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Characterizing a Putative Membrane Androgen Receptor in Glioma Cell Models

Emma Yuen

LOYOLA UNIVERSITY CHICAGO

CHARACTERIZING A PUTATIVE MEMBRANE ANDROGEN RECEPTOR IN GLIOMA CELL MODELS

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EMMA R. YUEN

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ABSTRACT

The goal of this current research was to explore the role of a putative membrane AR in modulating the viability of various models of glioma/astrocytoma, relative to normal (non-tumor) astrocytes. Our lab previously found that ligand binding to a putative membrane AR increases cell vulnerability to the metabolic/oxidative stressor, Iodoacetic acid (IAA), and suggested that the same may be true for the chemotherapeutic, temozolomide (TMZ). Given that the cellular damage elicited by these two compounds is through two distinct mechanisms, we proposed to more carefully characterize the "damage-promoting" consequence of ligand (Testosterone-BSA, T-BSA) binding to the membrane AR and determine if: 1) the consequence of T-BSA is mechanism (of cytotoxicity)-specific, and 2) if T-BSA exerts similar effects in primary ("healthy" or "normal") astrocytes relative to glial tumor cells (astrocytomas). Our data revealed that the effectiveness of T-BSA (and thus, engaging a putative membrane AR) is context specific, with the most robust effect (of sensitizing the cells to either IAA or TMZ) noted in C6 glioma cells. Moreover, our data suggest that T-BSA may have minimal impact on enhancing the effects of TMZ in "normal" astrocytes, suggesting the possibility that exploiting the membrane AR could enhance the demise of the tumor cells while leaving "normal" (and perhaps, healthy) astrocytes unscathed. While our data certainly support the need for additional studies, with complementary tools to interrogate the role of the putative membrane AR, the data also lay the foundation for the development of novel therapeutics targeting the membrane androgen receptor, the impact of which would be to facilitate the development of new

therapeutics for such devastating glial tumors as glioblastoma multiforme, for which the prognosis is very poor.

CHAPTER ONE

INTRODUCTION

In mammalian systems, androgens are classified as one of the six families of steroid hormones [11]. Testosterone, a principal androgen, is highly conserved among vertebrates as it plays an important role in evolution and development [12]. While testosterone is typically associated with its role in reproduction, androgens also affect a wide variety of tissues including skin, bone, muscle, and brain [1, 28]. Within these tissues, testosterone and its metabolite 5α-dihydrotestosterone (DHT) exert their effects primarily through binding with the androgen receptor (AR) [1,2]. The AR belongs to the nuclear hormone receptor super-family and characteristic of this receptor super-family, binding of androgens initiates a conformational change that allows the AR to be translocated into the nucleus to interact with DNA as a transcription factor [1,3,5]. This model of androgen action presumes the steroid is capable of freely crossing the plasma membrane to enter the cytoplasm where it can bind to and activate the AR [4,5]. This mechanism of intracellular AR activity is referred to as the "classical" or genomic model.

In addition to the classical AR, a growing body of evidence suggests that androgens are capable of eliciting non-genomic actions as well, likely through a receptor alternative to the AR. Distinct membrane receptors have been identified for other steroid hormones, which suggests a novel membrane androgen receptor may also exist [4]. The way by which androgens, through mechanisms that include potentially novel, putative membrane-associated receptors, are thought to elicit non-genomic actions is through activation of second messenger pathways, including the

extracellular signal regulated kinase 1/2 (ERK1/2) and PI3K-Akt pathways [13]. The ERK1/2 cascade is an integral signaling pathways that regulates a variety of cellular processes, including differentiation, proliferation, and survival, as well as apoptosis and stress response [17,18, 33, 35]. The PI3-K/Akt pathway is another integral signaling pathway which mediates several cellular functions such as angiogenesis, metabolism, growth and proliferation, survival, protein synthesis, and apoptosis [19, 36, 37].

Although androgens have previously been shown to play a role in neuroprotection, previous research from our laboratory suggests that ligand binding to a putative membrane androgen receptor may induce damaging effects in certain cell types [20,21,22]. Joshua Gatson et al. (2005) found evidence that androgens, particularly DHT, are capable of regulating ERK and PI3-K/Akt pathways through a novel membrane androgen receptor in rat glioma cells [21]. One aspect of these experiments aimed to address whether the effect of DHT was being mediated through the classical AR, or if a membrane AR may be involved [21]. It was shown that when cells were treated with DHT, there was a robust increase in the expression of both phosphorylated ERK and phosphorylated Akt; it was also shown that the effect of DHT on ERK phosphorylation was blocked by the classical AR antagonist flutamide, which may suggest the classical AR is mediating this change in ERK phosphorylation [21]. Notably, these experiments also showed that treatment with a membrane impermeable androgen conjugate, DHT-conjugated to bovine serum albumin (DHT-BSA), led to a substantial suppression of ERK and Akt phosphorylation. In contrast to treatment with DHT, the effect of DHT-BSA was insensitive to flutamide, suggesting a novel receptor may be involved [21]. In later work also done by Joshua Gatson (2007), this research was extended to primary cortical astrocytes to assess the effects of DHT-BSA on the cell's sensitivity to a metabolic/oxidative insult [20]. In these experiments, a

metabolic/oxidative insult was elicited by treatment with Iodoacetic Acid (IAA). IAA acts by inhibiting glyceraldehyde-3-phosphate dehydrogenase, a key glycolytic enzyme, resulting in a substantial decrease in cellular ATP and leading to cell death [20,26]. It was shown that combined treatment with DHT-BSA greatly enhanced the effect of IAA alone on induced cell death [20]. These experiments also showed that combined treatment with DHT-BSA and IAA greatly exacerbated the decline in levels of phosphorylated Akt, as well as decreasing overall expression of Akt [20].

As previously mentioned, the ERK and PI3-K/Akt pathways are tightly regulated and critical for cell maintenance/viability. Due to their importance, dysfunction of these signaling cascades can lead to various pathologies including neurodegenerative diseases and cancers [17, 18, 23, 29, 30, 31, 34]. In fact, it has been shown that activating mutations in the ERK cascade occurs in most cancer types and mutations in this pathway are a major oncogenic factor across all cancer types [17,18, 32]. Additionally, it has been shown that overexpression of Akt has an anti-apoptotic effect in many cell types, which can contribute to cancer pathology and poor prognosis, in part, due to a resulting increase in the resistance of the cancer to anti-tumor agents [23, 38, 39]. Conversely, inducing inhibition of PI3-K, thus reducing Akt activity, can block cellular proliferation while inhibition of ERK signaling pathways can restore tumor cells to a non-transformed state [23, 8]. With evidence from our lab suggesting that decreased expression of phosphorylated ERK and Akt can be elicited by application of the putative membrane-associated androgen receptor, DHT-BSA, we hypothesized that this mechanism could be exploited in the treatment of certain cancers.

To further explore the idea that cell vulnerability (specifically of cancer cells) can be influenced by ligand binding to a putative membrane AR, we extended our research into human

glioblastoma cells to assess whether treatment with membrane impermeable androgens enhances cell vulnerability in response to a chemotherapeutic. These studies utilized two human glioblastoma cell lines, A172 and T98G, to determine how treatment with a membrane impermeable androgen, testosterone-BSA (T-BSA), might affect cell viability in the presence of the chemotherapeutic Temozolomide (TMZ) [22].

TMZ is an alkylating drug that acts through methylation of the O^6 position of guanine, resulting in DNA lesions which are considered to be responsible for the cytotoxic effect. The presence of the DNA repair enzyme, O^6-methylguanine DNA methyltransferase (MGMT), can cause resistance to alkylating agents, such as TMZ, by removing the methyl group from the O^6 position of guanine [24, 42]. Our lab was interested in using both A172 and T98G glioblastoma cell lines due to their difference in responsiveness to TMZ, likely caused by their difference in expression of MGMT. A172 cells are considered TMZ-sensitive and express low MGMT activity, whereas T98G cells are considered TMZ-insensitive and express high MGMT activity [24, 25, 47]. Our lab showed that while treatment with TMZ alone showed a decrease in A172 cell viability, this effect was significantly enhanced with the administration of T-BSA; T98G cells, which were unresponsive to TMZ treatment alone, also showed a decrease in cell viability with the administration of T-BSA [22]. Further experiments showed treatment with T-BSA significantly decreased mRNA levels of MGMT in T98G cells, compared to T98G cells not treated with T-BSA [22]. Additionally, it was shown that inhibition of Akt resulted in decreased levels of MGMT protein [22]. Collectively, these results suggest that ligand binding to the putative membrane AR can influence cell responsiveness to cytotoxic insult, possibly through changes in MGMT expression mediated by the Akt pathway [22].

While the classical AR has been implicated in the protective effects of androgens, binding to the membrane AR, in contrast, appears to favor cell death; this may suggest that the balance between the classical AR and the membrane AR modulates the effect of androgens on cell function/viability. Given that binding to the membrane AR was associated with a decrease in both ERK1/2 and Akt phosphorylation, this suggests as a mechanism by which cell sensitivity to cytotoxic insult can be manipulated through exploitation of the "death-promoting" membrane AR. This proposes the idea that the putative membrane AR could potentially serve as a novel therapeutic target for treatment of glial tumors, specifically glioblastoma, by sensitizing cells to TMZ.

Gliomas are the most common primary brain tumors in adults, accounting for more than 70% of all primary central nervous system tumors [6]. Glioblastomas, the most frequent and most malignant subtype of glioma, account for 45.6% of primary malignant brain tumors [6,7]. Defining histopathologic features of glioblastoma include necrosis and microvascular proliferation and like other cancers, glioblastoma exhibits characteristic malignant phenotypes such as self-sustained proliferation, evasion of external growth control, and tissue invasion [7, 8, 40, 43]. Despite glioblastomas being highly invasive, capable of invading the surrounding brain parenchyma, they are typically confined within the central nervous system and do not metastasize [10].

While the frequency of glioblastoma is low (3 per 100,000) compared to cancers originating from other organs, glioblastoma is a highly aggressive cancer associated with significant morbidity and mortality [7,8]. The incidence rate for glioblastoma varies by both sex and age [16]. Compared to females, glioblastoma is 1.58 times more common in males [16]. The median age at diagnosis is 65 years and rates are highest in the age group of 75-84 years [16].

Glioblastoma often develop rapidly in elderly patients after short clinical history; the average time of progression from low-grade glioma to glioblastoma is 5.3 years [6, 9]. Patient survival is inversely associated with age; 5% of patients diagnosed with glioblastoma are alive after 5 years, but this measure decreases to 2% among patients aged 65 years and older [7].

Although glioblastoma prognosis is invariably fatal, standard treatment options, including surgical resection, radiation, and chemotherapy, may stabilize or improve quality of life and cognitive function for a period of time [14,10,15,16]. A glioblastoma diagnosis is initially achieved by neuroimaging followed by resection or biopsy of tumor tissue to establish a definitive diagnosis, and determine tumor grade and characteristics [15,7,10]. While maximal surgical resection aims to relieve mass effect, both resection and biopsy can present risks such as brain swelling, or disruption of neurological function [15]. Additionally, tumors may be deemed inoperable depending on their location in the brain [15,7]. After resection or biopsy, factors such as patient's age, performance status, and genotype should all be considered when discussing course of treatment [16]. If the patient is deemed fit, it is advised that patients undergo systemic therapies since infiltrating cells beyond the site of resection can further drive tumor progression, even when gross total resection appears to be confirmed by neuroimaging [7]. The standard approach for systemic therapy in treating glioblastoma is concurrent radiation and chemotherapy, followed by adjuvant chemotherapy [9, 14, 16, 41]. Radiotherapy is an important modality of treatment to improve both local control and survival, and radiation treatment with concurrent and adjuvant TMZ treatment is the standard of care treatment for glioblastoma [14,16]. TMZ is the standard first-line chemotherapeutic used to treat glioblastoma [15,16]. As previously mentioned, TMZ acts by inducing DNA damage to prevent tumor proliferation [24,14]. TMZ is administered orally with standard dosing at 75mg/m^2 with concurrent radiotherapy and 150-200mg/m^2 on days

1-5 every 28 days during six cycles of adjuvant treatment [7,16]. Due to its small molecular size and lipophilicity, TMZ is capable of penetrating the blood-brain barrier to enter the central nervous system (CNS) [14, 27, 46]. However, despite being able to enter the CNS, the concentration of TMZ is relatively low in brain tumor tissue – about 20% of plasma levels – which requires that the cancers being treated are very sensitive to TMZ [27, 46]. Additionally, there is no survival benefit to dose-intensified TMZ regimens, as higher doses are associated with higher toxicity and reduced quality of life [16, 7, 44] Treatment with TMZ can lead to significant side effects such as myelosuppression, especially thrombocytopenia and neutropenia, as well as lymphopenia which is long-lasting and is associated with reduced survival and poor response to immunologic interventions [16,27]. Unfortunately, first-line treatments often do no prevent recurrent glioblastoma and most patients experience disease progression after a median 7 to 10-month progression-free survival period [10, 45]. Treatment options for recurrent glioblastoma include further surgical resection, reirradiation, systemic therapies, and combined approaches; however, there is no established standard of care for recurrent glioblastoma and there is little evidence that current interventions prolong overall survival [16]. Although current treatment options do offer some benefit to prolonged patient survival, further discoveries of strategies which enhance the efficacy of these regimens are sorely needed. To this end, we were excited at the opportunity to better understand whether the previously characterized, cell death-promoting androgen receptor mechanism, could serve as a valuable adjuvant to the existing chemotherapy regimen.

The goal of this current research was to further explore a novel mechanism by which a putative membrane AR modulates cell function/viability. As noted above, our lab previously found that ligand binding to a putative membrane AR increases cell vulnerability to two different

cytotoxic agents, IAA and TMZ. Given that the cellular damage elicited by these two compounds is through two distinct mechanisms, ***one aim of this research was to determine if the "damage-promoting" effect of ligand binding to the membrane AR is mechanism (of cytotoxicity)-specific***. If the primary mechanism of the membrane AR is to reduce intracellular anti-oxidant defenses, or render the mitochondria more vulnerable, it is likely that ligand binding will have a significant effect on IAA-induced cytotoxicity. Alternatively, if the major mechanism by which the membrane AR elicits its effects is by reducing DNA repair capacity, then there may be more robust consequences in the presence of TMZ.

We, of course, accepted the possibility that ligand binding to the membrane AR is equally effective at enhancing cell death consequent to either IAA or TMZ. Given that engaging the membrane AR has been shown to reduce ERK and Akt signaling, two very broadly-impacting cell survival pathways, ligand binding may weaken cell viability signaling in such a "general" way to render the cell vulnerable to <u>any</u> cytotoxic insult. Understanding the mechanism by which the putative membrane AR facilitates changes in cell viability will help to further characterize the function of this novel receptor, and its potential utility in targeting it to treat different diseases.

Another aim of this research was to determine if engaging the membrane AR enhances sensitivity to cytotoxic insults in "normal", healthy primary astrocytes, or whether the death-promoting effects are specific to tumor cells. If the effect of engaging the membrane AR is limited to tumor cells, this would be very exciting since it would suggest the opportunity to selectively target cancer cells, while leaving healthy astrocytes around the tumor unscathed. This would be of great value since most chemotherapeutic regimens promote cell death in both the target cell (i.e., the cancer cell) as well as other, non-cancer cells.

As previously discussed, the current regimen for chemotherapy with TMZ often results in prolonged systemic exposure and harsh side effects, despite relatively low concentrations of TMZ in tumor tissue. Although chemotherapy with TMZ can significantly prolong survival, the patient's quality of life may deteriorate with treatment (partly due to "off-target" effects of TMZ), and therefore the benefit of treatment is modest. Ideally, improvements to current TMZ treatment would aim to reduce systemic toxicity, theoretically reducing side effects, and hopefully improving patient quality of life. Should the current research provide evidence that glioblastoma cell sensitivity to TMZ is enhanced through engaging the membrane AR, this could provide a mechanism through which lower doses of TMZ become effective. Therefore, this current research provides an opportunity to explore a novel mechanism by which the current standard of glioblastoma treatment can be improved.

