Inhibitory Effects of Human Serum Albumin-conjugated Superoxide Dismutase Mimetic on Breast Cancer Cell Proliferation

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Personal Statement

I began research the same way I began to speak or walk: imitation. I saw my parents as tall, strong superheroes strutting confidently around the world on two sturdy feet. My ultimate goal was to be like them, so I would cautiously relinquish the use of my hands as crawling devices, and take one wobbly step, then another, finally doing it, finally able to-- SPLAT. I am overeager with one of my steps and fall face-first onto the ground.

Sixteen years later, and I haven't changed much. My pursuit of knowledge remains wobbly and uncertain, and my parents remain my biggest role models. Both of them have pursued scientific research as a living, but their experience is not limited to their careers. Throughout my life, my parents have encouraged me to question the world, always indulging my constant inquiries of "Why?" or "How?" Furthermore, they taught me early on to acknowledge my mistakes, as those mistakes lead to success, often through paths previously unthought-of.

In my science classes, I had learned about the concept of research through famous individuals: Einstein, Newton, etc. Textbooks often described the achievements of these people as if they themselves did all the work, with no outside help. However, by the time I entered high school, textbooks had become less as sources of information and more as starting points. I knew that if I wanted to discover something, I had to take the initiative. I discovered an opportunity for high schoolers to pursue research at an accredited laboratory institution (Sanford Research). It was a lengthy time commitment, long enough that it seemed almost a waste of time if I failed. But I decided that the information and experience I would gain would far outweigh the cost of the time spent.

At Sanford, I met my mentor, Dr. Keith Miskimins. I had chosen his lab because I was particularly attracted to the concept of alternative treatment methods for cancer. His lab researched treatments using subject areas seemingly unrelated to cancer. As someone who was always connecting topics from different classes in school, the Miskimins lab seemed like the ideal place to experience the research world firsthand. I, fortunately, have not had any personal experience with cancer. However, this did not diminish my interest at all, for if I could help

anyone with my research, that would be enough. Cancer is a very elusive subject; my textbooks would often place approach the topic with a disclaimer that our knowledge of cancer was evolving. And through this, I became aware that though the collection of data itself is rigorous and exact, the actual formation of hypotheses and methods to test them is an unsure process. Specifically, the results we receive can be surprising and, at times, disappointing. I had many trips in the road during my first official laboratory experience. Learning the basic research techniques as well as the process of documenting everything made me feel again like a baby learning to walk.

However, I quickly learned that research is not a solitary process. My mentor and fellow lab members answered all my questions, and with their help, I formed a project of my own. The countless publications I delved into to familiarize myself with the area of breast cancer treatment assisted me through my own research. My parents instilled in me a work ethic and insatiable curiosity that propelled me through the data collection. Finally, the mistakes and disappointments I faced during my research experience challenged my thought processes. For example, my initial Western blotting produced blank strips of paper, not very exciting additions to my lab journal. However, I read publication after publication about specific antibodies that other researchers had used regarding the cancer treatment I was exploring, and I tried again. Though I was initially discouraged, I performed blot after blot, and finally, I received lines!

As cliché as it sounds, my advice to future researchers is to appreciate mistakes and deviations from your original plan. As you've probably learned, many of science's greatest accomplishments were accidents. But even little things like a Western blot can be solved through perseverance. If you feel discouraged, keep an open mind to alternative solutions. Additionally, find others who share your interest in research. Find opportunities in which you can explore research with experienced professionals. Discuss your interests with friends and family. But finally, stay confident in yourself, and know that when you begin your pursuit of research, you are helping to unravel one of the countless mysteries of our world, one baby step at a time.

Abstract

According to the American Cancer Society, about 1 in 8 women in the United States will develop breast cancer in their lifetime. Unlimited cell proliferation in the tumor area is a hallmark of cancer. A treatment that manipulates the levels of reactive oxygen species (ROS) in tumor cells is being developed. The drug SZ16 consists of a superoxide dismutase mimetic conjugated with human serum albumin. The aims of this study were to demonstrate the effects of SZ16 on the growth of breast cancer cells and to explain the mechanisms behind these effects. Cell culture was carried out by using 4T1, a mouse breast cancer line. Clonogenic assays were performed to determine the effects of SZ16 on cell proliferation, while flow cytometry and Western blotting were used to elucidate the mechanisms. Breast cancer cell colonies were decreased in both size and number following drug treatment. The levels of ROS and the expression of cell cycle protein cyclin D1 decreased after drug treatment, suggesting a correlation between ROS and the cell cycle in cancer cells. The results of the study suggest that the manipulation of ROS with SZ16 may contribute to possible alternative treatments of breast cancer by inhibiting cell proliferation.

Introduction

Breast cancer continues to be one of the most prevalent of cancers, acting as the "leading cause of cancer-related death for women worldwide" (DeSantis et al., 2015); current treatments of breast cancer include surgery, radiotherapy, and medication. In this study, a novel drug that targets the tumor by inhibiting cell proliferation is being examined. One of the hallmarks of cancer is unlimited proliferation. Cancer cells ignore signals that in normal cells maintain a consistent balance of growth and replication (Hanahan and Weinberg, 2011). This developing treatment, by inhibiting cell proliferation, is ridding breast cancer cells of a key factor and facilitating possible clearance of the tumor cells.

SZ16 is a novel drug composed of a human serum albumin-conjugated superoxide dismutase mimetic. SZ16 manipulates the levels of reactive oxygen species in the tumor microenvironment through its superoxide dismutase component. Reactive oxygen species (ROS) are oxygen-containing molecules with an unpaired electron, making ROS highly chemically reactive. ROS have been implicated in the stimulation of cancer cell proliferation, angiogenesis, metastasis, etc. (Hecht et al., 2016). ROS are naturally produced in cells, particularly as byproducts of the electron transport chain during cellular respiration. Therefore, cells contain enzymes that break down ROS, such as superoxide dismutase (SOD). Superoxide (O_2^-), a type of ROS, is broken down by SOD into hydrogen peroxide and oxygen. Catalase then enzymatically converts hydrogen peroxide into water and oxygen, completing the two-step detoxification of superoxide (Held, 2015). Because SZ16 contains a mimetic of SOD, SZ16 performs the catalytic function of SOD but is not the exact structure of natural SOD.

Human serum albumin (HSA) is the most abundant protein in the blood of all vertebrates and has a variety of functions, the most significant being that HSA can bind and transport hydrophobic compounds, including drugs, through the blood. The use of albumin as a drug carrier has become more common, especially with the success of Abraxane® (Merlot et al., 2014). Abraxane® is an albumin-bound form of paclitaxel, a treatment used for breast, lung, and advanced ovarian cancers. It has been shown that albumin-bound paclitaxel is more efficient at decreasing tumor volume than other formulations of paclitaxel (Desai et al., 2006). Albumin

seems to increase the amount of drug entering the tumor microenvironment, and this effect may also occur with SZ16. Binding the SOD mimetic with HSA may pose a more efficient method of delivering the drug to the tumor cells.

