetostat with a stock concentration 10mM (Selleckchem) was diluted with cell media to make dosages of 0.5 μM, 5 μM, 50 μM, and 100 μM. Volumes of dimethyl sulfoxide (DMSO) (Sigma Life Sciences) equal to corresponding drug dose were diluted with cell media to use as a vehicle control for tazemetostat. Cells were treated with tazemetostat or vehicle for 24 or 48 hours and cell viability was analyzed through an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay. 10 μL of MTT Reagent (R&D systems) was added to each well after either 24 or 48 hours of treatment exposure and incubated for 3 hours. After this period, 100 μL of Detergent Reagent (R&D Systems) was added to each well and the well plate was placed in a dark shelf for 3 hours. The plates were then read at 570 nm using BioTek Synergy 2 plate reader apparatus and Gen5 2.07 reading software. Each trial was conducted in triplicate. Data from cell viability assays was analyzed using GraphPad Prism 5 software. One-way ANOVA and Bonferroni's post test was used to determine significance ($p < 0.05$).

C. Western Blot Analysis

BT549 cells were plated in 100 mm culture dishes and treated with either 100 μM of tazemetostat or DMSO for 21 hours. Cells were then washed with PBS, scraped, and centrifuged for 5 minutes at 300 x g and 4°C in a ThermoScientific Sorvall ST 16R centrifuge. The supernatant was discarded and 50μL of Kinase Extraction Buffer (Abcam) was added onto the cell pellets and pipetted up and down. After 5 minutes, the samples were centrifuged again for 10

minutes at 13,000 rpm and 4°C in a ThermoScientific Sorvall Legend Micro 21R centrifuge. Supernatant was transferred into microfuge tubes and stored overnight at -80°C.

The next day, the protein lysate was thawed and the protein concentration determined by comparing it to a BSA (bovine serum albumin) protein standard with the BioRad Protein Assay Dye Reagent Concentrate. The appropriate volumes of each sample were used to provide 10 μg of protein. The volumes were equalized by the addition of RIPA buffer (ThermoScientific). Laemmli sample buffer (2x) equal to the sum of the RIPA buffer and protein volume was added to the samples. The samples were heated in boiling water for 5 minutes. Precision Plus Protein Kaleidoscope Standards (BioRad), vehicle sample, and drug sample were loaded onto 4-15% SDS-PAGE gradient gels (BioRad) in two sets. Proteins were electrophoresed through the gel at 150 V and then transferred onto a BioRad Trans-Blot Turbo Transfer Pack 0.2 μM PVDF (polyvinylidene difluoride) membrane using the BioRad Trans-Blot Turbo Transfer System. The membrane was split between the two sets and membranes were incubated in blocking buffer [3% dilution of BSA Fraction V (Roche Diagnostics) in TBST (Tris-buffered saline containing 137 mM NaCl, 4 mM KCl, 18 mM Tris, pH 8, 0.1% Tween20 (Sigma)] for 1 hour prior to incubation in the primary antibody at 1:1000 dilution in blocking buffer. Membranes were incubated in their respective primary antibodies overnight at 4°C. The primary antibodies used were mouse anti-caspase 3 (cleaved) antibody (Novus) and rabbit anti-survivin antibody **(**Novus).

Following incubation, the membranes were washed three times for 10 minutes each in TBST and incubated in a 1:10,000 dilution of secondary antibody in blocking buffer for 45 minutes. The secondary antibodies used were peroxidase-conjugated affinipure donkey anti-mouse IgG (Jackson ImmunoResearch) for the caspase-3 membrane and peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) for the survivin

membrane. After 45 minutes, the membranes were washed again thrice for 10 minutes each in TBST. Membranes were incubated in ThermoScientific SuperSignal West Pico PLUS Chemiluminescent Substrate kit, exposed to CL-Xposure film (ThermoScientific), and developed using the AFP Imaging Mini-Medical Series developer to detect cleaved caspase-3 and survivin expression. ImageJ software was used to determine band intensity.