CHAPTER TWO

METHODOLOGIES

Cell Culture.

Rat glioma cells (C6; ATCC, Manassas, VA) are a glial cell strain cloned from a rat glial tumor induced by N-nitrosomethylurea by Benda et al. [54, 55]. For the current research C6 cells were grown and maintained in DMEM media without sodium pyruvate (Gibco Laboratories, Montgomery County, MD) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. TMZ-sensitive human glioblastoma cells, derived from glioblastoma in a 53-year-old male (A172; ATCC) [56, 57], were grown and maintained in DMEM media without sodium pyruvate (Gibco) supplemented with 10% FBS and 1% Penicillin-Streptomycin. TMZ-insensitive human glioblastoma cells, derived from glioblastoma multiforme in a 61-year-old male (T98G; ATCC) [58], were grown and maintained in EMEM (ATCC) supplemented with 10% FBS and 1% Penicillin-Streptomycin. Primary astrocytes were obtained from cortical tissue of 2-day-old mouse pups (BrainBits LLC, Springfield, IL) [59]. For the current research primary astrocytes were grown and maintained in DMEM with sodium pyruvate (Gibco) supplemented with 10% FBS and 1% Penicillin-Streptomycin. All cell types were maintained at 37ºC in a humidified incubator containing 5% CO_2.

24 hours before experiments, cell culture media was changed to DMEM media without sodium pyruvate (C6; A172), EMEM media (T98G), or DMEM media with sodium pyruvate (primary astrocytes) supplemented with 10% charcoal-stripped FBS (CS-FBS) and 1%

Penicillin-Streptomycin. Charcoal-stripped FBS was used to avoid the potential confound of endogenous steroid hormones that may be present in non-charcoal stripped FBS. For experiments, cells were plated on black-wall, clear bottom 96-well plates one day prior to treatment at a concentration of 10,000 cells per well.

Treatment of Cells with IAA.

To evaluate the cytotoxic effects of Iodoacetic Acid (IAA; Sigma-Aldrich, St. Louis, MO) cells were treated with either vehicle control (CS-FBS media) or IAA [1uM, 3uM, 10uM, 30uM, 100uM] for 6 hours to assess dose response and generate an EC_{50} value for IAA.

Treatment of Cells with IAA and T-BSA.

Once an EC_{50} value was obtained for IAA, cells were treated with either vehicle control (CSFBS media), or co-application of IAA [EC_{50} value, which was ~10uM] and Testosterone (T)-3-(O-carboxy-methyl)oxime: BSA (T-BSA; Steraloids Inc., Newport RI) [0.5uM, 1.0uM, 10uM] for 6 hours to assess the effects of cytotoxic insult and androgens on cell viability. These concentrations of T-BSA were based on prior work conducted in the Singh lab, and derived from considerations of both noted efficacy and solubility constraints of the compound. Additional control groups included IAA alone [10uM], T-BSA [0.5uM, 1.0uM, 10uM] alone, and Bovine Serum Albumin (BSA; Sigma-Aldrich, St. Louis, MO) [10uM] (control for T-BSA). 100uM IAA was included as a positive control, noting that this concentration of IAA kills approximately 100% of the cells.

Treatment of Cells with TMZ.

To evaluate the cytotoxic effects of the chemotherapeutic Temozolomide (TMZ; Sigma-Aldrich, St. Louis, MO), cells were treated with either vehicle control (CS-FBS media) or TMZ [0.1mM, 0.25mM, 0.35mM, 0.45mM, 0.6mM, 0.75mM, 1mM, 5mM] for 48 hours to assess

concentration-response curve and generate EC_{30} and EC_{50} values for TMZ. Our lab has shown a relative EC_{50} for TMZ in TMZ-sensitive human glioblastoma at approximately 500uM; notably, 500uM TMZ has relevant equivalence to the dose administered clinically to patients at 200mg/m^2 [22]. We chose to look at the EC_{30} concentration is to assess whether lower doses of TMZ have increased efficacy under conditions where a presumed ligand for the membrane AR (i.e. T-BSA) is also present.

Co-Treatment of Cells of TMZ and T-BSA.

Based on the obtained EC_{30} and EC_{50} values for TMZ, cells were treated with either vehicle control (CSFBS media), or the co-application of TMZ (Sigma-Aldrich) at EC_{30} and EC_{50} concentrations [0.2mM; 0.5mM] and T-BSA (Steraloids Inc.) [0.1uM, 1.0uM, 10uM] for 48 hours to assess the effects of cytotoxic insult and androgens on cell viability. Additional control groups included TMZ [0.2mM, 0.5mM] alone, T-BSA [0.1uM, 1.0uM, 10uM] alone, and BSA alone (Sigma-Aldrich) [10uM] (control for T-BSA). 5mM TMZ was also included as a positive control, noting that this concentration of TMZ kills approximately 100% of the cells.

Treatment of Cells with TMZ and T-BSA Pre-Treatment.

To assess the effect of "priming" the cells by binding to the putative membrane-associated androgen receptor (mAR), cells were initially treated with T-BSA [0.1uM, 1.0uM, 10uM] for 24 hours, followed by application of TMZ [0.2mM, 0.5mM] for an additional 24 hours. Control groups included TMZ alone [0.2mM, 0.5mM], T-BSA alone [0.1uM, 1.0uM, 10uM], BSA alone [10uM] (control for T-BSA), and 5mM TMZ (as the positive "kill" control).

Cell Viability Assay.

After treatment with the above-referenced compounds for the specified times, cell viability was assessed by using the Live/Dead Viability/Cytotoxicity Kit from Invitrogen

(Invitrogen, Carlsbad, CA) which includes two components, calceinAM and EthD-1; the assay was conducted according to the manufacturer's instructions. Following the specified cell treatments, for the specified duration, media was removed, and cells were washed with 250uL of PBS. To create the working solutions for the cell viability assay, 20uL of the supplied 2mM EthD-1 stock solution was added to 10mL PBS and mixed thoroughly, giving an approximately 4uM EthD-1 working solution. 5uL of the supplied 4mM calceinAM stock solution was then added to the 10mL EthD-1 solution and mixed thoroughly, yielding an approximately 2uM calcein AM and 4uM EthD-1 working solution. 100uL of fresh PBS was then added to each well, followed by the application of 100uL of the calceinAM and EthD-1 working solution to each well, yielding a final volume of 200uL per well, containing a final concentration of 1uM calceinAM and 2uM EthD-1. The plate was then incubated at 37ºC in a humidified incubator containing 5% CO_2 for 30 minutes. Fluorescence was measured at an excitation wavelength of 485nm and emission wavelength of and 530nm for calceinAM, and 530nm excitation and 645nm emission wavelengths for EthD-1.

Statistical Analysis.

Raw fluorescence values were first normalized to the control/baseline levels, yielding data represented as "percent of control". The data from at least three independent experiments were then analyzed (each experiment representing an "n" of 1) using an analysis of variance (ANOVA), followed by a Tukey's *post hoc* analysis to assess group differences. The data were presented in a bar graph depicting the mean ± S.E.M (GraphPad Software, San Diego, CA). Statistical significance was inferred if the probability of a Type I error ("false positive", α, was less than 0.05 (i.e., $p < 0.05$). Each experiment was conducted a minimum of three separate times.

CHAPTER THREE

RESULTS

C6 Cells are Responsive to Iodoacetic Acid (IAA).

As an initial step to establish the experimental model, we determined the concentration of IAA required to elicit approximately 50% cell death. Using the calcein AM assay as an indicator of cell viability, a concentration-response curve was generated for IAA, following 6 hr treatment, in C6 cells (Fig. 1). This duration of treatment was based on prior work conducted and published by the Singh lab [20, 21]. The data revealed an effective concentration (EC_{50}) of approximately 10uM, which was used in subsequent experiments.

T-BSA Enhances the Cytotoxic Effect of IAA in C6 Cells.

In C6 cells, treatment with 10uM IAA alone for 6 hours significantly reduced cell viability (to 59.5% viable cells) compared to the vehicle control (consisting of 10% charcoal-stripped fetal bovine serum (CSFBS) containing media), whose viability was set at 100%) ($p<0.0001$) (Fig. 2). The application of 10uM T-BSA along with 10uM IAA resulted in a further decrease in cell viability (down to 15.8%) relative to cells treated with 10uM IAA alone ($p<0.0001$). Co-treatment of 0.5uM T-BSA or 1uM T-BSA with 10uM IAA, however, did not show significant change in cell viability compared to cells treated with 10uM IAA alone, suggesting that the effect was concentration dependent. Treatment with T-BSA alone at any of the concentrations tested [0.5uM, 1uM, or 10uM] did not have any significant effect on cell viability compared to the vehicle control. Neither treatment with 10uM BSA alone, nor co-

treatment of 10uM BSA with 10uM IAA had any significant effect on cell viability compared to vehicle control.

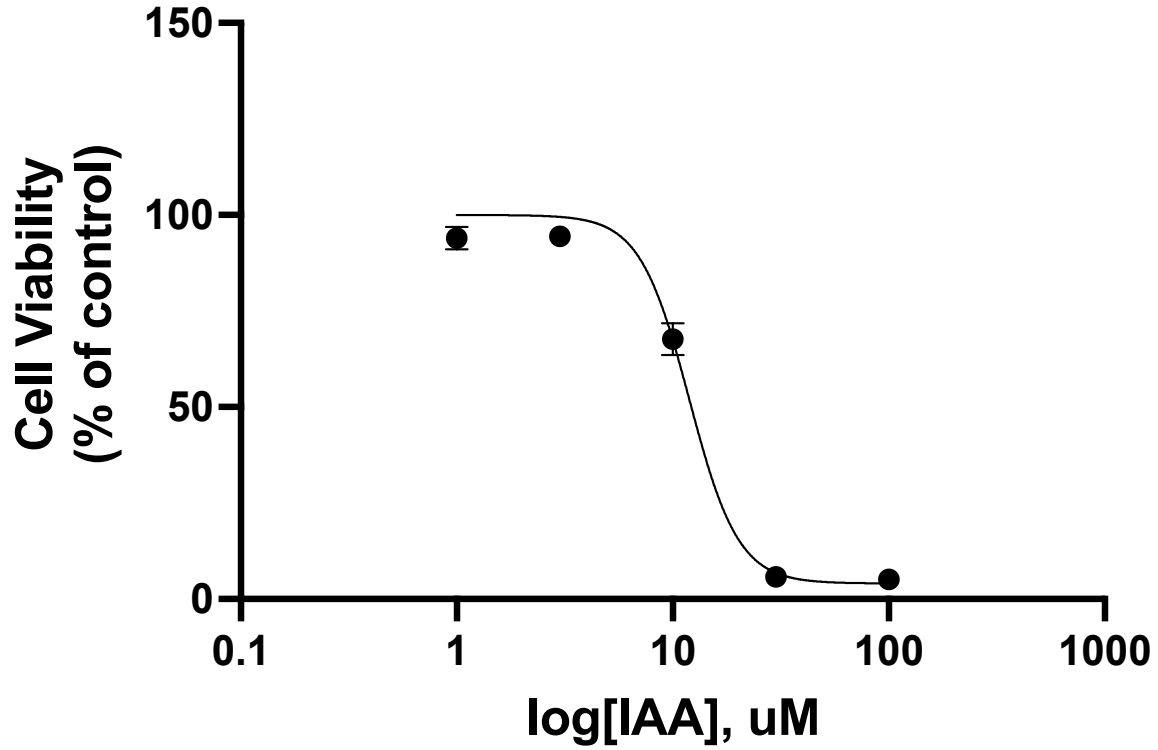

Figure 1. Concentration-Response for IAA-Induced Cytotoxicity in C6 Cells. C6 cells were plated at 10,000 cells/well 24 hrs prior to treatment with IAA (6hr). C6 cells showed a concentration-dependent response to IAA where the half maximal effective concentration (EC_{50}) to elicit cytotoxicity was 11.91uM. Cell viability was assessed using a calceinAM assay (n=3).

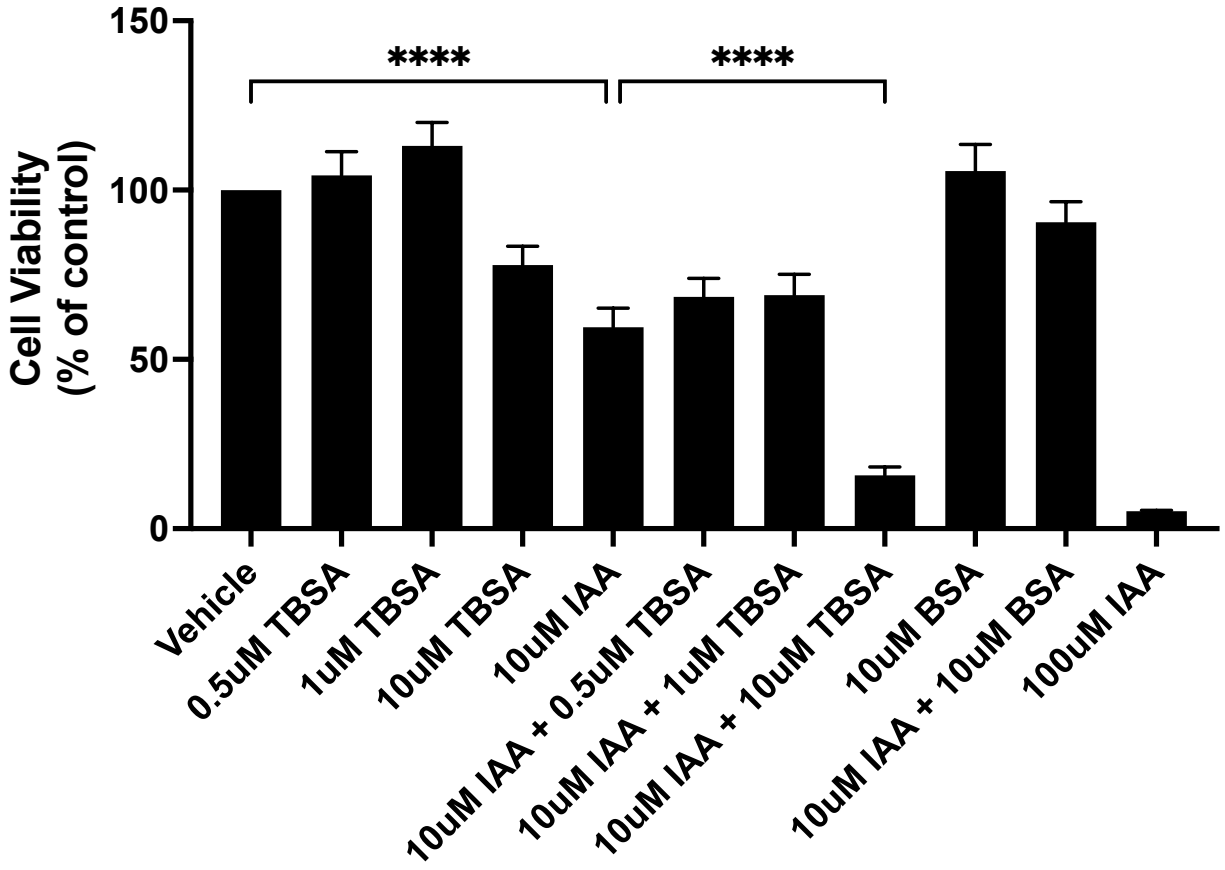

Figure 2. T-BSA Enhances the Cytotoxic Effect of IAA in C6 Cells. C6 cells were plated at 10,000 cells/well 24 hrs prior to treatment. Cells were co-treated with IAA and T-BSA for 6hrs. Treatment with 10uM IAA alone significantly reduced cell viability compared to the vehicle control (10% CSFBS media) after 6 hrs ($p<0.0001$). Co-treatment of 10uM T-BSA with 10uM IAA showed significantly greater reduction in cell viability compared to treatment with IAA alone ($p<0.0001$). In contrast, co-treatment of IAA with the lower concentrations of 0.5uM T-BSA and 1uM T-BSA did not enhance IAA-induced cell death. Treatment with T-BSA alone did not have any significant effect on cell viability. 100 uM IAA was used as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application. Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control which is set at 100% (n=4). ****: $p< 0.0001$

C6 Cells are Responsive to Temozolomide (TMZ).