SZ16 potentially inhibits cancer cell proliferation by manipulating the levels of reactive oxygen species in the tumor microenvironment. Specifically, decreasing the levels of ROS seems to cause cells to leave the cell cycle. The cancer cells may enter a state of senescence, or "permanent cell-cycle arrest" (Campisi and di Fagagna, 2007). A feature of almost all cancers is elevated levels of reactive oxygen species, usually as a result of increased metabolic activity and respiration. ROS may act as second messengers in cell signaling pathways that promote cell proliferation (Storz, 2005). ROS also affect the levels of cell cycle regulatory proteins, such as cyclin D1, that are present in the cell when the cell transitions from G1 to S phase in the cell cycle (Burch and Heintz, 2005). Thus, decreasing ROS levels may decrease the levels of cyclins and decrease the rate of proliferation of cancer cells.

A related topic is the idea of hypoxia. Hypoxia is a condition of abnormally low oxygen levels, and most tumors show hypoxic conditions. Hypoxia poses multiple problems, contributing to resistance to chemotherapy, radiotherapy and other treatments (Wilson and Hay, 2011). Hypoxia induces transcription factors including HIF-1α. SOD has been shown to decrease levels of HIF-1α, suggesting that superoxide contributes to HIF-1α production and possibly hypoxia itself (Liou and Storz, 2010). SZ16, with its superoxide dismutase mimetic component, may target the hypoxia that is prevalent in cancer cells, further eliminating a key characteristic of tumor cells.

In this study, the effects of SZ16 were observed on breast cancer cells. The consequences of administering human serum albumin-conjugated superoxide dismutase mimetic were examined. The study aimed to discover SZ16's effects on cell proliferation in cancer cells, particularly inhibitory effects. The study further examined the possible mechanisms behind these effects, focusing on the role of ROS in controlling the defining characteristics of cancer cells.

Materials and Methods

Chemicals

SZ16 (human serum albumin-conjugated superoxide dismutase mimetic) and human serum albumin (HSA) were provided by SynZyme Technologies LLC®, Sioux Falls, SD. SZ16 was provided as a 10% solution and the human serum albumin was provided at 25%. Saline solution was used to dilute the stock human serum albumin to a concentration of 10%.

Cell Culture

Mouse breast cancer cell line 4T1 (CRL-2539™) was purchased from ATCC®. 4T1 cells closely mimic human breast cancer cells in terms of growth potential. Mouse oropharyngeal epithelial cell line was previously derived from C57BI/6 mouse oropharyngeal epithelium. The cells were retrovirally transduced with human papillomavirus-16 proteins E6 and E7 and the oncogene H-Ras resulting in an immortalized cell line known as MEER (Spanos et al., 2009). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.2% amphotericin. Cells were incubated under conditions of 37°C and 5% CO_2.

Clonogenic assay

The clonogenic assay is based on the ability of cells to proliferate into a colony and how treatment will affect this colony growth (Franken et al., 2006). 4T1 breast cancer cells were plated on 6-well plates and incubated overnight in DMEM. Cells were then treated with either SZ16 (3 μM, 6 μM, 12 μM) or human serum albumin at the same concentrations. Cells were also treated with only DMEM media to serve as control. After incubation for 5 days, media was aspirated and cells rinsed in phosphate buffered saline (PBS). Cells were fixed in 70% ethanol, rinsed with PBS, and stained with Coomassie blue dye. After further extensive rinsing with PBS, pictures of the plates were taken using an Alpha Imager (AlphaInnotech®, Santa Clara, CA).

Cell viability assay

4T1 cells were plated on black 96-well plates with clear bottoms and treated with either SZ16 or human serum albumin (or only DMEM media as control). After incubation for 3 days, cells were

incubated with Sytox Green fluorescent dye (Thermo Scientific®) for 15 minutes at room temperature to detect dead cells. Fluorescence was detected by using a SpectraMax® M5 fluorescence plate reader at a λex/em = 485 nm/535 nm with a 515 nm cutoff. Cells were then permeabilized with 6% Triton X-100 and fluorescence was again detected by using the fluorescence plate reader to detect total cells.

Senescence-associated β-galactosidase assay

Cells in the senescent state produce a higher level of lysosomal β-galactosidase than normally growing cells (Eccles and Li, 2012). Senescence β-Galactosidase Staining Kit (Cell Signaling Technology®) was used to identify senescent cells after drug treatment. 4T1 cells were plated and treated with either SZ16 or human serum albumin (or only DMEM media as control). After incubation for 3 days, media was aspirated and cells were rinsed with PBS. MEER cells were plated and treated with DMEM media. After incubation for 24 hours, MEER cells were exposed to 30 grays of radiation to induce senescence as a positive control. After further incubation, media was aspirated and cells were rinsed with PBS. Cells were fixed in Fixative Solution (Cell Signaling Technology®) for 10 minutes at room temperature. Cells were rinsed in PBS and treated with β-Galactosidase Staining Solution (Cell Signaling Technology®), incubated overnight in a dry incubator with no CO_2. The plates were observed under an inverted bright-field microscope at a total magnification of 100X (Olympus® IX71). The lab technician assisted with use of the microscope. Blue-green staining indicated positive results of senescence.

Flow cytometry

4T1 cells were plated in 24-well plates and treated with either SZ16 or human serum albumin (or only DMEM media as control). After incubation for 1 day, media was aspirated and cells were rinsed with PBS. Cells were then incubated with 2', 7'-dichlorofluorescein diacetate (DCF-DA) for 15 minutes. 2', 7'-dichlorofluorescein diacetate is a cell-permeant indicator for reactive oxygen species that is nonfluorescent until it enters the cell, where it is converted to the fluorescent DCF (Held, 2015). Media was aspirated and cells were rinsed again with PBS. Cells were trypsinized and analyzed on a BD Accuri™ C6 Flow Cytometer on channel FL1. The lab technician gave assistance in utilizing the Flow Cytometer.