Actin expression was used to standardize protein loading. Membranes used to detect cleaved caspase-3 or survivin were rinsed in methanol (Fisher Scientific), washed six times with DI (deionized) water, and incubated in 0.1M HCl for 5 minutes to strip them free of primary antibody. Then the stripped membranes were washed six times with TBST and incubated in blocking buffer for 30 minutes. The membranes were then incubated in anti-β-actin mouse monoclonal antibody (Sigma Life Sciences) at a 1:2500 dilution in blocking buffer for an additional 30 minutes. The membranes were washed three times for 10 minutes each in TBST followed by a 30-minute incubation in peroxidase-conjugated affinipure donkey anti-mouse IgG at 1:10,000 dilution in blocking buffer. After 30 minutes, the membranes were washed again thrice for 10 minutes each in TBST. Membranes were incubated inThermoScientific SuperSignal West Pico PLUS Chemiluminescent Substrate kit, exposed to film, and developed using the AFP Imaging Mini-Medical Series developer to detect β-actin expression. ImageJ software was used to determine band intensity.

IV. Results and Discussion

A. Tazemetostat has optimal efficacy at a high dose of treatment

Tazemetostat was administered to cells at doses ranging from 0-100 μM and cells were grown for either 24 or 48 hours. MTT absorbance values were used as an indication of cell viability. Tables 1 and 2 show average absorbances of cells treated with different drug doses for varying intervals, while Figure 3 is a graphical representation of this data. Tazemetostat had minimal effect on BT549 cells at lower doses. Absorbance readings of tazemetostat-treated cells were similar to vehicle absorbance levels upto 50 μM regardless of the length of treatment, but decreases substantially at 100 μM for both treatment lengths. Overall, absorbance, and by extension cell viability, decreases in a dose-dependent manner. This was observed in cells treated for both 24 and 48 hours. An interesting point to note is that the margin of error of the data also becomes smaller at the 100 μM dose indicating lower variability at this dose. About 60% of cells were eliminated after 24 hours of exposure to tazemetostat while this number jumps to about 75% after 48 hours of exposure.

Treatment	Experiment 1	Experiment 2	Experiment 3	Mean ± SEM
Untreated	0.186	0.259	0.406	0.284 ± 0.065
DMSO	0.196	0.283	0.473	0.317 ± 0.082
0.5 µM Taz	0.175	0.29	0.436	0.300 ± 0.076
DMSO	0.196	0.278	0.434	0.303 ± 0.07
5 µM Taz	0.236	0.284	0.427	0.316 ± 0.057
DMSO	0.211	0.225	0.399	0.278 ± 0.061
50 µM Taz	0.167	0.216	0.479	0.287 ± 0.097
DMSO	0.153	0.192	0.419	0.255 ± 0.083
100 µM Taz	0.118	0.075	0.178	0.124 ± 0.03

Table 1: Average MTT assay absorbances after 24 hours of treatment.

Treatment	Experiment 1	Experiment 2	Experiment 3	Mean ± SEM
Untreated	0.287	0.436	0.657	0.46 ± 0.108
DMSO	0.298	0.476	0.641	0.472 ± 0.099
0.5 µM Taz	0.314	0.471	0.653	0.479 ± 0.098
DMSO	0.277	0.485	0.63	0.464 ± 0.102
5 µM Taz	0.304	0.47	0.683	0.486 ± 0.11
DMSO	0.265	0.415	0.674	0.451 ± 0.12
50 µM Taz	0.231	0.46	0.578	0.423 ± 0.102
DMSO	0.263	0.417	0.668	0.449 ± 0.118
100 µM Taz	0.147	0.077	0.101	0.108 ± 0.021

Table 2: Average MTT assay absorbances after 48 hours of treatment.