As conducted with IAA, prior to determining whether T-BSA influenced the cell death-promoting effects of the chemotherapeutic, TMZ, we determined the concentration of TMZ required to elicit approximately 50% cell death in C6 cells. As in all our experiments, the calcein AM assay was used to gauge cell viability. The concentration-response curve generated for TMZ in C6 cells following 48 hr treatment (a time point used previously by the Singh lab) revealed a half maximal effective concentration (EC_{50}) of approximately 900uM (Fig. 3) and was used in subsequent experiments.

T-BSA Enhances the Cytotoxic Effect of IAA in C6 Cells.

In C6 cells, treatment with 900uM TMZ alone significantly reduced cell viability (to 67%) compared to the vehicle control (comprised of 10% CSFBS media) (100%) after 48 hours ($p<0.0001$) (Fig. 4). Co-treatment of 10uM T-BSA with 900uM TMZ enhanced the cytotoxic effects of TMZ alone to 45% viable cells ($p<0.0001$). Neither co-treatment of 0.1uM or 1uM T-BSA with 900uM TMZ significantly changed cell viability compared to cells treated with 900uM TMZ alone. Treatment with 10uM T-BSA alone significantly decreased cell viability (75%) compared to vehicle control, although this effect appeared to be inconsistent within C6 cells. Treatment with 0.1uM or 1uM T-BSA alone did not have any significant effect on cell viability. While in these particular studies, 10uM BSA alone showed a modest, but statistically significant, decrease in cell viability (to 89.2% of control) compared to the vehicle control; 10uM BSA did not alter the effect of TMZ.

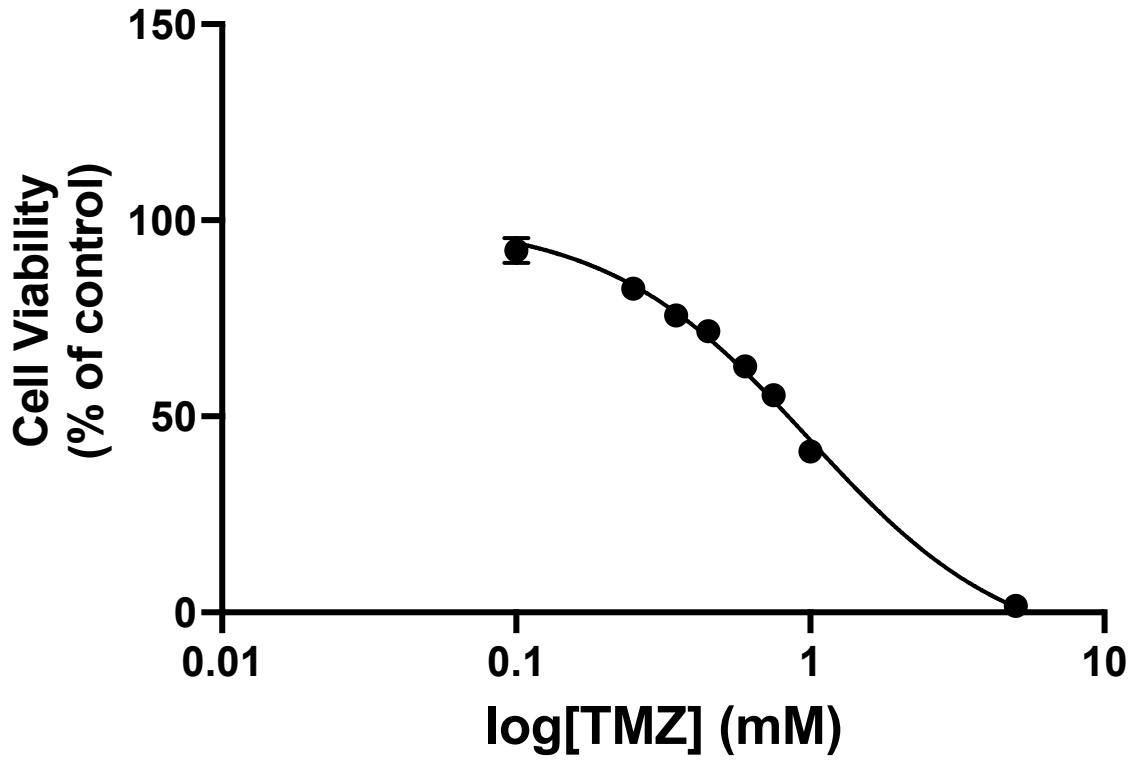

Figure 3. Concentration-Response for TMZ-Induced Cytotoxicity in C6 Cells. C6 cells were plated at 10,000 cells/well one day prior to treatment with TMZ (48hr). C6 cells showed a concentration-dependent response to TMZ where the half maximal effective concentration (EC_{50}) required to elicit cell death was approximately 0.988mM. Cell viability was assessed using a calceinAM assay (n=3).

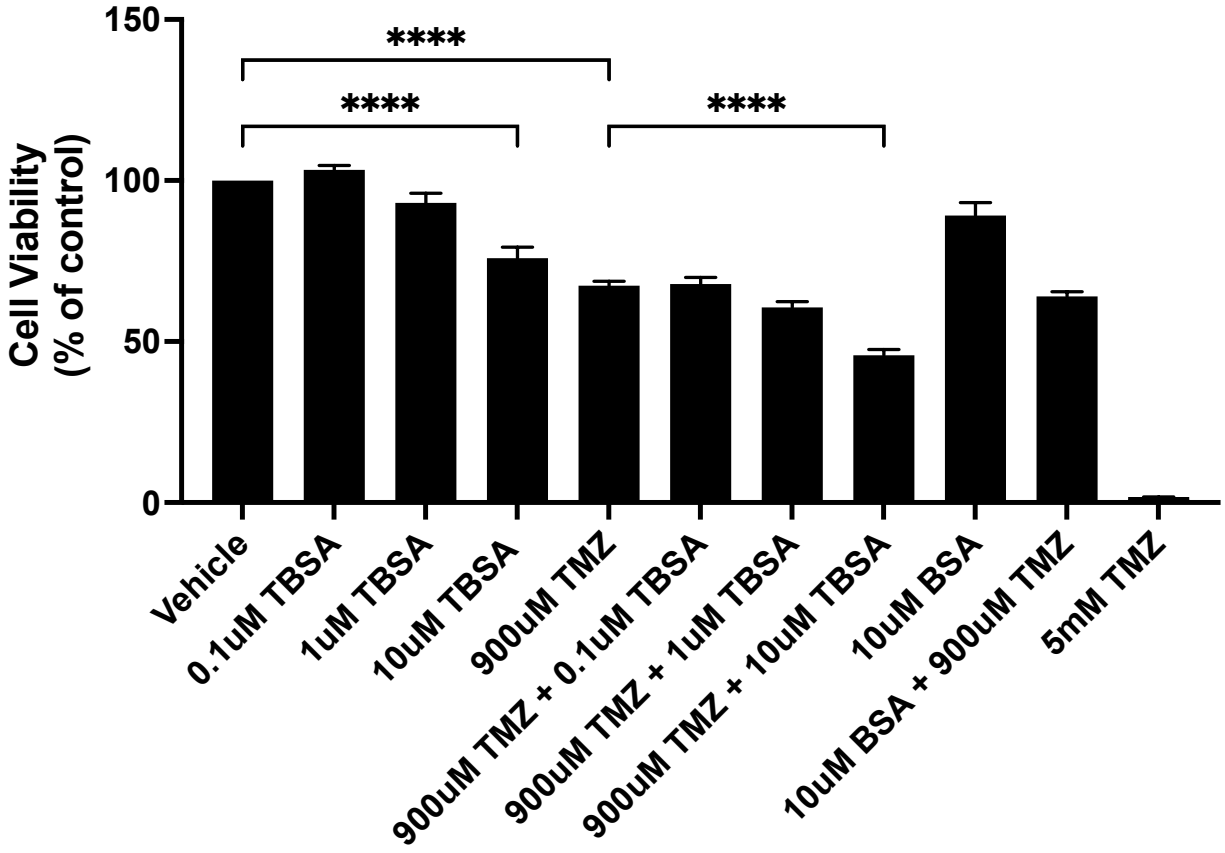

Figure 4. T-BSA Enhances the Cytotoxic Effects of TMZ in C6 Cells. C6 cells were plated at 10,000 cells/well one day prior to treatment. Cells were co-treated with TMZ and T-BSA for 48hrs. Treatment with 900uM TMZ alone significantly reduced cell viability compared to the vehicle control (10% CSFBS media) after 48 hrs ($p<0.001$). Co-treatment of 10uM T-BSA with 900uM TMZ showed significant enhancement of decreased cell viability compared to treatment with TMZ alone ($p<0.0001$). Co-application of either 0.1uM T-BSA or 1uM T-BSA with 900uM TMZ did not significantly decrease cell viability relative to TMZ treatment alone. Treatment with 10uM T-BSA alone significantly decreased cell viability, whereas treatment with 0.1uM or 1uM T-BSA alone did not have any significant effect on cell viability. 5mM TMZ served as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application. Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control which is set at 100% (n=3). ****: $p<0.0001$

A172 Cells are Responsive to TMZ.

Figure 5 shows the results of our concentration response analysis for TMZ in A172 cells. The analysis confirms that A172 cells are responsive to TMZ after a 48-hour treatment and revealed a half maximal effective concentration (EC_{50}) of approximately 500uM. In an effort to determine if sublethal concentrations of TMZ could also be augmented by T-BSA in subsequent experiments, the EC_{30} values were also determined and served as the basis for subsequent experiments. The EC_{30} value was found to be approximately 200uM.

T98G Cells are Responsive to TMZ.

Figure 6 shows the results of our concentration response analysis for TMZ in the chemotherapy insensitive cell line, T98G. Following the conduct of a concentration response analysis for TMZ in T98G cells, we determined the half maximal effective concentration (EC_{50}) to be approximately 750uM, with the EC_{30} being approximately 300uM. This relatively higher EC_{50} (and EC_{30}) values are consistent with the literature that suggests the T98G cells are less sensitive to TMZ [24, 25, 47].

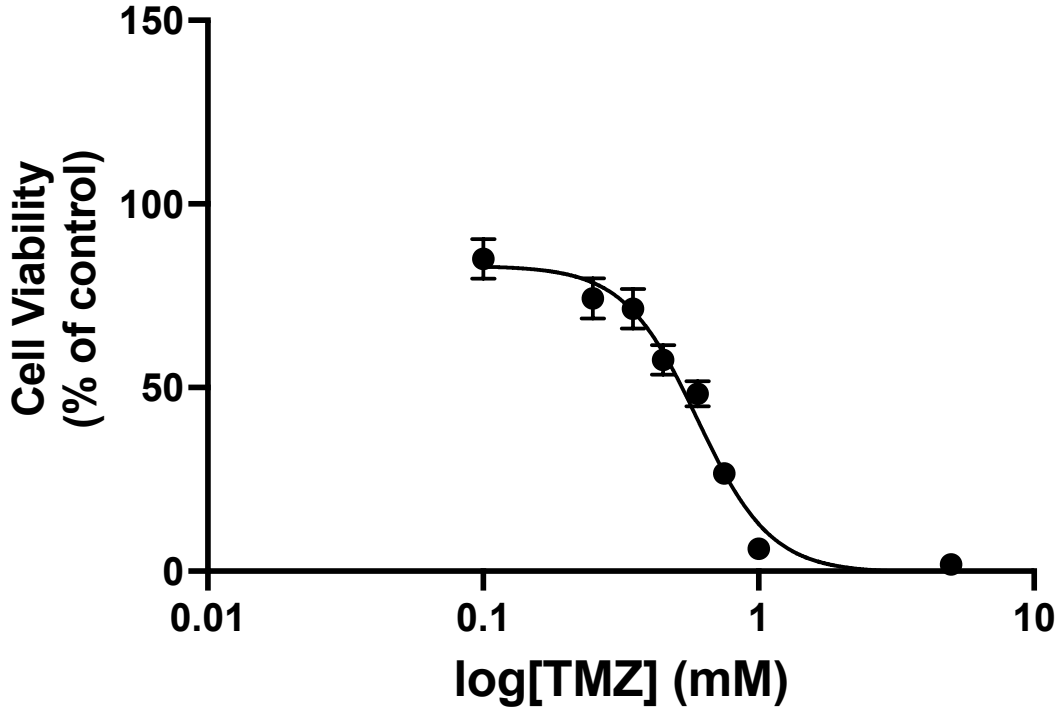

Figure 5. Concentration-Response for TMZ-Induced Cytotoxicity in A172 Cells. A172 glioblastoma cells were plated at 10,000 cells/well 24 hrs prior to treatment with TMZ (48hr). A172 cells showed a concentration-dependent response to TMZ where the half maximal effective concentration (EC_{50}) required to elicit cytotoxicity was 0.599mM and EC_{30}=0.256mM. Cell viability was assessed using a calceinAM assay (n=3).

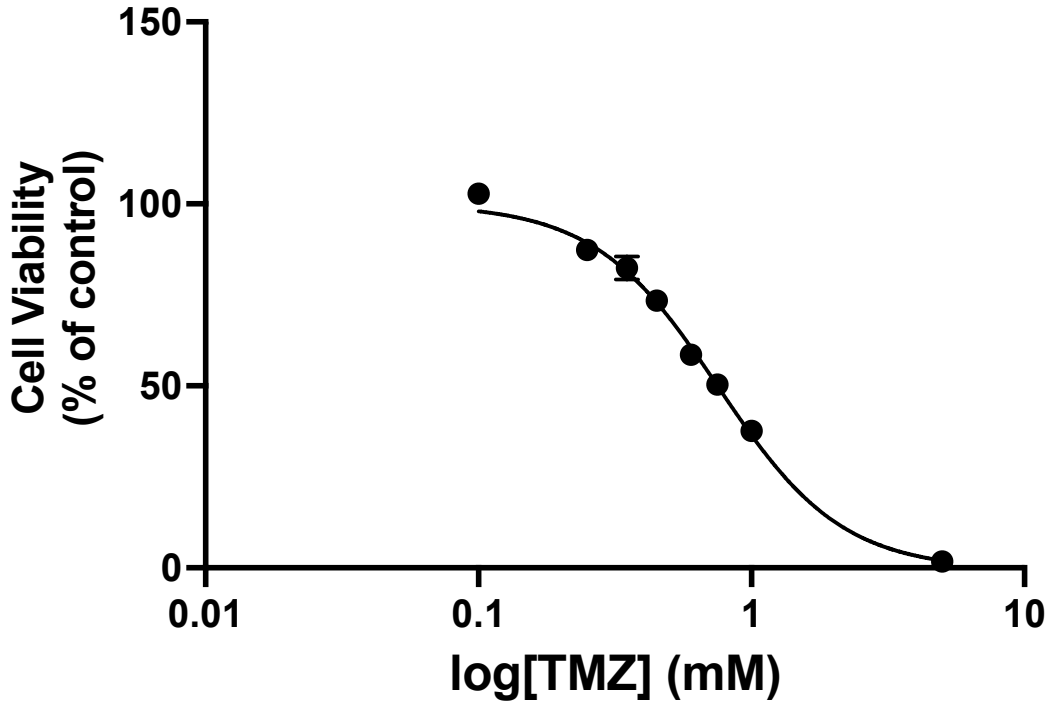

Figure 6. Concentration-Response for TMZ-Induced Cytotoxicity in Chemotherapy Resistant T98G Cells. T98G cells were plated at 10,000 cells/well one day prior to treatment with TMZ (48hr). T98G cells showed a concentration-dependent response to TMZ where the half maximal effective concentration (EC_{50}) to elicit cytotoxicity was 0.756mM and EC_{30}=0.324mM. Cell viability was assessed using a calceinAM assay (n=4).