Western blotting

Cells were plated in 35 mm dishes and treated with SZ16 or human serum albumin. After incubating for 1 day, medium was aspirated and cells rinsed with PBS. Cells were then lysed by addition of 1x sodium dodecylsulfate (SDS) sample buffer (2.5 mM Tris-HCl (pH 6.8), 2.5% SDS, 100 mM dithiothreitol, 10% glycerol, 0.025% bromophenol blue). Lysates were thawed, centrifuged, and sonicated. Sypro orange was used to check relative protein density in lysates. Equal protein amounts were loaded on gels. Proteins were transferred onto membranes using a Bio-Rad Trans-blot apparatus. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour. The membrane was then incubated with the following primary antibodies overnight at 4°C. Antibody against Cyclin D1 (2292, 1:1000 dilution in 5% non-fat dry milk in TBST) was purchased from Cell Signaling Technology®. Antibody against HIF-1α (ab113642, 1:1000 dilution) was purchased from Abcam®. After multiple washings of the membrane in TBST, the membrane was incubated with the species-appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour. After further washings of the membrane in TBST, proteins were detected on the UVP BioSpectrum® Imaging System using chemiluminescent substrate. Secondary horseradish peroxidase-linked anti-mouse and anti-rabbit IgG antibodies were purchased from Pierce Biochemical®. The membrane was further incubated with β-actin antibody to demonstrate equal loading of sample in each lane. Antibody against β-actin (A5441, 1:10000 dilution) was purchased from Sigma®.

Statistics

Data were expressed as means ± SEM (standard error of the mean). P-values were calculated using student t-tests. P-values less than 0.05 indicated significance.

Results

Clonogenic assay

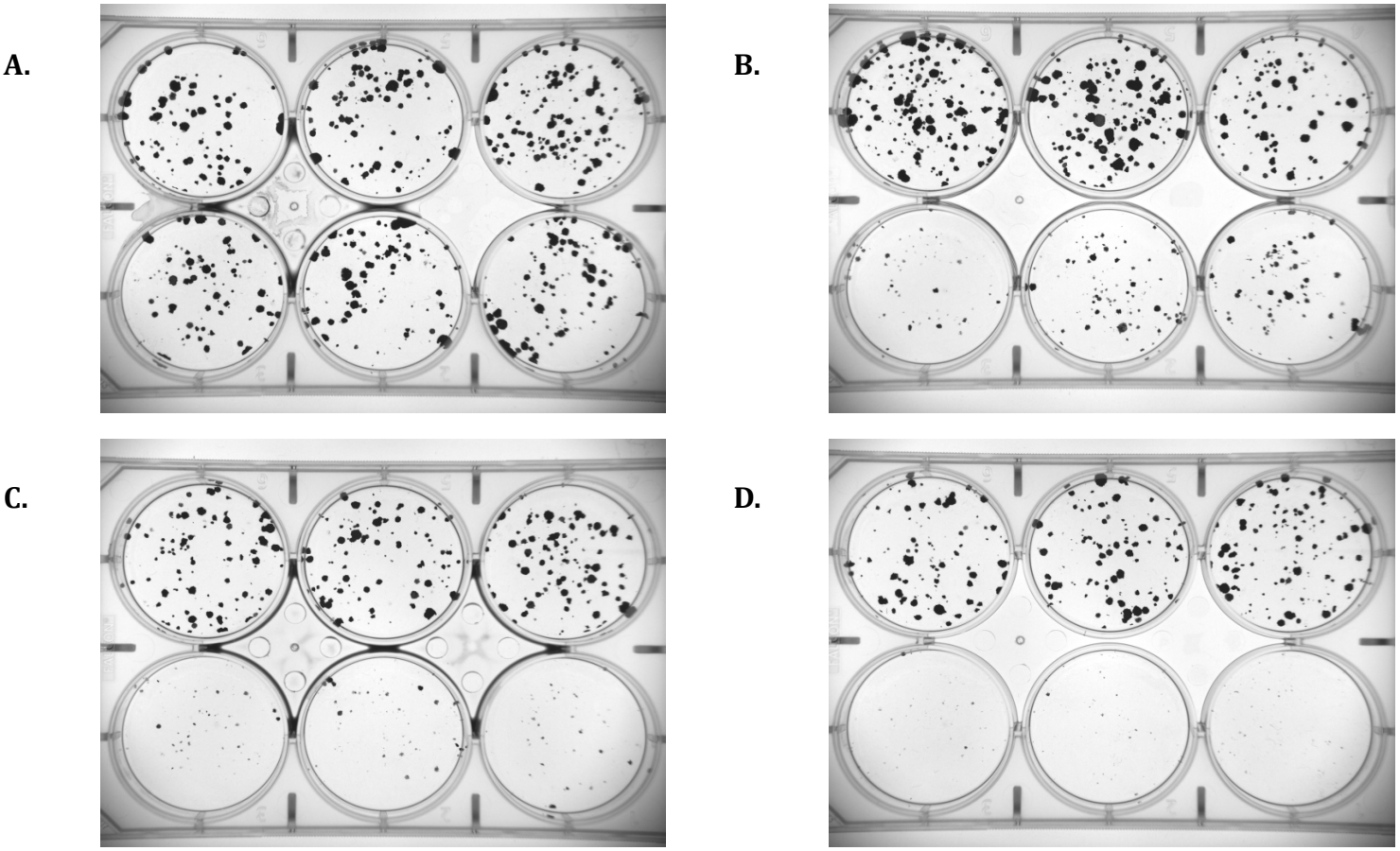
One of the study's aims was to examine the effects of a human serum albumin-conjugated superoxide dismutase mimetic (SZ16) on 4T1 breast cancer cell proliferation. Specifically, SZ16 inhibited 4T1 cell proliferation; this inhibition was dose-dependent at the scale of cell colonies. Clonogenic assays showed SZ16 significantly decreased cell colony size and number after drug treatment compared to HSA treatment alone (Fig. 1-1.1). Colonies grown in only DMEM showed the natural progression of colony formation for the 4T1 cell line. Cells treated with only HSA grew into colonies similar in size and number to the DMEM control, while cells treated with SZ16 grew into fewer colonies smaller in size than the DMEM control, indicating that SZ16 inhibits colony formation and cell proliferation. SZ16's inhibitory effects on colony formation also appeared to be dose-dependent, as is graphically depicted and mathematically shown through statistical tests (Fig. 1-1.2). Student t-tests were performed and p-values were greater than 0.05 at the 3 μM concentration, while p-values at the 6 μM and 12 μM concentrations were lower than 0.01, compared with HSA alone. Thus, at the lowest concentration the difference between HSA and SZ16 was not significant, while at the higher concentrations the differences were highly significant.

Cell viability assay

A cell viability assay involving Sytox Green® was then used to directly determine SZ16's effects on cell growth and survival. SZ16 decreased the number of live cells in a dose-dependent manner (Fig. 2-2.3). Interestingly, SZ16 treatment showed lower dead cell fluorescence compared to HSA-only treatment (Fig. 2-2.2). This may be due to the fact that SZ16 decreased the number of total cells, which would therefore decrease the number of cells available to die (Fig. 2-2.1). This implies that SZ16 may not be directly causing cell death, but rather causing cells to not proliferate in the first place. Furthermore, while SZ16's inhibitory effects on both total cell count and live cell count are dose-dependent, SZ16's effects on dead cell count do not seem to be dose-dependent, further supporting the idea that SZ16's mode of action is not inducing cell death but inhibiting cell proliferation.