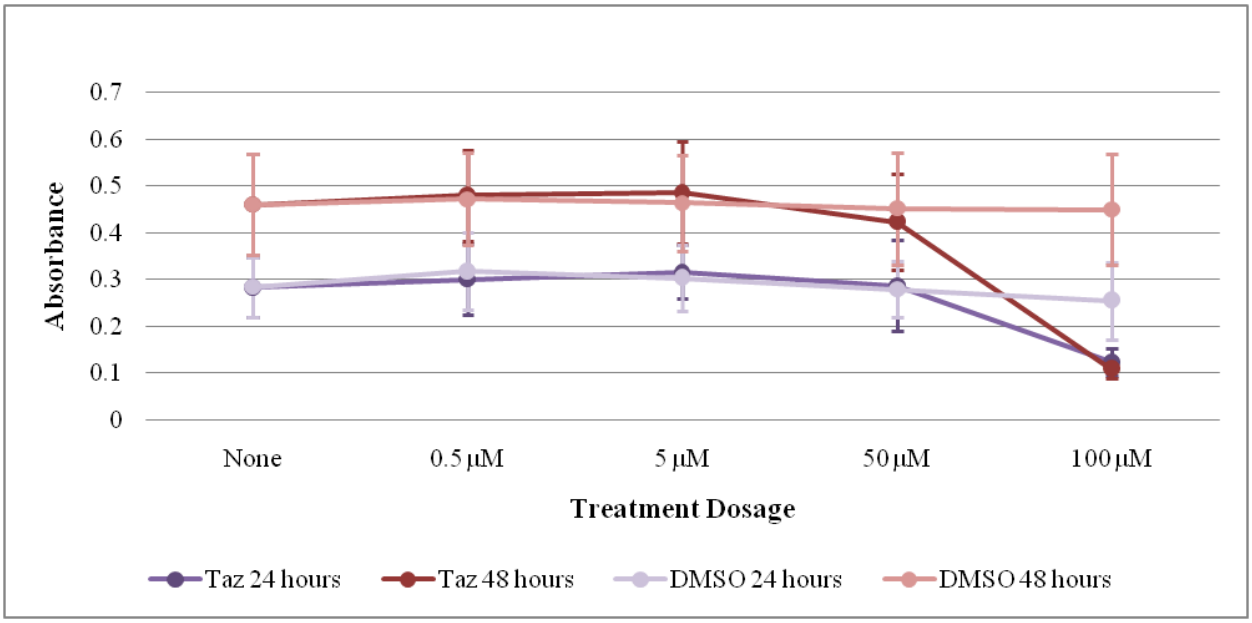

Figure 3: Dose- and time dependent effects of tazemetostat (taz) and vehicle after 24 and 48 hours of treatment.

B. Tazemetostat activates the apoptotic pathway of BT549 cells via caspase-3 cleavage

Tazemetostat decreased the viability of BT549 cells, but the mechanism through which this was accomplished remained unclear. Since most cells seemed to be dead by 48 (or even 24) hours, protein expression levels were analyzed after only 21 hours of drug exposure. Cleaved caspase-3 expression was analyzed to explore this process further. Most caspases are proteases that need to be cleaved to be activated. Once activated, they function in one of three ways, depending on the specific type of caspase it is: initiate apoptosis, execute apoptosis, or notify the immune system to clean up dead cells (6). Executioner caspases work by degrading DNA structures, cytoskeleton proteins, and other crucial protein substrates. Caspase-3 is a lead player

among executioner caspases. The abundance of cleaved caspase-3 was investigated to determine if tazemetostat activated the BT549 cells' apoptotic pathway to eliminate them (Figure 4). Western blot analysis revealed that substantial activation of caspase-3 was present in cells treated with tazemetostat as opposed to vehicle-treated cells which did not show a band indicating there was little to no caspase-3 cleavage in these cells. β-actin expression was used as a housekeeping gene to ensure equal amounts of protein samples were loaded into the control and treatment lanes during gel electrophoresis. Figure 4 also shows that β-actin expression was similar in both lanes indicating caspase-3 was indeed cleaved at higher levels due to tazemetostat treatment.