T-BSA Does Not Enhance the Cytotoxic Effect of TMZ in A172 Cells When Administered Together.

In A172 cells, treatment with the EC_{30} concentration of TMZ (200uM) alone did not have an effect on cell viability, whereas treatment with 500uM of TMZ alone significantly decreased cell viability (to 54.5% of control) (Fig. 7). Neither 0.1uM, 1uM, nor 10uM T-BSA when co-applied with either 200uM or 500uM TMZ significantly altered cell viability compared to cells treated with either 200uM or 500uM TMZ alone, which supports the conclusion that binding to the putative membrane AR does not augment cytotoxicity of the chemotherapeutic when co-administered. In these studies, treatment with T-BSA alone at any of the tested concentrations [0.1uM, 1uM, 10uM] showed no significant effect on cell viability compared to the vehicle control. 10uM BSA when administered alone did not have any influence on cell viability. Moreover, 10uM BSA did not alter the effect of either 200uM or 500uM TMZ when administered alone.

T-BSA Does Not Enhance the Cytotoxic Effect of TMZ in T98G Cells When Administered Together.

Figure 8 shows that in the chemotherapeutic (TMZ) resistant T98G cells, treatment with 200uM of TMZ alone did not have an effect on cell viability, whereas treatment with 500uM of TMZ alone significantly decreased cell viability (to 66.8% of control). Neither concentrations of T-BSA (0.1uM, 1uM, or 10uM) altered the effects of either 200uM or 500uM TMZ. As noted in the A172 cells, there was also no discernable effect when cells were treated with T-BSA alone, at any of the concentrations tested [0.1uM, 1uM, 10uM]. The osmolarity-based control (to account for the bulky BSA molecule associated with testosterone molecules) for T-BSA (10uM BSA)

also did not have an effect, either when administered by itself or when co-applied with 200uM or 500uM TMZ.

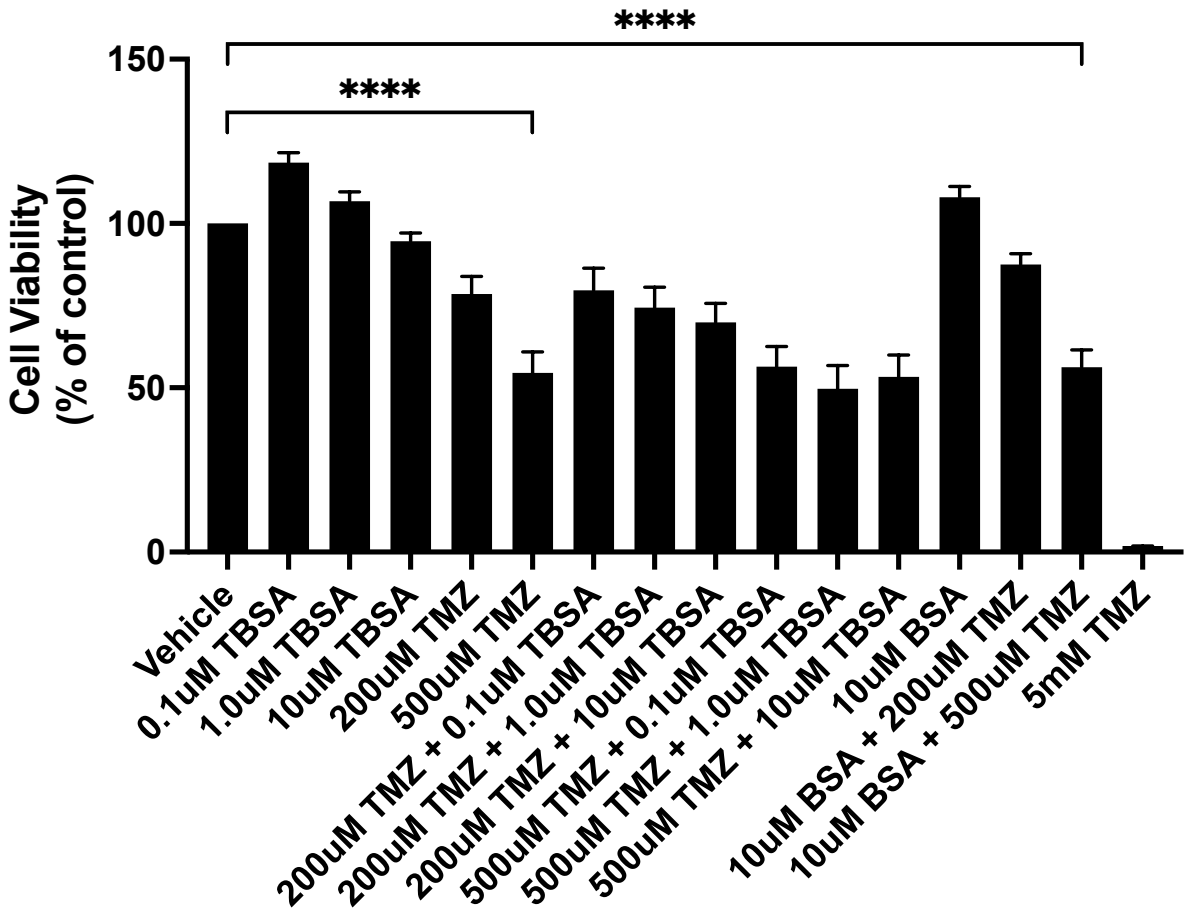

Figure 7. T-BSA Does Not Enhance TMZ-induced Cytotoxicity in A172 Cells When Administered Together. A172 cells were plated at 10,000 cells/well 24 hrs prior to treatment, after which the cells were treated with both TMZ and T-BSA (co-application) for 48hrs. Treatment with 200uM of TMZ alone did not have an effect on cell viability, whereas treatment with 500uM of TMZ alone significantly decreased cell viability compared to the vehicle control (10% CSFBS media) after 48 hrs ($p<0.0001$). Cells co-treated with T-BSA and TMZ, however, did not result in any further decline in cell viability compared to cells treated with TMZ alone. Treatment with T-BSA alone, at either 0.1, 1 or 10 uM, did not have any significant effect on cell viability. Co-treatment of 10uM BSA, the osmolarity-based control for T-BSA, with 500uM TMZ did not alter the effect of TMZ alone. 5mM TMZ served as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application. Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control, which is set at 100% (n=3). ****: $p<0.0001$

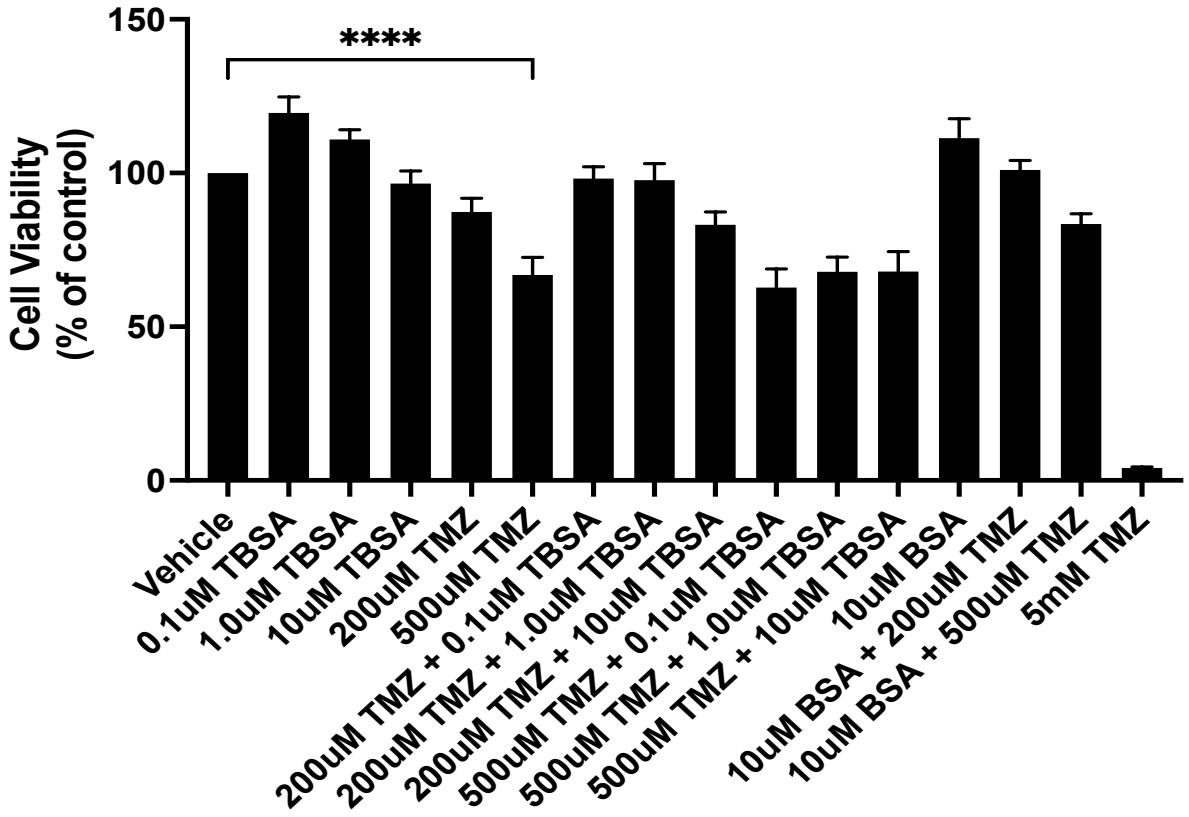

Figure 8. T-BSA Does Not Enhance TMZ-induced Cytotoxicity in T98G Cells When Administered Together. T98G cells were plated at 10,000 cells/well 24 hrs prior to treatment, after which the cells were treated with both TMZ and T-BSA (co-application) for 48hrs. Treatment with 200uM of TMZ alone did not have an effect on cell viability, whereas treatment with 500uM of TMZ alone significantly decreased cell viability compared to the vehicle control (10% CSFBS media) after 48 hrs ($p<0.0001$). Co-treatment of either 0.1, 1, or 10uM T-BSA with either 200uM or 500uM TMZ did not significantly affect cell viability compared to cells treated with 200uM or 500uM TMZ alone. Treatment with T-BSA alone, at either 0.1, 1 or 10 uM, did not have any significant effect on cell viability. 5mM TMZ served as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application. Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control which is set at 100% (n=3). ****: $p< 0.0001$

Pre-treatment of T-BSA Does Not Affect Cell Viability Consequent to 24hr TMZ Treatment in A172 Cells.

We surmised that co-application of the membrane AR ligand (T-BSA) with the chemotherapeutic may not give the compound sufficient time to elicit the appropriate cell death-sensitizing effects and thus, "set the stage", for enhanced vulnerability to TMZ. As such, we tested whether pre-treatment with T-BSA would be more likely to augment the cytotoxicity (or sensitize) the human glioblastoma cell models to TMZ. And whereas the concentration response analysis for the effect of TMZ on A172 cells used the paradigm of 48 hr exposure to TMZ, we chose to also evaluate the impact of T-BSA pretreatment on A172 cells treated for a shorter duration of time with TMZ (i.e., 24 hrs). In subsequent experiments, we also evaluated the effect of T-BSA pre-treatment on the "usual" 48hr treatment with TMZ.

Figure 9 reveals that in A172 cells, treatment with 200uM or 500uM TMZ alone did not affect cell viability compared to the vehicle control after 24hrs (Fig. 9), a result that wasn't entirely unexpected since prior (unpublished) work from the Singh lab revealed that the ideal time point for TMZ to elicit cell death was 48 hr post treatment. However, we initially hypothesized that the T-BSA might sensitize the cells to TMZ such that its cytotoxic effects could be noticeable even at shorter periods of (TMZ) treatment. The data showed that pre-treatment with T-BSA does not enhance the effect of TMZ (or sensitize the cells to TMZ). Cells that were pre-treated with T-BSA [0.1uM, 1uM, 10uM] for 24 hours prior to being treated with either 200uM or 500uM TMZ for an additional 24 hours showed no significant change in cell viability compared to cells treated with either 200uM or 500uM TMZ alone. While cells treated with 0.1uM and 10uM T-BSA alone showed no significant effect on cell viability compared to the vehicle control, treatment with 1uM T-BSA alone showed fluorescence values that were statistically higher than

the vehicle control ($p<0.005$), the basis for which is not clear and will require further investigation. Neither treatment with 10uM BSA alone, nor co-treatment of 10uM BSA with 200uM TMZ or 500uM showed significant effect on cell viability compared to control.

Pre-Treatment with T-BSA Failed to Augment TMZ-Induced Cytotoxicity in A172 Cells.

In an effort to be consistent with the "ideal" duration of TMZ treatment, we extended our pre-treatment paradigm analysis to include 48 hr post-TMZ treatment. In this situation, we noted, as in prior experiments, that only the 500uM concentration of TMZ resulted in a statistically significant decrease in cell viability (to 56.7% of control, Fig. 10). Through statistical analysis of group differences, we did, however, find that cells pre-treated with 1uM T-BSA for 24 hours prior to being treated with 200uM TMZ for an additional 48 hours showed significant increase in cell viability compared to cells treated with 200uM TMZ alone. But since the 200uM TMZ alone group was not statistically different from the vehicle treated control, we suggest that this result should not be over-interpreted, but rather, the significant "protective" effect seen in this treatment condition is more likely a statistical anomaly, rather than a true protective effect of T-BSA. Treatment with T-BSA alone [0.1uM, 1uM, 10uM] showed no significant effect on cell viability compared to the vehicle control. Furthermore, and consistent with other experiments, treatment with 10uM BSA alone had no effect on cell viability, either when administered alone or in conjunction with TMZ.