Illustrations

1.1



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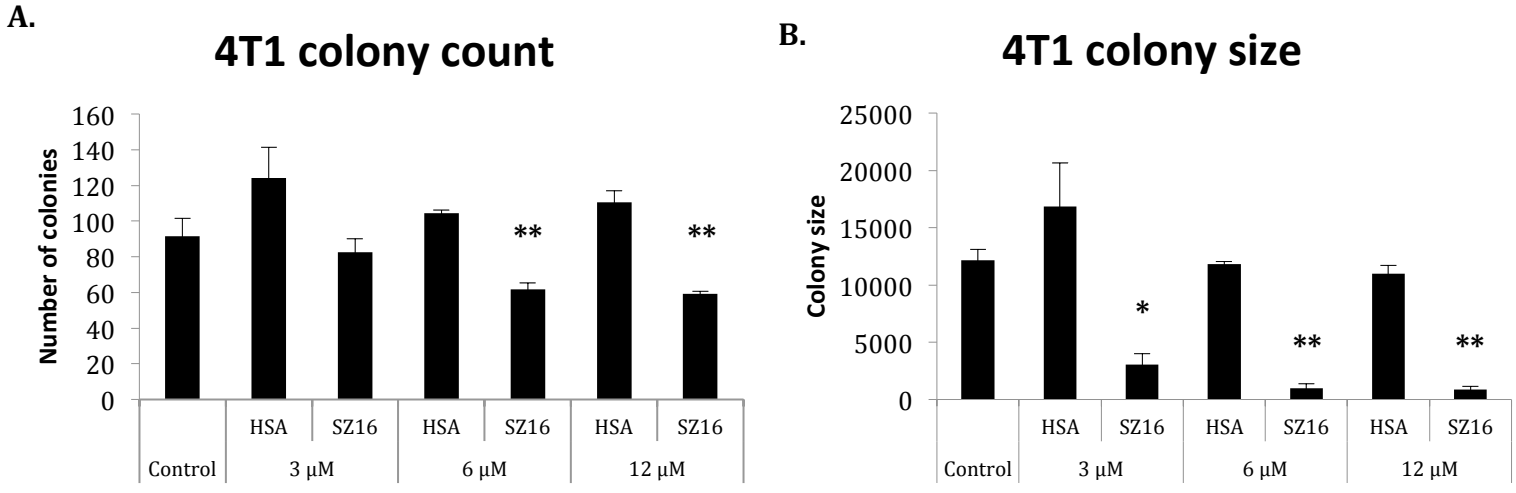
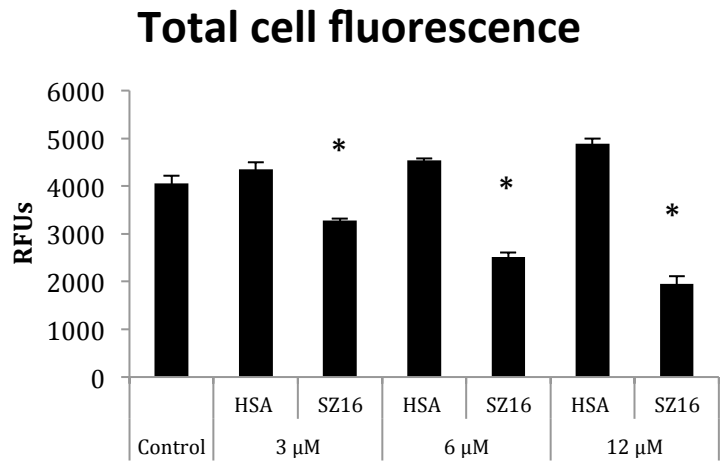


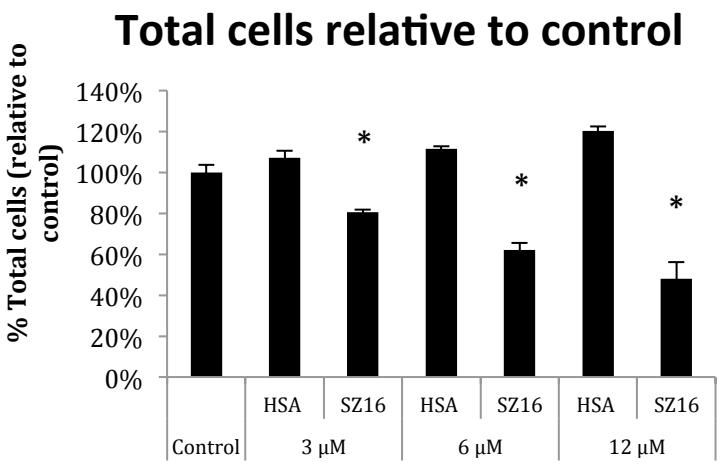
Figure 1. SZ16 inhibits proliferation of 4T1 breast cancer cell colonies.

1.1 A. 4T1 cells were plated with only DMEM to serve as control. **B.** 4T1 cells were treated with either (top row) HSA 3 µM, (bottom row) SZ16 3 µM. **C.** (top row) HSA 6 µM, (bottom row) SZ16 6 µM. **D.** (top row) HSA 12 µM, (bottom row) SZ16 12 µM

1.2 A. 4T1 cells were treated with SZ16 at the indicated concentrations for 5 days. Cell colonies were counted manually using alpha imager. **B.** Cell colony size was calculated by alpha imager. Data is representative of three independent experiments; n=3 per group; Error bars represent standard error of the mean. (* = $p < 0.05$, ** = $p < 0.01$ vs. HSA)