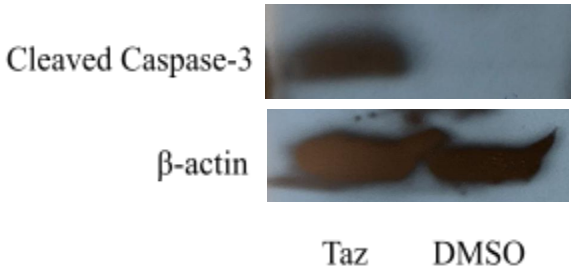

Figure 4: Representative film of cleaved caspase-3 and β-actin abundance in taz- and DMSO-treated cells. Taz = Tazemetostat.

C. *Tazemetostat reduces survivin expression in BT549 cells further suggesting apoptotic pathway activation in these cells.*

Survivin is an apoptotic protein inhibitor that functions by blocking the activity of various caspases. Elevated survivin levels can also result in chemoresistance and higher aggressiveness of tumors (21). Lower levels of survivin are expected from apoptotic cells. Western blot analysis was conducted to determine if the BT549 cells were indeed undergoing apoptosis when treated with tazemetostat. The results revealed substantially lower levels of

survivin present in cells treated with tazemetostat as opposed to vehicle-treated cells (Figure 5). β-actin expression was used as a housekeeping gene to ensure equal amounts of protein were loaded. Bands were quantified and showed an approximate 30% decrease in survivin expression in cells treated with tazemetostat (Figure 6). Overall, this data suggests that tazemetostat-treated cells did indeed have lower levels of survivin expression.

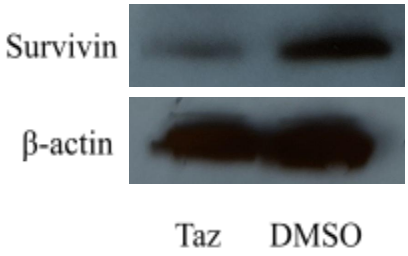

Figure 5: Representative film of survivin and β-actin abundance in taz- and vehicle-treated cells. Taz: tazemetostat.

(a)

Experiment	DMSO	Tazemetostat
#1	31704	27455
#2	43318	26503
#3	36825	15924

(b)