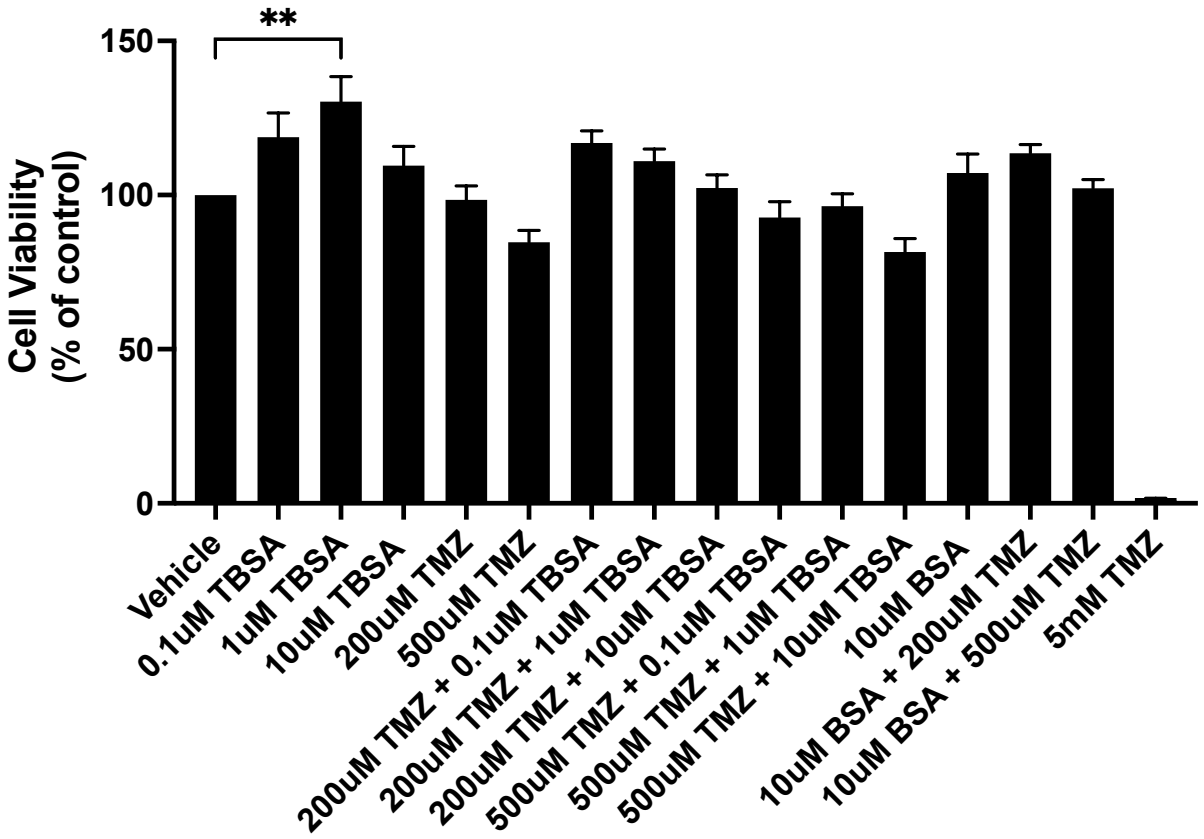

Figure 9. Pre-treatment of T-BSA Does Not Affect Cell Viability Consequent to 24hr TMZ Treatment in A172 Cells. A172 cells were plated at 10,000 cells/well 24 hrs prior to treatment. A172 cells were initially treated with T-BSA at various concentrations for 24hrs before being additionally treated with 200uM or 500uM TMZ for 24hrs. Cells treated with TMZ alone showed no significant decrease in cell viability 24hrs after treatment compared to vehicle control (10% CSFBS media). Pre-treatment with T-BSA [0.1uM, 1uM, 10uM] for 24 hrs prior to being treated with either 200uM or 500uM TMZ for an additional 24 hrs showed no significant change in cell viability. Cells treated with 0.1uM and 10uM T-BSA alone showed no significant effect on cell viability compared to the vehicle control, whereas treatment with 1uM T-BSA alone showed a significant increase in cell viability compared to the vehicle control ($p<0.005$). 5mM TMZ served as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application. Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control which is set at 100% (n=3). **: $p<0.005$

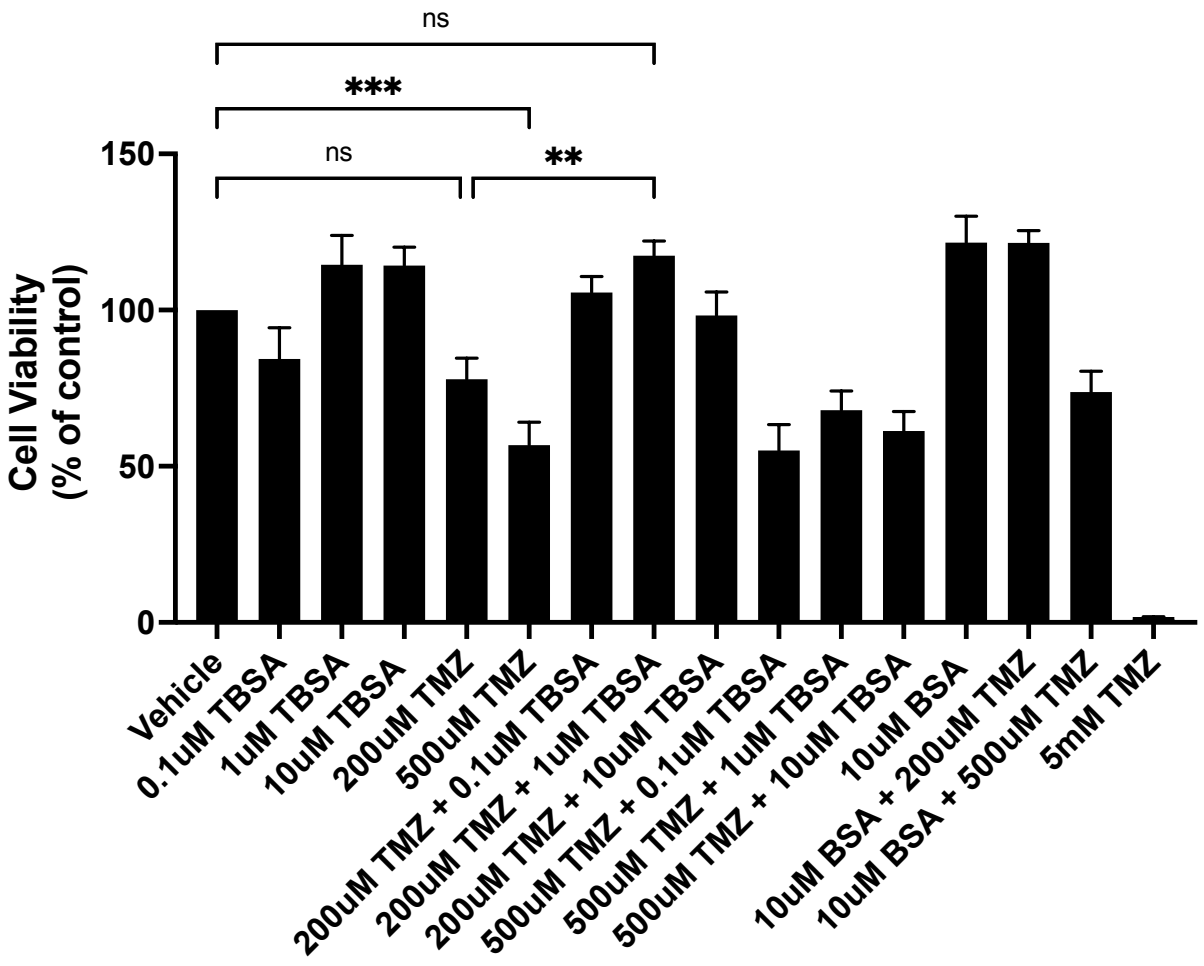

Figure 10. T-BSA Does Not Enhance A172 Cell Death Elicited by 48hr TMZ. A172 cells were plated at 10,000 cells/well 24 hrs prior to treatment. A172 cells were initially treated with T-BSA at various concentrations for 24hrs before treatment with either 200uM or 500uM TMZ, in the continued presence of T-BSA, for an additional 48hrs. Treatment with 200uM TMZ did not affect cell viability compared to the vehicle control (10% CSFBS media), whereas treatment with 500uM TMZ showed significant decrease in cell viability compared to the vehicle control after 48hrs ($p<0.001$). Cells pre-treated with 1uM T-BSA showed significant increase in cell viability following treatment with 200uM TMZ after 48hrs, compared to cells treated with 200uM TMZ alone ($p<0.005$). Treatment with T-BSA alone, at either 0.1, 1 or 10 uM, did not have any significant effect on cell viability. 5mM TMZ served as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control which is set at 100% (n=3). ns: no significance; **: $p<0.005$; ***: $p<0.001$

Pre-Treatment of T-BSA Followed by 24hr Treatment with TMZ Enhances the Cytotoxic Effect of TMZ in T98G Cells.

We extended the analysis of T-BSA pretreatment to the T98G cells. We found that while treatment of cells with 500uM TMZ showed a modest, though statistically significant, decrease in cell viability (86.2% of control) compared to the vehicle control after 24hrs ($p<0.05$), pre-treatment with T-BSA [0.1uM, 1uM, 10uM] for 24 hours prior to 500uM TMZ (for an additional 24 hours) showed significant enhancement of cytotoxicity (59.8%, 69.5%, and 65.6% of control, respectively for 0.1, 1 and 10uM T-BSA) compared to cells treated with 500uM TMZ alone (Figure 11). In contrast, pre-treatment with T-BSA did not alter the effect of 200uM TMZ. Neither treatment with T-BSA alone [0.1uM, 1uM, 10uM] of 10uM BSA alone showed any effect on cell viability.

Pre-Treatment of T-BSA Followed by 48hr Treatment with TMZ Does Not Enhance the Cytotoxic Effect of TMZ in T98G Cells.

As done with A172 cells, we also evaluated the effect of T-BSA pre-treatment on the effect of 48 hr TMZ treatment in T98G cells. Whereas treatment with 500uM TMZ showed a significant decrease in cell viability (67.2%) compared to the vehicle control after 48hrs ($p<0.0001$) (Fig. 12), cells pre-treated with T-BSA [0.1uM, 1uM, 10uM] for 24 hours did not enhance the effect of either the 200 uM or 500uM concentrations of TMZ.

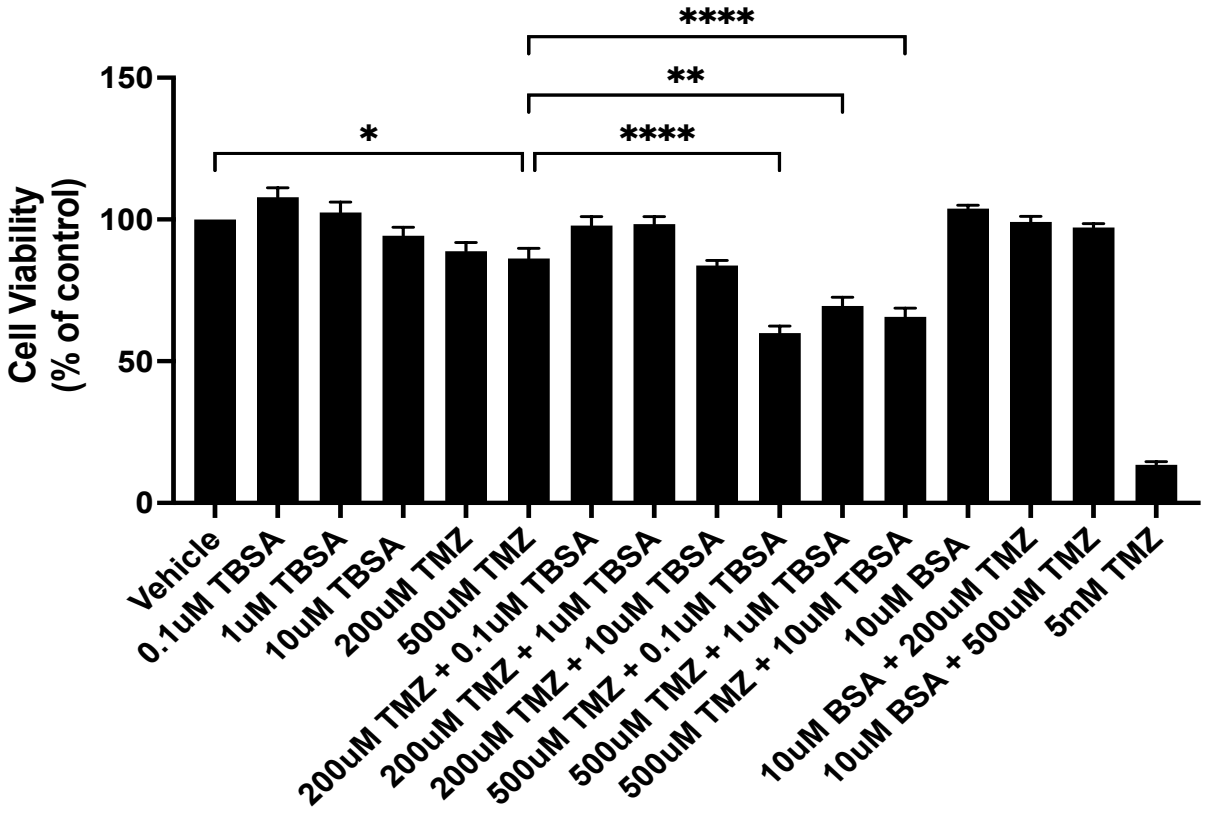

Figure 11. Pre-treatment With T-BSA Enhances the Cytotoxic Effect of TMZ in T98G Cells. T98G cells were plated at 10,000 cells/well 24 hrs prior to treatment. T98G cells were initially treated with T-BSA at various concentrations for 24hrs followed by treatment with 200uM or 500uM TMZ for an additional 24hrs, in the continued presence of T-BSA. Treatment with 200uM TMZ did not affect cell viability compared to the vehicle control (10% CSFBS media), whereas treatment with 500uM TMZ showed a statistically significant, decrease in cell viability compared to the vehicle control after 24hrs ($p<0.05$). Pre-treatment with T-BSA significantly enhanced the decrease in cell viability compared to 500uM TMZ alone ($p<0.0001$; $p<0.005$; $p<0.0001$). Treatment with T-BSA alone, at either 0.1, 1 or 10 uM, did not have any significant effect on cell viability. 5mM TMZ served as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application. Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control which is set at 100% (n=3). *: $p<0.05$; **: $p<0.005$; ****: $p<0.0001$

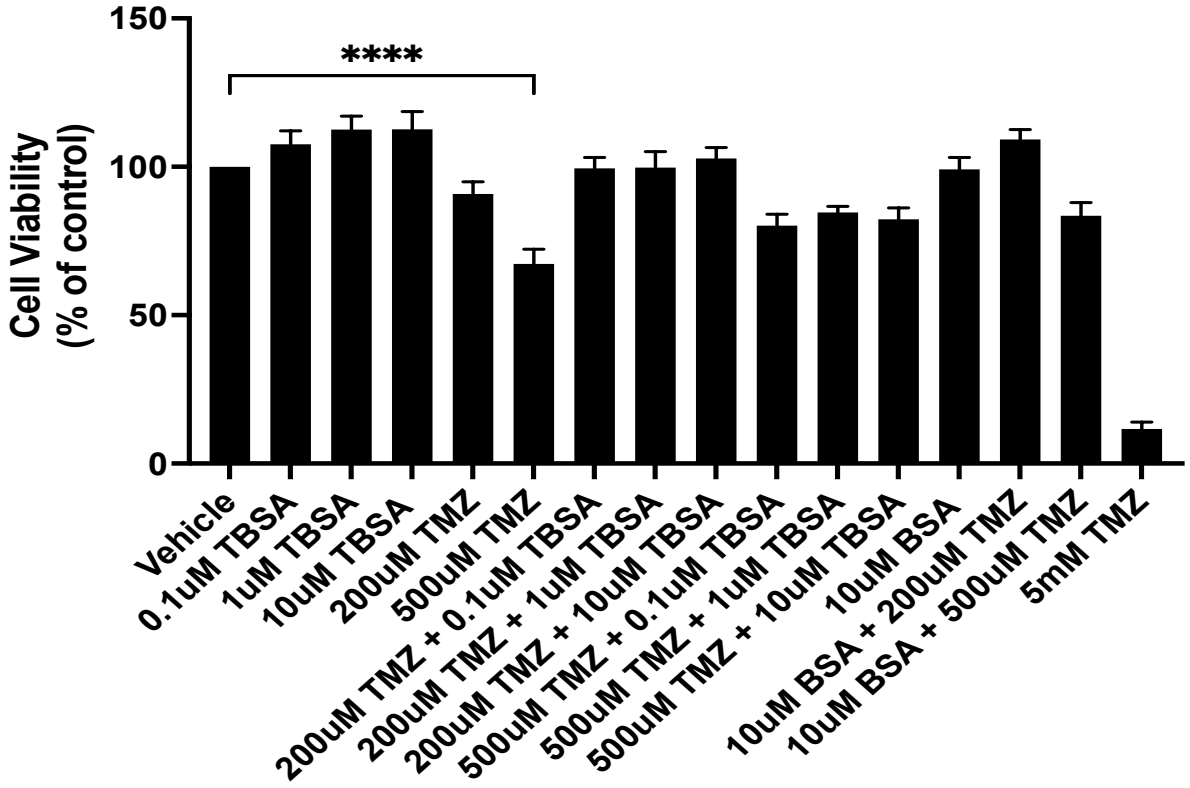

Figure 12. Pre-treatment With T-BSA Does Not Enhance the Cytotoxic Effect of TMZ After 48hrs in T98G Cells. T98G cells were plated at 10,000 cells/well 24 hrs prior to treatment. T98G cells were initially treated with T-BSA at various concentrations for 24hrs before being additionally treated with 200uM or 500uM TMZ for 48hrs. Treatment with 200uM TMZ did not affect cell viability compared to the vehicle control (10% CSFBS media), whereas treatment with 500uM TMZ showed a significant decrease in cell viability compared to the vehicle control after 48hrs ($p<0.0001$). Pre-treatment with T-BSA did not alter the effects of TMZ on cell viability. Treatment with T-BSA alone, at either 0.1, 1 or 10 uM, did not have any significant effect on cell viability. 5mM TMZ served as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application. Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control which is set at 100% (n=3). ****: $p<0.0001$

A172 Cells are Responsive to IAA.