2.1





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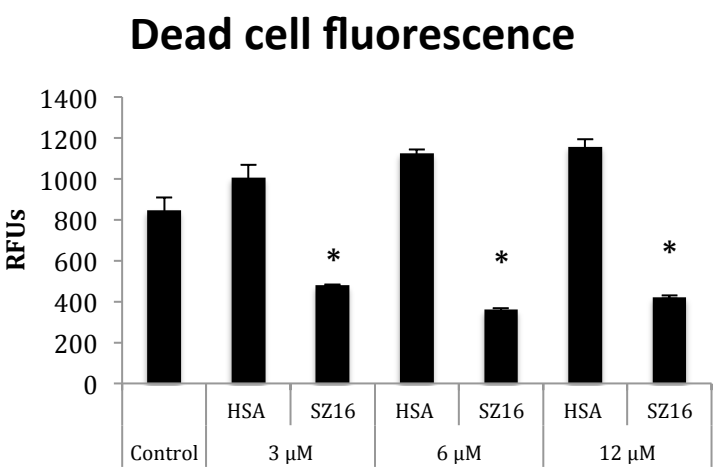
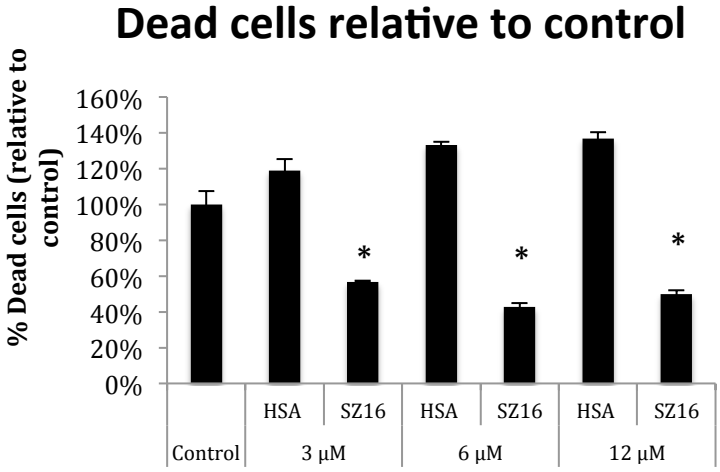
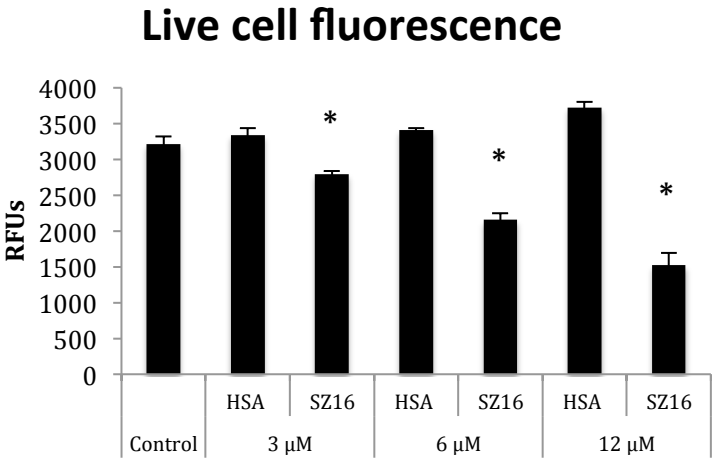




2.3



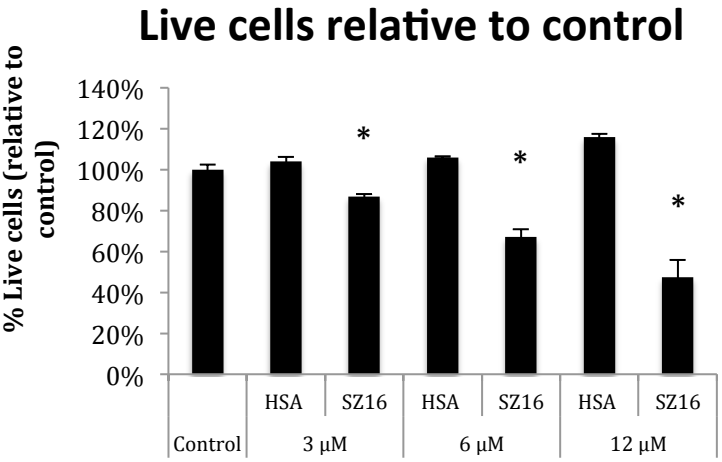


Figure 2. SZ16 inhibits proliferation, not viability, of 4T1 cells. Cells were treated with Sytox Green® and analyzed using a fluorescence plate reader, yielding the amount of dead cells. Cells were then permeabilized with Triton X-100 and analyzed again using a fluorescence plate reader, yielding the amount of total cells. Dead cell numbers were subtracted from total cell numbers to yield live cell numbers.

2.1 Total cell numbers after SZ16 and HSA treatment. **2.2** Dead cell numbers after SZ16 and HSA treatment. **2.3** Live cell numbers after SZ16 and HSA treatment. RFUs (relative fluorescence units) were recorded for each treatment. Data is representative of three independent experiments; n=3 per group; Error bars represent standard error of the mean. (* = $p < 0.01$ vs. HSA)

Senescence-associated β-galactosidase assay

The senescence-associated β-galactosidase assay takes advantage of the accumulation of lysosomal β-galactosidase specifically present in senescent cells (DiMaio et al., 2006). Using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology®), SZ16-treated cultures were found to have more senescent cells compared to HSA-treated cultures (Figs. 3A and 3B). Additionally, the positive control using MEER cells exposed to 30 grays of radiation showed that the kit functioned normally (Fig. 3D). The SZ16-treated cells showed similar patterns of senescence as the positive control cells, indicating that the staining found in the SZ16-treated cells was not a false positive. Interestingly, cells treated only with DMEM media showed high staining, but there are hints to the possibility of a false positive in that instance (Fig. 3C). The DMEM media-treated cells were highly confluent, which may have led to cells entering a senescent state, as it has been shown that excessive cell contact induces senescence (Ho et al., 2011). Furthermore, the staining pattern in these DMEM media-treated cells was irregular compared to the positive control. In this senescence assay, while quantifications were not performed, observations seemed to show that SZ16's effect on senescence was not dose-dependent, or at least not as clearly dose-dependent as in the clonogenic or cell viability assays. The staining observed in the 12 μM concentrations of both HSA and SZ16 (Fig. 3) showed negligible differences to the 3 μM and 6 μM concentrations (not shown).