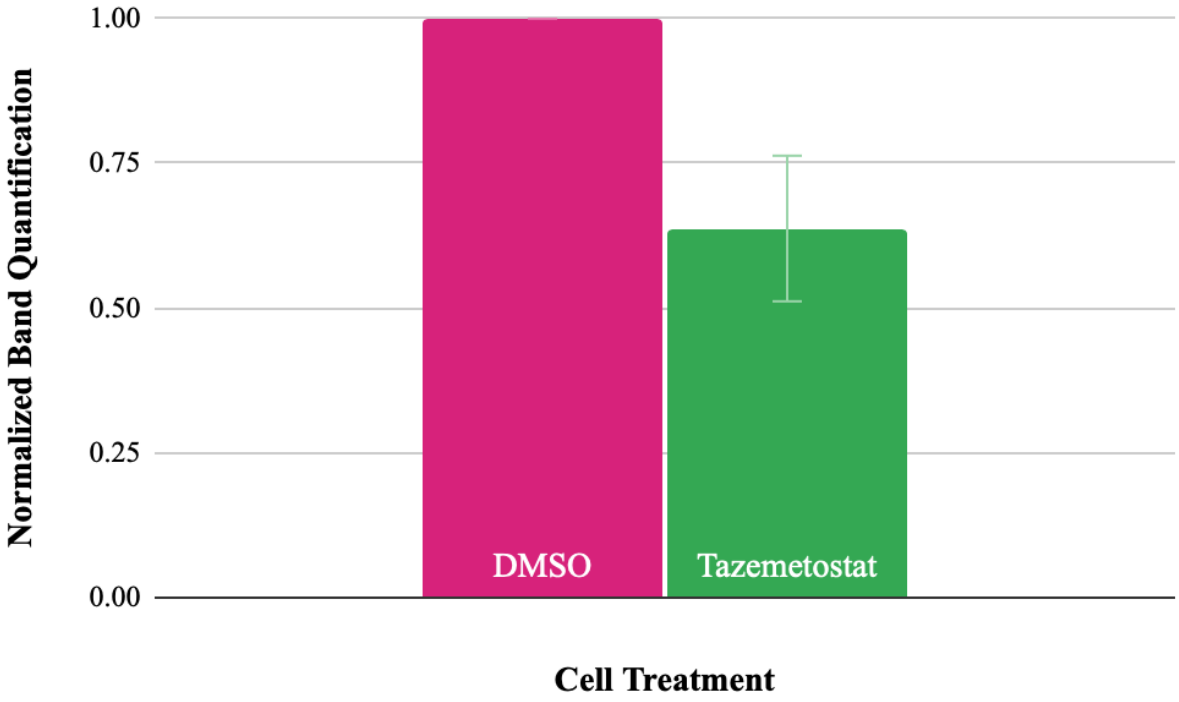

Figure 6: Normalized western blot band quantifications testing for survivin abundance. (a) The area of bands for each treatment group is shown in pixels and was measured using ImageJ software. (b) Graphical representation of band quantifications.

VI. Conclusion

Tazemetostat is a chemotherapeutic drug currently being used/tested in various types of cancers including lymphomas, sarcomas, and rhabdoid tumors (23). Its primary mode of action is through the inhibition of EZH2-mediated trimethylation, a generally overactivated process in many cancers including TNBC. Although limited research has been done on the applications of tazemetostat use in TNBC, it has been shown to reverse chemoresistance and obstruct metastasis. The goal of this study was to determine the efficacy of tazemetostat on BT549 TNBC cells.

Two key observations were made through this research. Tazemetostat was effective at high doses administered over 24-48 hours. Exposing cells to treatment for longer time periods progressively led to lower cell viability as evidenced by the fact that about 15% more cells were killed when exposed to tazemetostat for 48 hours as opposed to 24 hours. Some cells might have survived due to differences in the stage of the cell cycle the cell was in or due to variable efficacy of tazemetostat. Further treatment might be necessary to completely eliminate residual cells.

Second, tazemetostat shows lethality in BT549 cells by activating their apoptotic pathways. This was discerned by the increased abundance of cleaved caspase-3 protein and decreased expression of survivin in tazemetostat-treated cells compared to those given vehicle treatment. It is possible that other pathways are also affected considering the vast functional range of EZH2. Based on these results, tazemetostat may be a promising targeted therapy option to pursue in treatment of TNBC. Further *in vitro* and *in vivo* studies are needed to validate this claim.

VII. Future Directions

There are several ideas that future experiments could explore. First, time intervals longer than 48 hours could be tested to determine if longer exposure improves efficacy. Conversely, cells could be treated with a lower dose of tazemetostat for an extended period of time to ascertain whether similar efficacy could be reached at a lower dose.These experiments would provide a better indication of the time- and dose-dependent effects of tazemetostat. Second, treatment protocols could be repeated using healthy breast tissue cells to examine the specificity of the drug to cancer cells. The current study primarily focused on the efficacy of tazemetostat on BT549 cancer cells. Although the drug showed promising results, it would not be feasible to use if it severely harmed wild-type breast tissue cells as well. Third, complementary drug combinations could be tried to deduce if the residual levels of BT549 cells could be further diminished. Cancer cells that are left behind could cause recurrence that is resistant to treatment. Eliminating as much of the tumor(s) as possible in the first cycle of treatment could give the best chances of long-term survival for the patient.

VIII. Acknowledgements

I would like to thank Dr. Marilyn Odom for guiding me through each step of my thesis journey from experimental design to thesis draft edits and always answering my infinite questions. I would also like to thank Dr. Chris Barton and Dr. Jennifer Thomas for their support in this project and the feedback they have given me so I could write my best possible thesis. Finally, I am grateful to Belmont University's Honors Program for giving me the opportunity to embark on this journey.

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