While the gold standard chemotherapeutic used in the treatment of glioblastoma is TMZ, we wanted to determine if the modest (or lack of) effect of T-BSA was attributed to the type of insult, noting that T-BSA robustly enhanced the cytotoxicity of the metabolic/oxidative insult, IAA, in C6 glioma cells. As in prior experiments, we first conducted a concentration response analysis for IAA in A172 cells (Fig. 13). The data demonstrate that A172 cells are indeed responsive to IAA, after a 6-hour treatment, with the half maximal effective concentration (EC_{50}) required to elicit cytotoxicity being approximately 75uM. This concentration was used for subsequent experiments where IAA was applied to A172 cells.

T-BSA Does Not Enhance the Cytotoxic Effect of IAA in A172 Cells.

In A172 cells, treatment with 75uM IAA alone, for 6 hrs, significantly decreased cell viability (46%) compared to vehicle control ($p<0.0001$) (Fig. 14). Co-treatment of T-BSA [0.1uM, 1uM, 10uM] with 75uM IAA did not significantly affect cell viability compared to cells treated with 75uM IAA alone. As noted in previous experiments, treatment with 10uM BSA alone did not affect cell viability compared to vehicle control, and nor did it alter the effect of IAA.

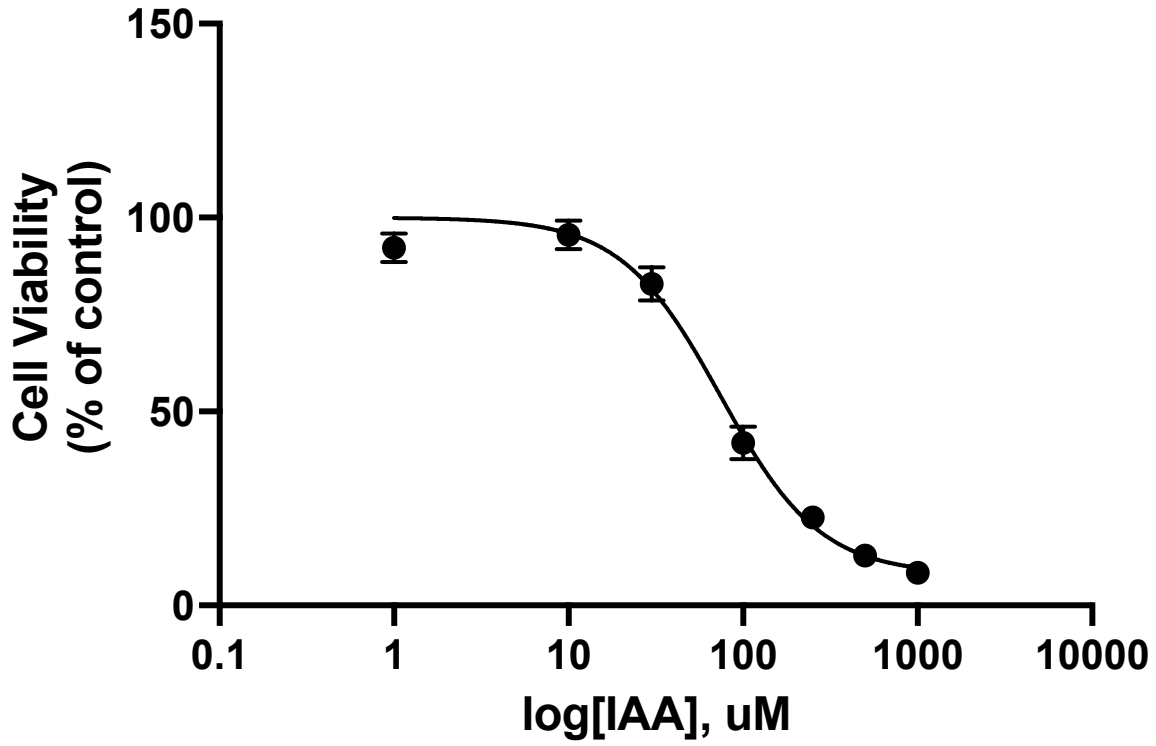

Figure 13. Concentration-Response for IAA-Induced Cytotoxicity in A172 cells. A172 cells were plated at 10,000 cells/well 24 hrs prior to treatment with IAA (6hr). A172 cells showed a concentration-dependent response to IAA where the half maximal effective concentration (EC_{50}) to promote cell death was 74.24uM. Cell viability was assessed using a calceinAM assay (n=5).

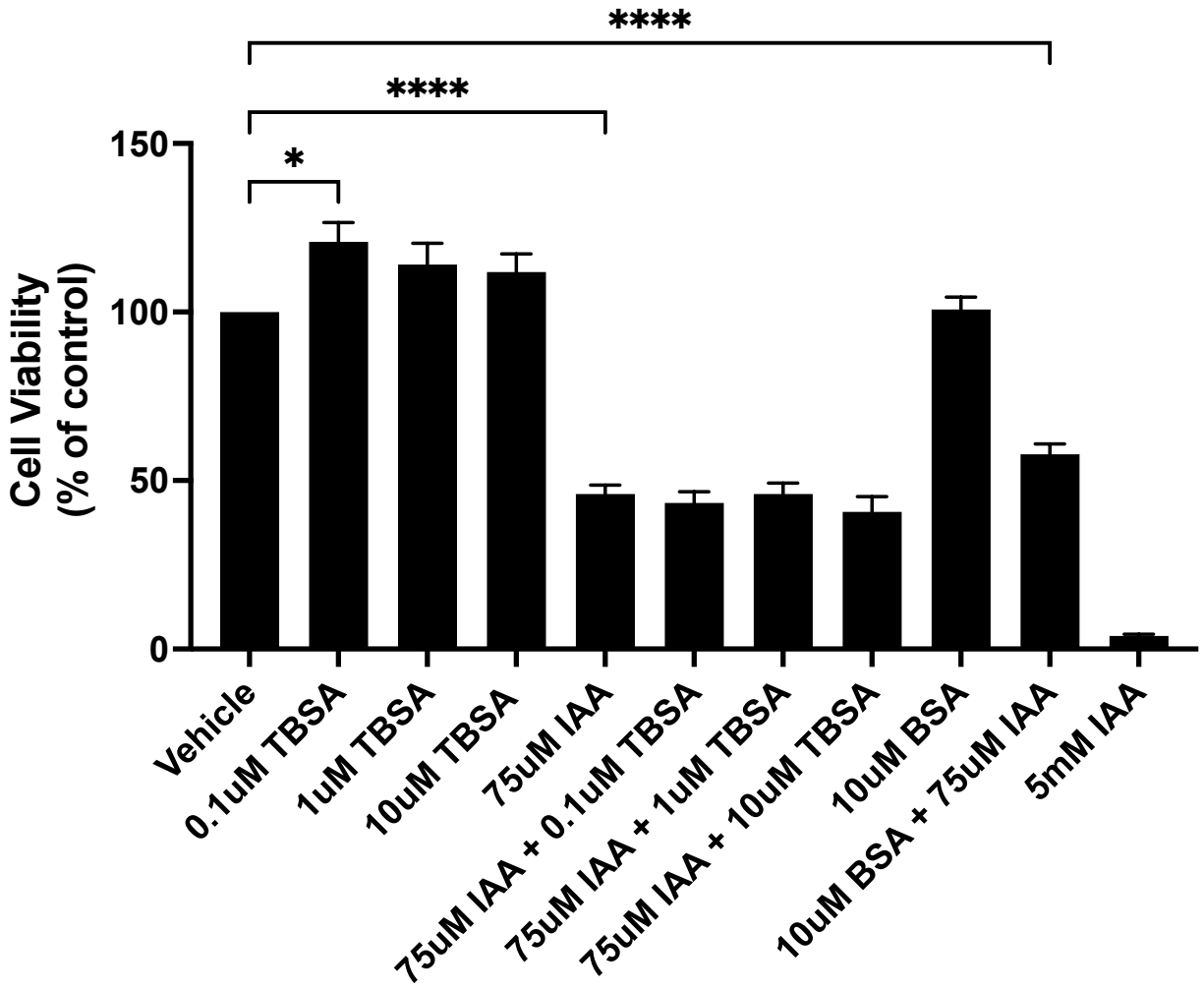

Figure 14. T-BSA Does Not Enhance the Cytotoxic Effect of IAA in A172 Cells. A172 cells were plated at 10,000 cells/well 24 hrs prior to treatment. Cells were co-treated with IAA and T-BSA for 6hrs. Treatment with 75uM IAA alone significantly decreased cell viability compared to vehicle control (10% CSFBS media) after 6hrs ($p<0.001$). Co-treatment with T-BSA and 75uM IAA did not result in greater cell death relative to that elicited by IAA alone. Cells treated with 0.1uM T-BSA alone significantly increased cell viability compared to the vehicle control ($p<0.05$), whereas treatment with 1uM or 10uM T-BSA alone did not significantly affect cell viability compared to the vehicle control. Co-treatment of 10uM BSA with 75uM IAA significantly decreased cell viability compared to vehicle control ($p <0.0001$). 5mM IAA served as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application. Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control which is set at 100% (n=3). *: $p<0.05$; ****: $p<0.0001$

T98G Cells are Responsive to IAA.

As conducted with A172 cells, we also determined the concentration response of T98G cells to IAA (Fig. 15). The data verifies that T98G cells are also responsive to IAA after a 6-hour treatment with the half maximal effective concentration (EC_{50}) required to elicit cell death as being approximately 80uM.

Co-Treatment of T-BSA with IAA Increases Cell Viability in T98G Cells.

In T98G cells, treatment with 75uM IAA alone failed to elicit a reduction in cell viability compared to vehicle control after 6hrs (Fig. 16). Given that T98G cells are relatively resistant to TMZ, these data may suggest that T98G cells have enhanced cell-survival associated mechanisms in place that render them more resistant to cytotoxic insult, more generally. Alternatively, the narrow concentration response range noted in Figure 15 may suggest that even though 75uM was close to the EC50 value of ~80uM, we may have ended up with a sublethal concentration of IAA. Nevertheless, we proceeded with this concentration to determine whether treatment with T-BSA might alter the effect of a sublethal concentration of IAA and transform it into a cytotoxic effect. We found that neither concentration of T-BSA, when applied concomitantly with IAA, resulted in a reduction in cell viability.

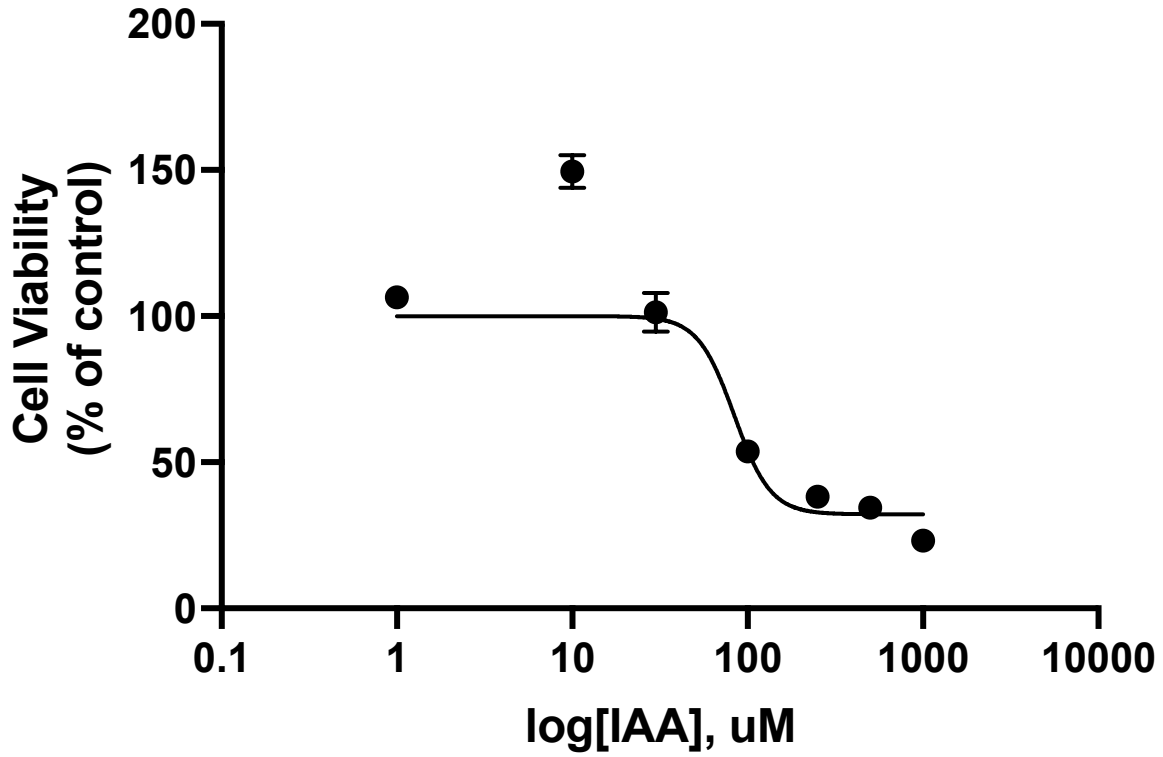

Figure 15. Concentration-Response for IAA-Induced Cytotoxicity in T98G cells. T98G cells were plated at 10,000 cells/well 24 hrs prior to treatment with varying concentrations of IAA (6hr). T98G cells showed a concentration-dependent response to IAA where the half maximal effective concentration (EC_{50}) is 83.81uM. Cell viability was assessed using a calceinAM assay (n=5).