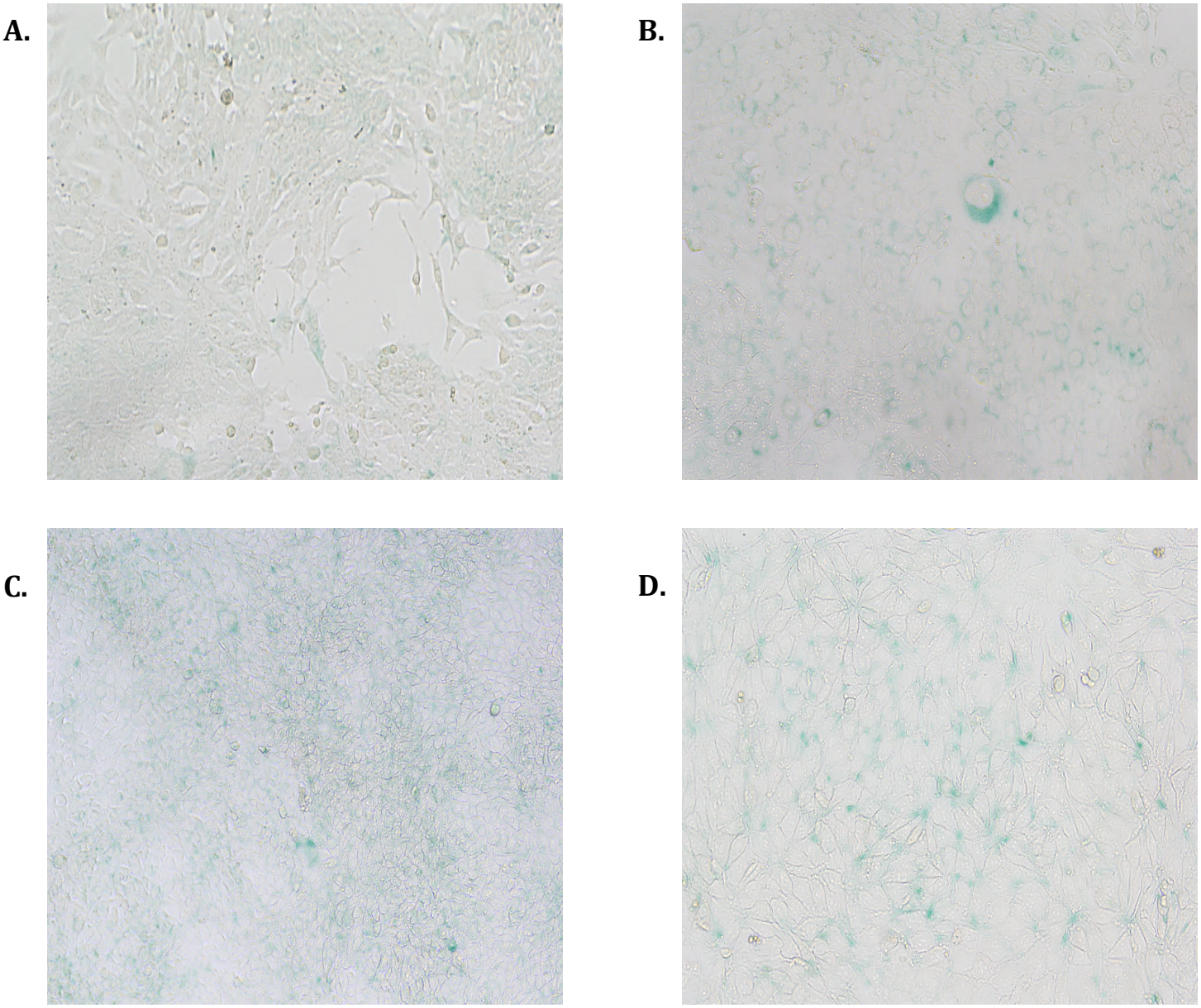


Figure 3. SZ16 induces senescence in 4T1 cells.
Green staining indicated senescent cells.
A. Cells were treated with human serum albumin at 12 μM concentration;
B. Cells were treated with SZ16 at 12 μM concentration;
C. Cells were plated with only DMEM to serve as control;
D. Cells were exposed under 30 grays UV radiation as positive control (MEER cells)
Images were taken with inverted bright-field microscope at 100x total magnification.
Images are from same experiment.

Flow cytometry

Several assays were performed to elucidate the mechanisms by which SZ16 inhibits cell proliferation. Flow cytometry was used to examine the concentration of reactive oxygen species (ROS) in cells. Using DCF, flow cytometry showed that SZ16-treated cells contained lower levels of ROS than HSA-treated cells or DMEM media-treated cells (Fig. 4). SZ16's effects are dose-dependent, though interestingly this is accentuated because at increased dosages, the levels of ROS increase in HSA-treated cells.

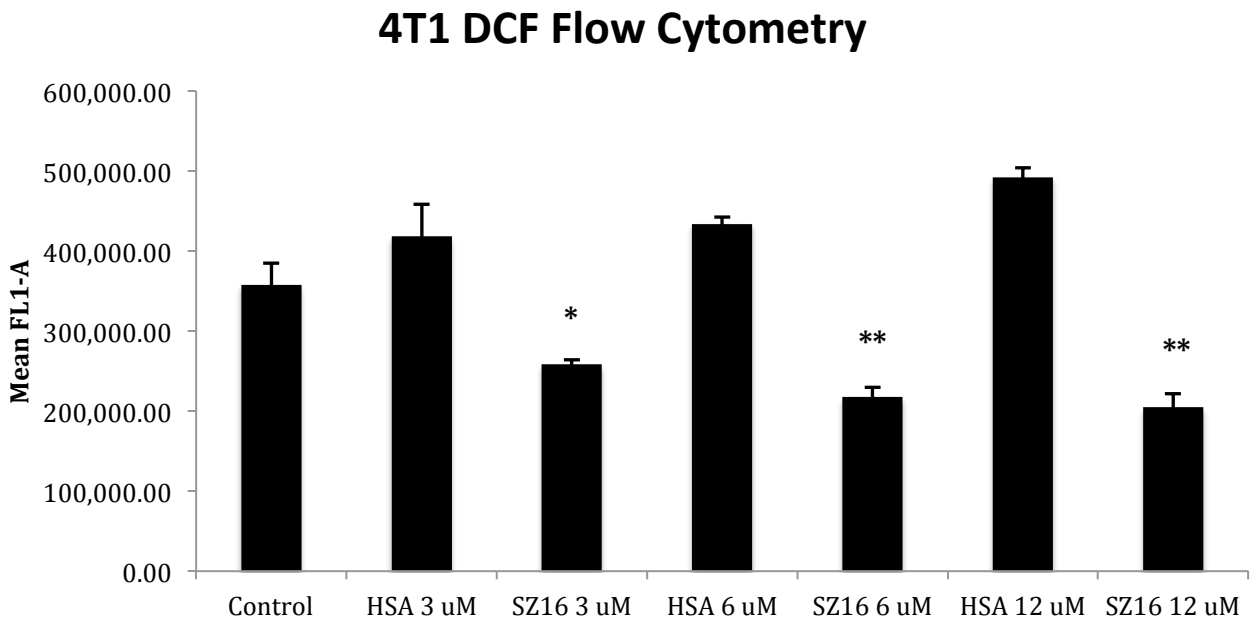


Figure 4. SZ16 decreases levels of reactive oxygen species in 4T1 cells. The levels of ROS were detected by DCF in control and drug-treated groups; n=3 for each group. Data is representative of three independent experiments; * = $p < 0.05$, ** = $p < 0.01$ vs. HSA

Western blotting

Western blotting showed SZ16's effects on the levels of different proteins possibly involved in the mechanisms behind SZ16's inhibition of cell proliferation. Cyclin D1 is a protein required for progression through the G1 phase of the cell cycle; additionally, cyclin D1 has been shown to be an indicator of the effects of reactive oxygen species on the cell cycle (Burch and Heintz, 2005). Treatment with SZ16 decreased the levels of cyclin D1 in 4T1 cells compared to treatment with HSA or DMEM media control (Fig. 5). As the flow cytometry data showed, SZ16 decreases the levels of ROS in 4T1 cells. Therefore, a correlation exists between the effects of SZ16 on ROS and cyclin D1. A decrease in cyclin D1 levels also indicates that cells are not proceeding through the cell cycle, which is corroborated by the cell viability assays.