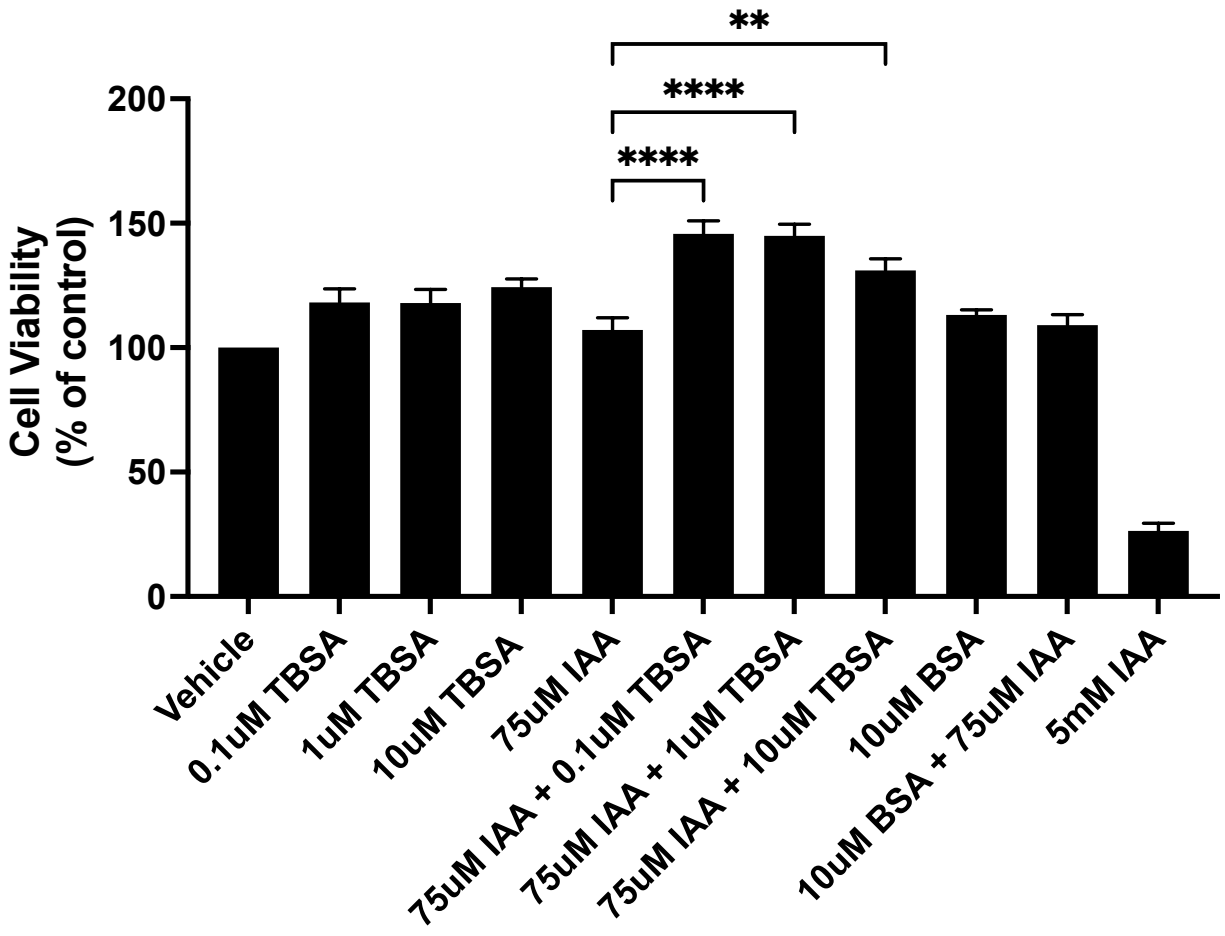

Figure 16. Co-Treatment of T-BSA and IAA Does Not Enhance the Effect of IAA Alone in T98G Cells. T98G cells were plated at 10,000 cells/well 24 hrs prior to treatment. Treatment with 75uM IAA alone does not affect cell viability compared to vehicle control (10% CSFBS media) after 6hrs Cells co-treated with T-BSA and 75uM IAA showed significant increases in cell viability compared to cell treated with 75uM IAA alone ($p<0.0001$; $p<0.0001$; $p<0.005$). Treatment with T-BSA alone, at either 0.1, 1 or 10 uM, did not have any significant effect on cell viability. 5mM IAA served as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application. Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control which is set at 100% (n=3). **: $p<0.005$; ****: $p<0.0001$

Primary Astrocytes are Responsive TMZ.

A key question this thesis poses is whether any effects of T-BSA also occur in "healthy" (i.e., non-tumor) astrocytes. As conducted in prior experiments, in other cells and using different cytotoxic agents, we first conducted a concentration-response analysis for the effect of TMZ in primary astrocytes (Fig. 17). The concentration response verifies that primary astrocytes were indeed responsive to TMZ after a 48-hour treatment with the half maximal effective concentration (EC_{50}) required to elicit cell death being approximately 475uM and the EC_{30} being approximately 200uM.

Co-Treatment of 1uM T-BSA Enhances the Cytotoxic Effect of 200uM TMZ in Primary Astrocytes.

In primary astrocytes, treatment with 200uM of TMZ alone did not have an effect on cell viability, whereas treatment with 500uM of TMZ alone significantly decreased cell viability (78.8%) compared to the vehicle control after 48 hours ($p<0.0001$) (Fig. 18). Co-treatment of 1uM T-BSA with 200uM TMZ showed a modest, though statistically significant, decrease in cell viability (82.2%) compared to cells treated with 200uM TMZ alone ($p<0.005$). Co-treatment of 0.1uM or 10uM T-BSA, however, together with 200uM TMZ did not significantly affect cell viability compared to cells treated with 200uM TMZ alone. Co-treatment of T-BSA [0.1uM, 1uM, 10uM] with 500uM TMZ did not significantly affect cell viability compared to cells treated with 500uM TMZ alone.

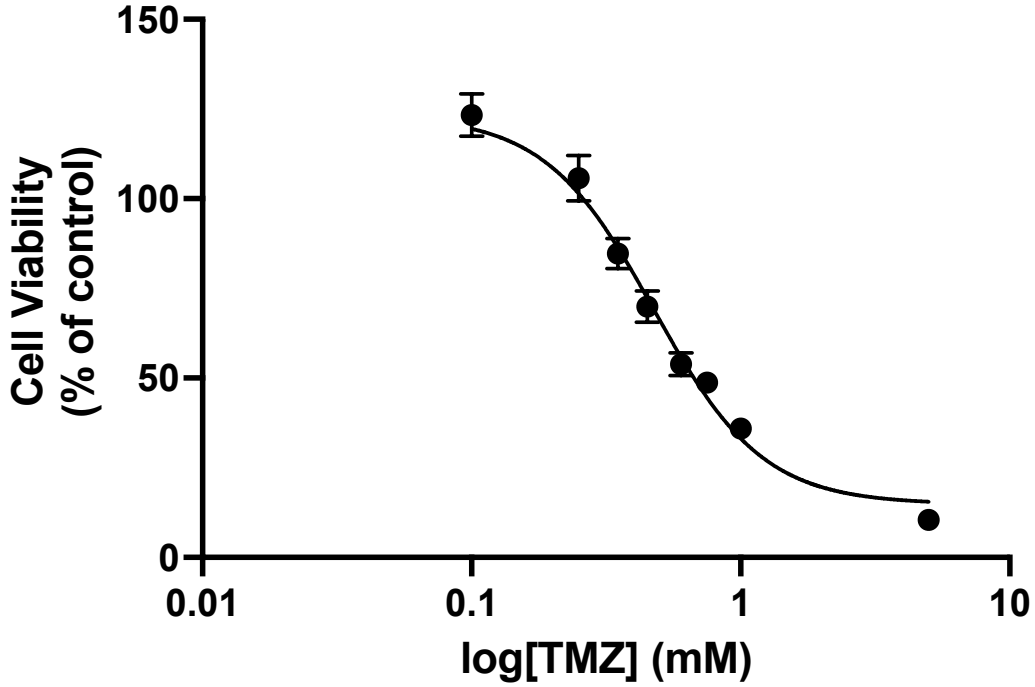

Figure 17. Concentration-Response for TMZ-Induced Cytotoxicity in Primary Astrocytes. Primary astrocytes were plated at 10,000 cells/well 24 hrs prior to treatment with TMZ (48hr). Primary astrocytes showed a concentration-dependent response to TMZ where the half maximal effective concentration (EC_{50}) required to elicit cytotoxicity was 0.476mM and EC_{30}=0.204mM. Cell viability was assessed using a calceinAM assay (n=3).

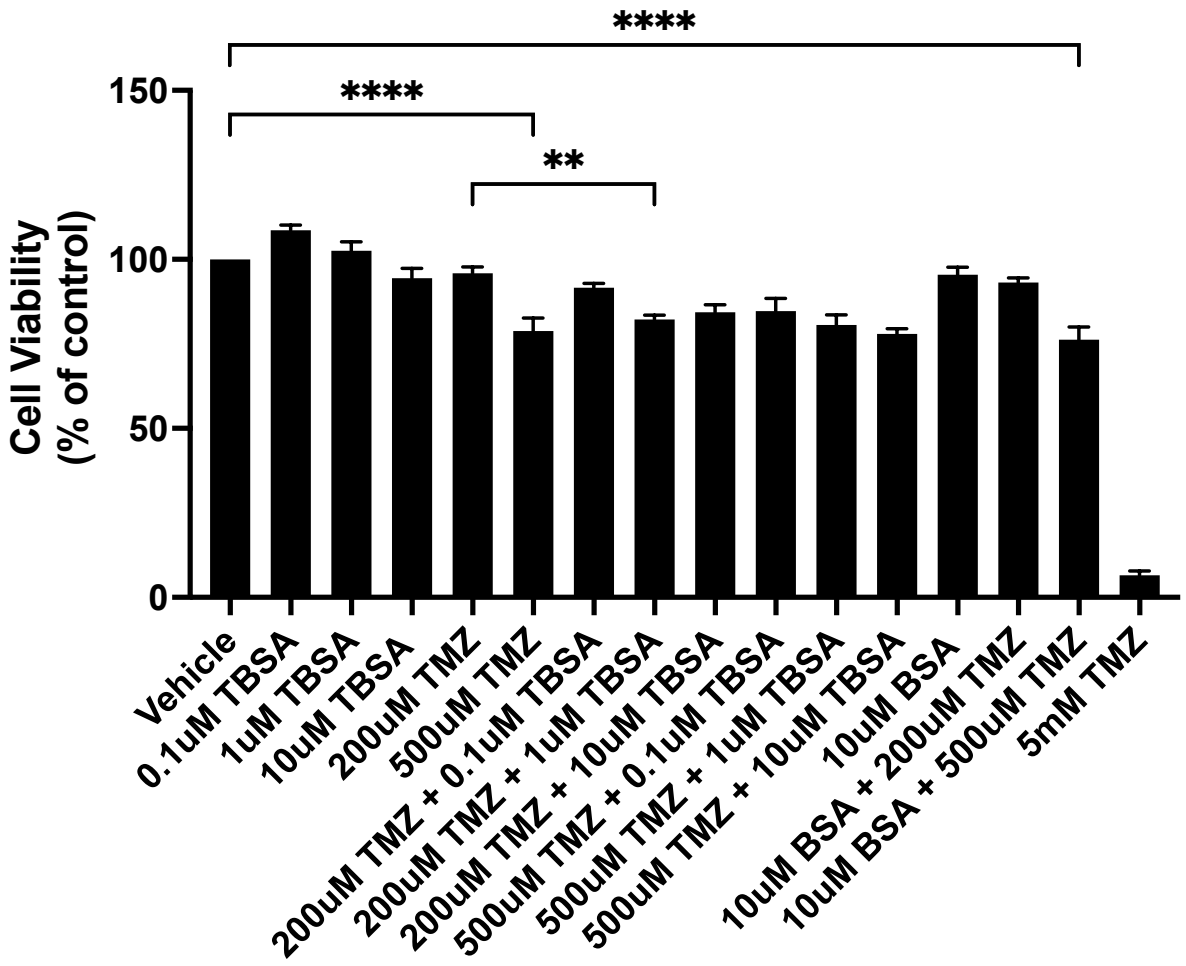

Figure 18. T-BSA Modestly Enhanced the Cytotoxic Effect of 200uM TMZ in Primary Astrocytes. Primary astrocytes were plated at 10,000 cells/well 24 hrs prior to treatment, followed by co-application of both TMZ and T-BSA for 48hrs. Treatment with 200uM of TMZ alone did not have an effect on cell viability, whereas treatment with 500uM of TMZ alone significantly decreased cell viability compared to the vehicle control (10% CSFBS media) after 48 hrs ($p<0.0001$). Cells co-treated with 1uM T-BSA and 200uM TMZ showed significant decrease in cell viability compared to treatment with 200uM TMZ alone ($p<0.005$). Co-treatment of 0.1uM or 10uM T-BSA with 200uM TMZ did not significantly affect cell viability compared to cells treated with 200uM TMZ alone. Co-treatment of T-BSA [0.1uM, 1uM, 10uM] with 500uM TMZ did not significantly affect cell viability. Co-treatment of 10uM BSA with 500uM TMZ showed significant decrease in cell viability compared to the vehicle control ($p<0.0001$). 5mM TMZ served as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application. Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control which is set at 100% (n=3). **: $p<0.005$; ****: $p<0.0001$

Pre-Treatment of T-BSA Followed by 24hr Treatment with TMZ Does Not Affect Cell Viability in Primary Astrocytes.

In primary astrocytes, treatment with 200uM or 500uM TMZ alone did not affect cell viability compared to the vehicle control after 24hrs (Fig. 19), in contrast to that noted following 48 hr TMZ treatment (Fig. 18). Pre-treatment with T-BSA, using either of the concentrations tested (0.1, 1 or 10uM) failed to influence the effects of TMZ. Although the following data supports the hypothesis that T-BSA does not enhance the cytotoxic effects of TMZ in "normal" astrocytes, it is again with the caveat that TMZ did not elicit cytotoxic effects after 24 hours.

Pre-Treatment of T-BSA Followed by 48hr Treatment with TMZ Does Not Enhance the Cytotoxic Effect of TMZ in Primary Astrocytes.

In primary astrocytes, treatment with 200uM TMZ did not affect cell viability compared to the vehicle control after 48hrs, whereas treatment with 500uM TMZ showed a significant decrease in cell viability (52.4%) compared to the vehicle control (100%) after 48hrs ($p<0.0001$) (Fig. 20), consistent with what we describe above, in that 500uM TMZ for 48hr is the concentration and duration of treatment at which we see consistent cytotoxicity in most cell types studied. In these experiments, cells that were pre-treated with T-BSA [0.1uM, 1uM, 10uM] for 24 hours prior to being treated with 500uM TMZ for an additional 48 hours showed no significant decreases in cell viability compared to cells treated with 500uM TMZ alone. These results suggest that T-BSA does not promote cytotoxicity in primary ("healthy") healthy astrocytes.

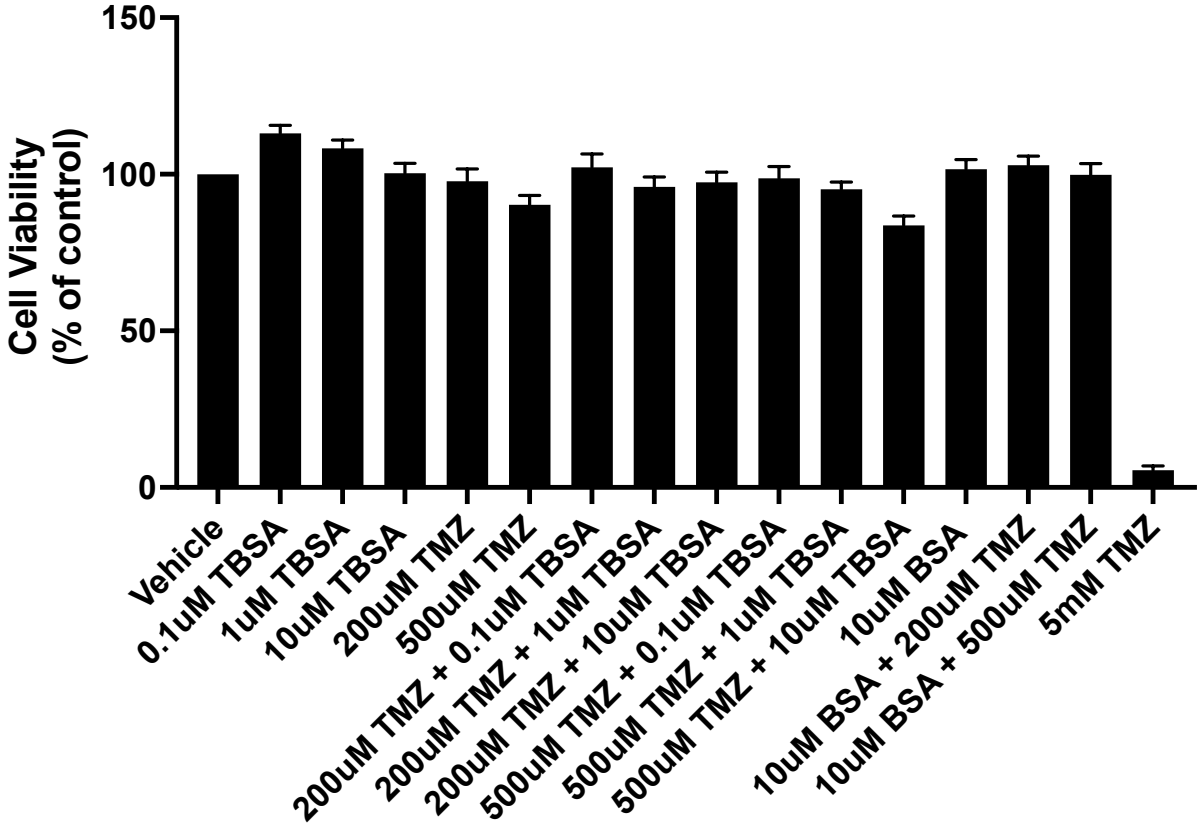

Figure 19. Pre-treatment With T-BSA Does Not Affect Cell Viability After 24hrs in Primary Astrocytes. Primary astrocytes were plated at 10,000 cells/well 24 hrs prior to treatment. Primary astrocytes were initially treated with T-BSA at various concentrations for 24hrs before being additionally treated with 200uM or 500uM TMZ for 24hrs. Cells treated with TMZ alone showed no significant decrease in cell viability 24hrs after treatment compared to vehicle control (10% CFBS media). Pre-treatment with T-BSA did not alter the effects of TMZ on cell viability. Treatment with T-BSA alone, at either 0.1, 1 or 10 uM, did not have any significant effect on cell viability. 5mM TMZ served as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application. Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control which is set at 100% (n=3).