Western blotting was also used to explore the effects of SZ16 on hypoxia. Superoxide dismutase, the main catalytic activity of SZ16, is known to decrease hypoxia and hypoxia-induced transcription factors such as HIF-1α (Liou and Storz, 2010). In turn, hypoxia is one of the characteristics of the tumor microenvironment, so decreasing hypoxia attacks one of the fundamental properties of the tumor. Western blots show that SZ16 treatment caused decreased levels of HIF-1α in 4T1 cells compared to HSA treatment or DMEM media treatment control (Fig. 6).

Quantifications used data from one Western blotting experiment for each primary antibody tested, so SEM could not be calculated and error bars were not formed.

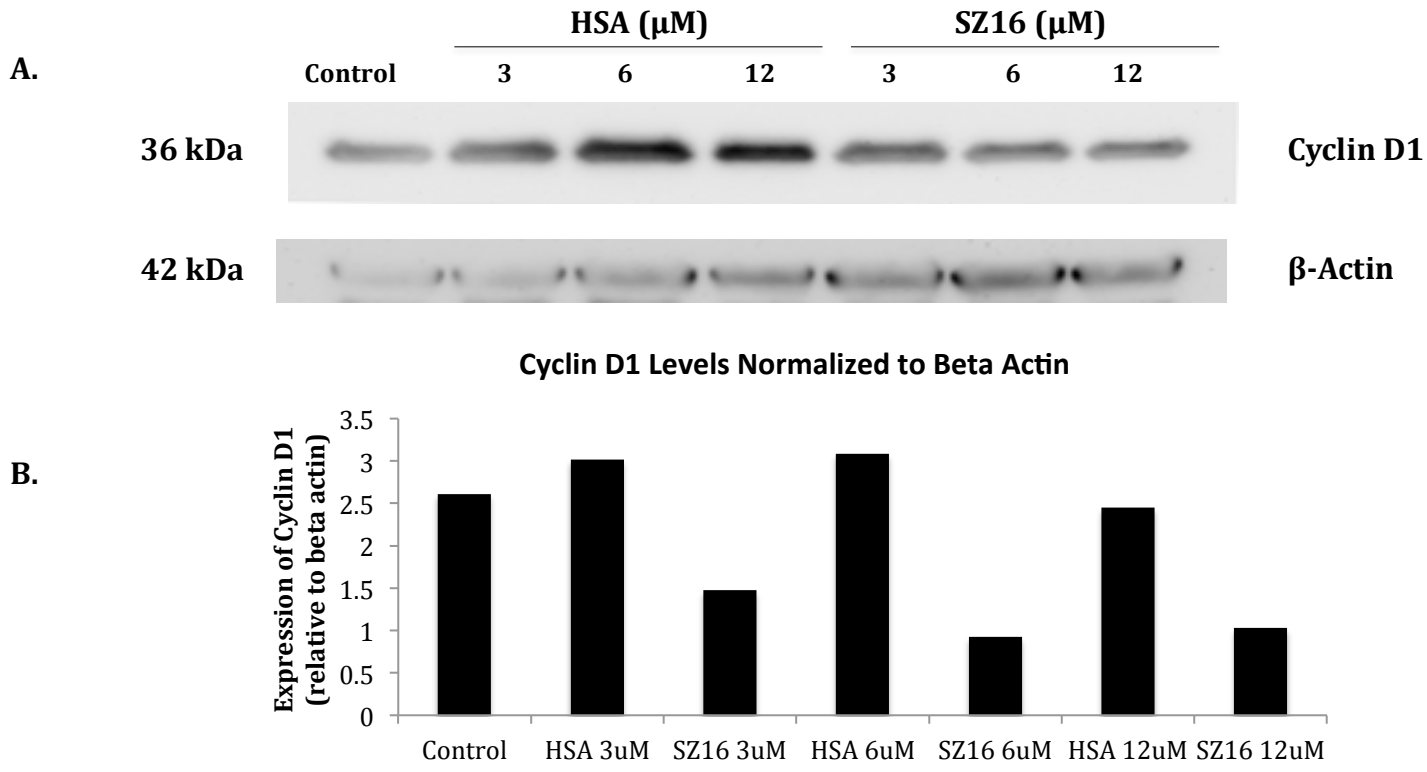


Figure 5 Cyclin D1 protein expression in control and drug-treated groups
A. Representative Western Blot images
B. Expression levels of Cyclin D1 relative to beta actin; The expression of Cyclin D1 decreased after SZ16

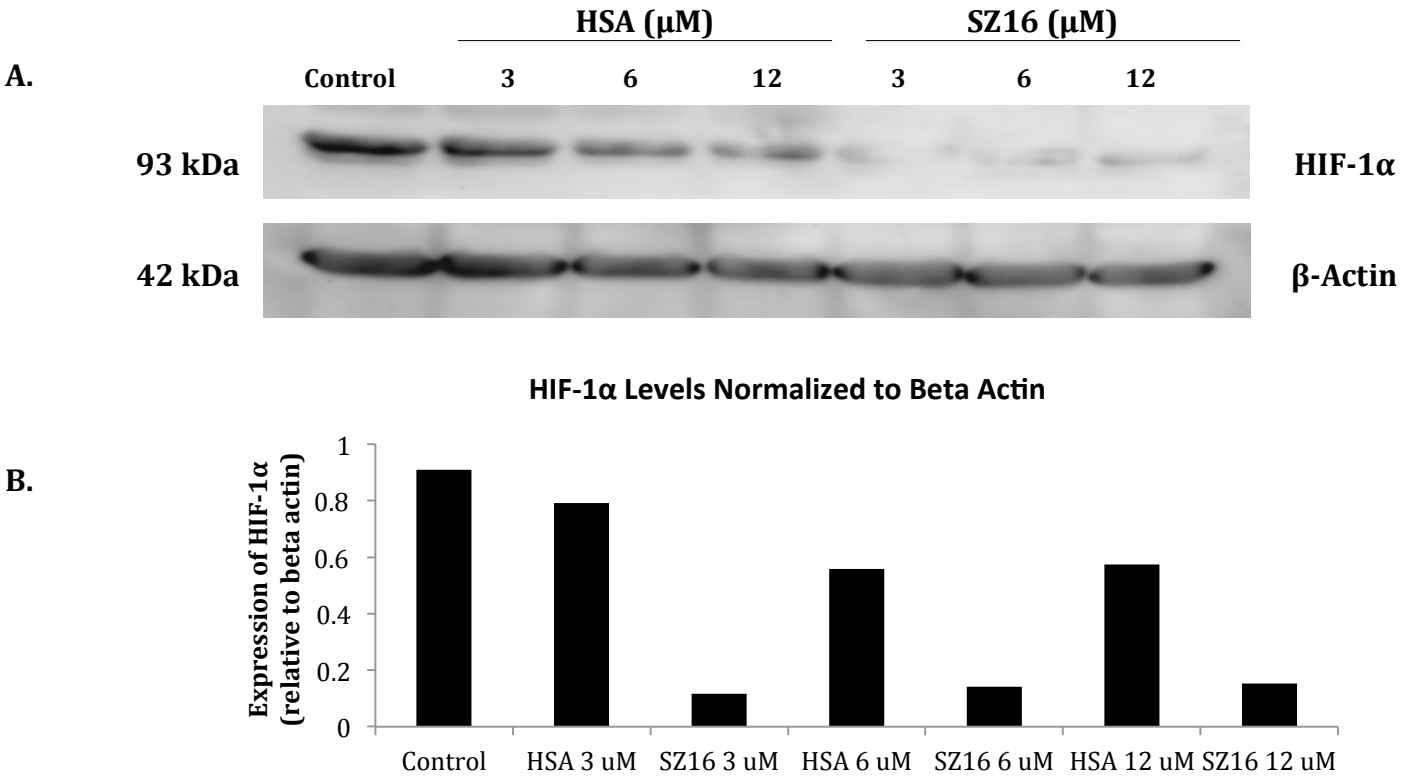


Figure 6 HIF-1α protein expression in control and drug-treated groups
A. Representative Western Blot images
B. Expression levels of HIF-1α relative to beta actin; The expression of HIF-1α decreased after SZ16 treatment.

Discussion

Recent publications have shown the effects of superoxide dismutase on cancer or human serum albumin on cancer. The effects of conjugation of albumin with superoxide dismutase on cancer were unknown. The present study explored the effects of the conjugation on breast cancer cells through SZ16, a human serum albumin-conjugated superoxide dismutase mimetic. *In vitro* experiments were performed to illustrate SZ16's effects on breast cancer cells. This study found that SZ16 inhibits 4T1 breast cancer cell proliferation in a dose-dependent manner. This was observed from cell colony formation to individual cell growth. However, rather than directly inducing cell death, SZ16 seems to be preventing cells from growing and dividing. A hallmark of cancer is unlimited proliferation, and SZ16 is nullifying this characteristic in breast cancer cells. The study explored the possibility that SZ16 may be inducing senescence in breast cancer cells rather than killing the cells. Senescence is a state of non-proliferation, which matches the effects of SZ16 shown in other assays. SZ16-treated cells appear to develop senescence at a higher rate than control cells. Thus, a rationale for why SZ16 inhibits cell proliferation is that the drug may be inducing senescence in these cells, causing the cells to exit the cell cycle.

The study also looked at the mechanisms behind the effects of SZ16 on breast cancer cells. Superoxide dismutase has been shown to manipulate the cell cycle by regulating a redox cycle within the cell cycle (Sarsour et al., 2014). Previous findings have shown that the levels of cell cycle proteins such as cyclin D1 decrease with increased levels of superoxide dismutase. This study confirms these findings as shown by the Western blotting for cyclin D1. With SZ16 treatment, the levels of cyclin D1 in 4T1 breast cancer cells decreased, which may in turn have decreased cell proliferation.

SZ16 may be affecting a redox cycle within the cell cycle, similar to SOD itself. SZ16 is decreasing the levels of reactive oxygen species in 4T1 breast cancer cells, as shown by flow cytometry experiments. Higher-than-normal levels of ROS are characteristic of cancer cells, possibly acting as second messengers in cancer cell signaling cascades (Storz 2005). These cell signaling cascades may be linked to progression through the cell cycle; thus, decreasing the levels of ROS will prevent cancer cells from utilizing ROS to maintain their oncogenic traits.

Additionally, the study touched upon the concept of hypoxia. With SZ16 treatment, breast cancer cells showed decreased levels of HIF-1α, a transcription factor induced by hypoxia. Cancer cells are also characterized by hypoxic conditions in the tumor microenvironment, which can lead to tumor proliferation and resistance to therapy. Lessening hypoxia in the tumor may be another component of the mechanisms by which SZ16 inhibits breast cancer cell proliferation.

Conclusions and Future Work

The two aims of this study were to examine novel drug SZ16's effects on breast cancer cell proliferation and to elucidate the mechanisms by which these effects occur. SZ16, a human serum albumin-conjugated superoxide dismutase mimetic, inhibits breast cancer cell proliferation in a dose-dependent manner. Rather than inducing cell death, SZ16 is attacking one of the fundamental hallmarks of cancer: unlimited proliferation. SZ16 may be causing breast cancer cells to enter a state of senescence and cease proliferation. The study also poses a possible model for how SZ16 may be inhibiting cancer cell growth and division. With SZ16 treatment, levels of cyclin D1 decrease in breast cancer cells. Cyclin D1 has been implicated in a redox cycle within the cell cycle, and SZ16's superoxide dismutase component may be affecting this redox cycle by decreasing the levels of reactive oxygen species in breast cancer cells. This may prevent cancer cells from progressing through the G1 phase into S phase of the cell cycle, thus effectively inhibiting proliferation. SZ16 has also been shown to manipulate hypoxia within the tumor microenvironment. By decreasing the levels of hypoxia in breast cancer cells, SZ16 is attacking another characteristic of cancer cells, which may further contribute to inhibiting proliferation.

Future research will seek to confirm SZ16's specific role in manipulating the cell cycle. Flow cytometry can be performed using fluorescent dyes such as propidium iodide to observe where SZ16-treated cells might exit the cell cycle, whether at the G1 phase as hypothesized or elsewhere (Pozarowski and Darzynkiewicz, 2004). Additional trials of the Western blotting of cyclin D1 and HIF-1α would confirm trends already observed. Western blotting of the other cyclin proteins as well as cell cycle inhibitors such as p27 may also help clarify the specific points at which SZ16 is affecting the cell cycle in breast cancer cells. Another important aspect

that will involve further experimentation involves the human serum albumin portion of SZ16. HSA may be allowing SZ16 a mechanism of entry into the tumor microenvironment, and SZ16 may be able to cause the effects summarized above because of its conjugation with HSA. Finally, it might be instructive to test the effects of SZ16 on other cell lines and possibly other types of cancer to learn if SZ16's inhibitory effects are unique to breast cancer cells alone or universal to inhibiting all cancer cell growth.

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