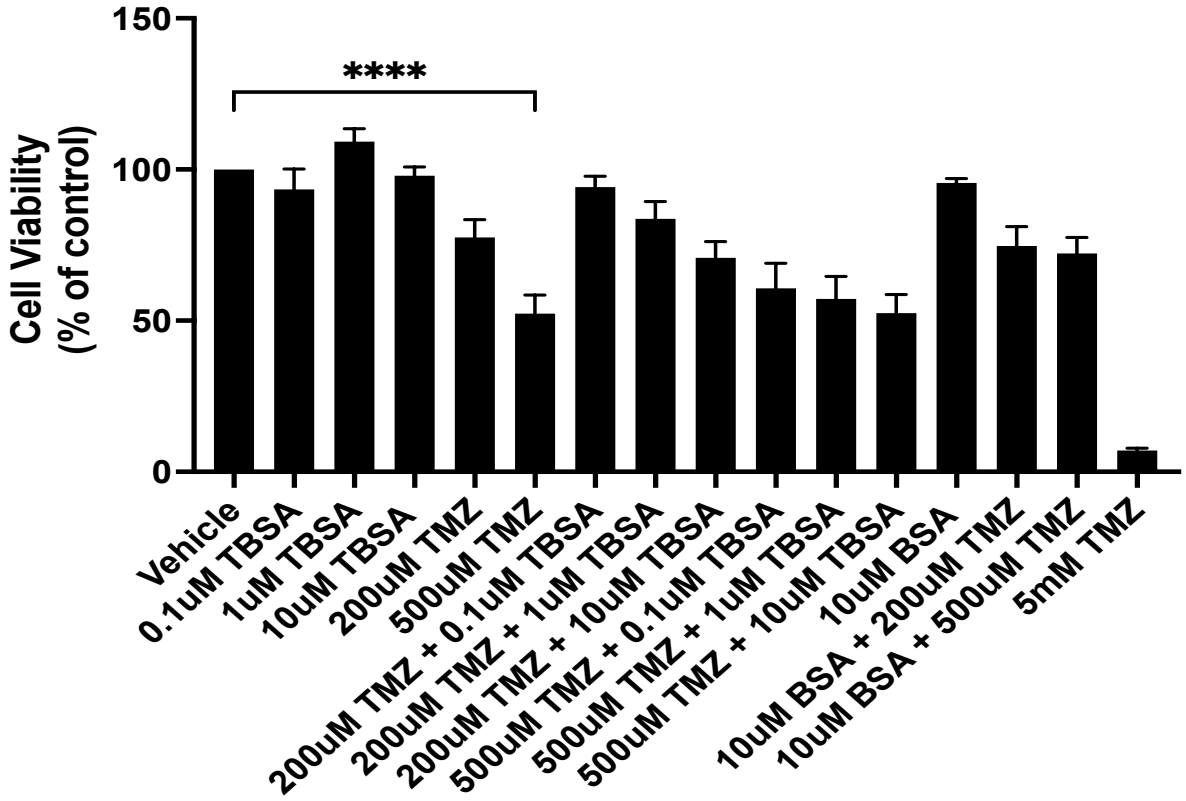

Figure 20. Pre-treatment With T-BSA Does Not Enhance the Cytotoxic Effect of TMZ in Primary Astrocytes. Primary astrocytes were plated at 10,000 cells/well 24 hrs prior to treatment. Primary astrocytes were initially treated with T-BSA at various concentrations for 24hrs, followed by treatment with 200uM or 500uM TMZ for an additional 48hrs, in the continued presence of T-BSA. Treatment with 200uM TMZ did not affect cell viability compared to the vehicle control (10% CSFBS media), whereas treatment with 500uM TMZ showed a significant decrease in cell viability compared to the vehicle control after 48hrs ($p<0.0001$). Pre-treatment with T- BSA did not alter the effects of TMZ on cell viability. Co-treatment of 10uM BSA with 500uM TMZ showed significant decrease in cell viability compared to the vehicle control. Treatment with T-BSA alone, at either 0.1, 1 or 10 uM, did not have any significant effect on cell viability. 5mM TMZ served as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application. Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control which is set at 100% (n=3). ****: $p<0.0001$

Primary Astrocytes are Responsive to IAA.

Consistent with our paradigm of analysis, we also sought to characterize if T-BSA has an impact on IAA-induced cytotoxicity in primary astrocytes. First, in our concentration response analysis for the effect of IAA in primary astrocytes, we found that primary astrocytes are responsive to IAA after a 6-hour treatment, with the half maximal effective concentration (EC_{50}) required to elicit cytotoxicity as being approximately 40uM. This was the concentration used in subsequent experiments.

T-BSA Does Not Enhance the Cytotoxic Effect of IAA in Primary Astrocytes.

Figure 22 shows that in primary astrocytes, treatment with 40uM IAA alone significantly decreased cell viability (62.8% of control) compared to vehicle control after 6hrs ($p<0.0001$). Co-treatment of T-BSA [0.1uM, 1uM, 10uM] with 40uM IAA did not significantly affect cell viability compared to cells treated with 40uM IAA alone, and suggest that T-BSA is neither directly cytotoxic to primary astrocytes, nor does it sensitize the cells to the effects of IAA.

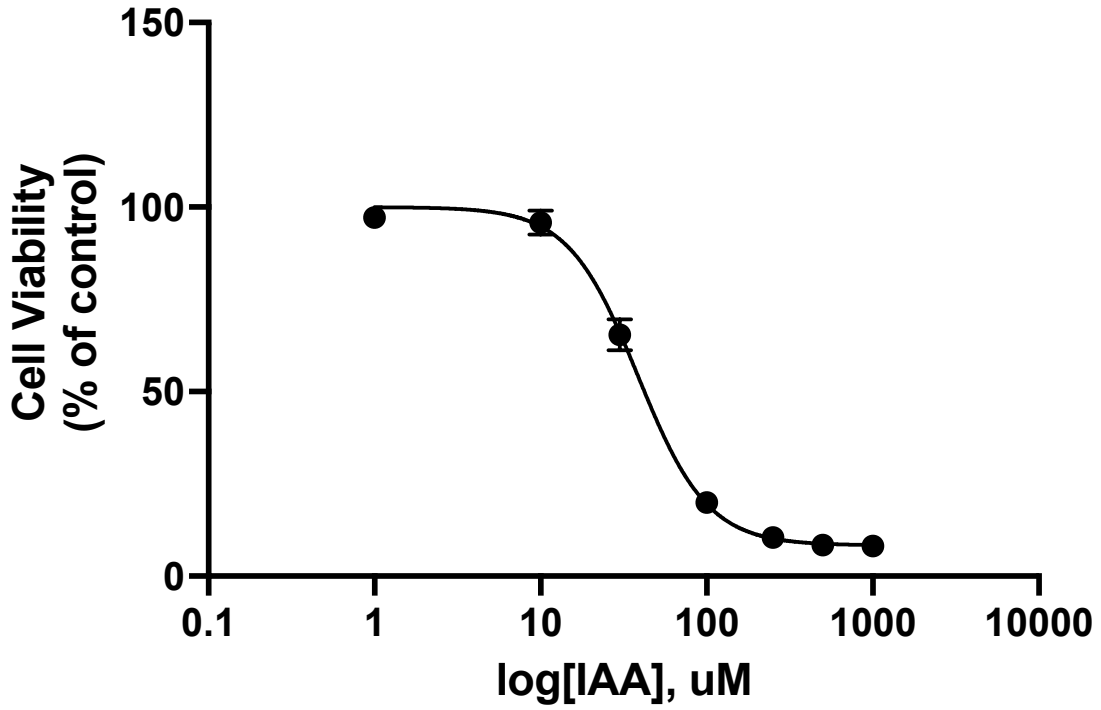

Figure 21. Concentration-Response for IAA-Induced Cytotoxicity in Primary Astrocytes. Primary astrocytes were plated at 10,000 cells/well 24 hrs prior to treatment with IAA (6hr). Primary astrocytes showed a concentration-dependent response to IAA where the half maximal effective concentration (EC_{50}) required to elicit cell death was 38.59uM. Cell viability was assessed using a calceinAM assay (n=3).

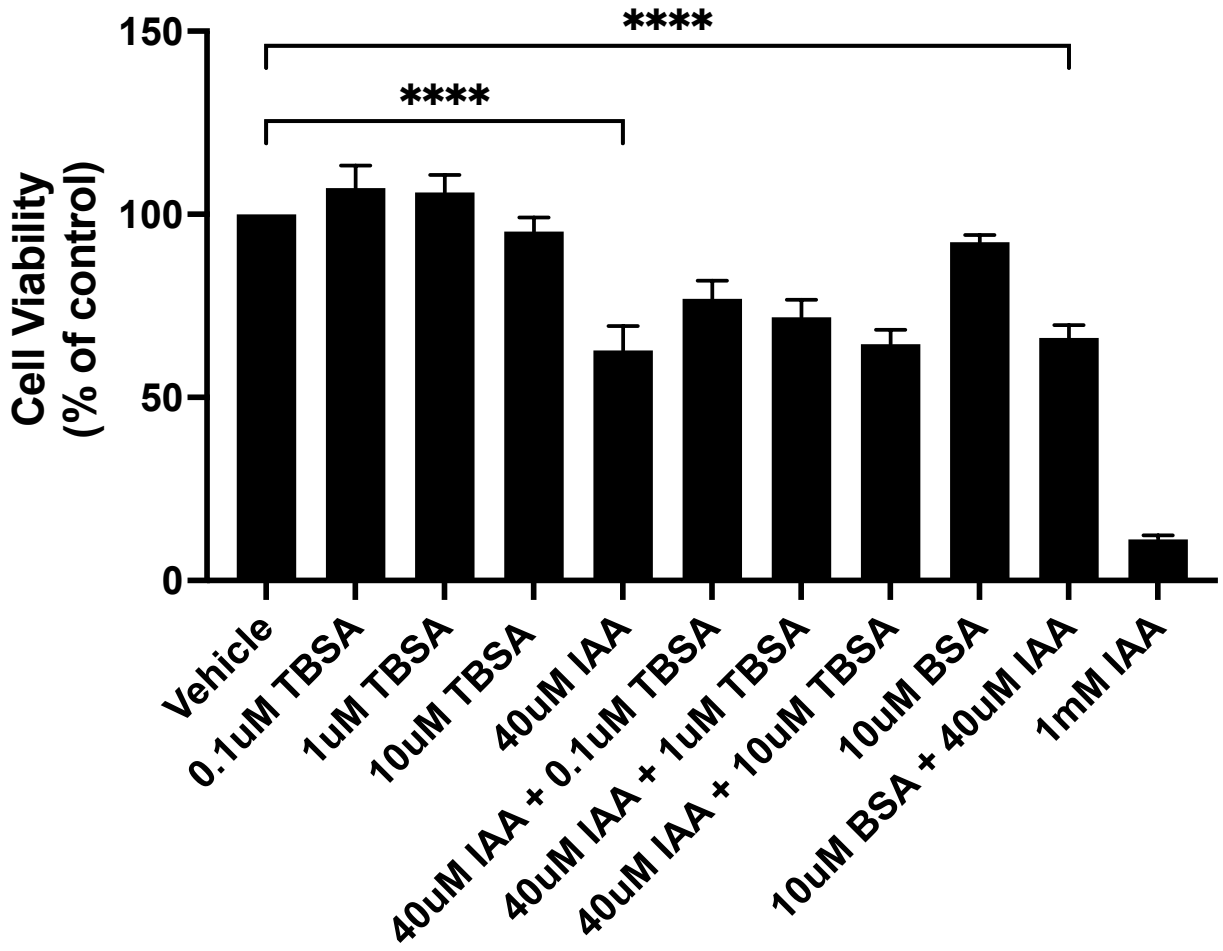

Figure 22. T-BSA Does Not Enhance the Cytotoxic Effect of IAA in Primary Astrocytes. Primary astrocytes were plated at 10,000 cells/well 24 hrs prior to treatment. Cells were co-treated with IAA and T-BSA for 6hrs. Treatment with 40uM IAA alone significantly decreased cell viability compared to vehicle control (10% CSFBS media) after 6hrs ($p<0.0001$). Co-treatment with T-BSA and 75uM IAA did not further reduce cell viability, relative to treatment with IAA alone. Treatment with T-BSA alone, at either 0.1, 1 or 10 uM, did not have any significant effect on cell viability. Co-treatment of 10uM BSA with 40uM IAA significantly decreased cell viability compared to vehicle control ($p<0.0001$). 1mM IAA served as a positive control for maximal cell death, while the 10uM BSA alone group served as the control for TBSA application. Cell viability was assessed using a calceinAM assay. Data were normalized to vehicle control which is set at 100% (n=3). ****: $p<0.0001$

Summary of Results.

Through the experiments completed, we learned that:

1) T-BSA by itself is generally non-cytotoxic to either the glioma cells or primary astrocytes
2) The ability of T-BSA to sensitize (or augment) cytotoxicity associated with IAA or TMZ is most noted in C6 glioma cells
3) T-BSA did augment the effect of TMZ in the chemotherapy-resistant T98G cells, but timing of the treatment appeared to be a key factor.
4) T-BSA did not sensitize primary astrocytes to either IAA or TMZ

CHAPTER FOUR

DISCUSSION

The data presented here demonstrate that each cell type utilized in this research exhibit distinct responsiveness to not only the cytotoxic compounds IAA and TMZ, but also to the putative membrane androgen receptor (membrane AR) ligand, T-BSA. Indeed, one major goal of this research was to explore the how ligand binding to the putative membrane AR influences cell viability/vulnerability in the presence of a cytotoxic insult. As previously mentioned, it has been suggested that ligand binding to the membrane AR may induce damaging effects in certain cell types, enhancing the effects of an introduced cytotoxic insult [20,21,22]. In the presence of two distinct types of cellular insult, our lab has previously shown that binding to the membrane AR through membrane-impermeable compounds is associated with a decrease in the phosphorylation of the pro-survival signaling endpoints, ERK1/2 and Akt, as well as reduced expression of the DNA repair enzyme, MGMT. Based on these observations, we anticipated that suppressing inherent cell protection programs (e.g., reducing the activity of pro-survival pathways, or reducing DNA repair capacity) would render cells more vulnerable. As such, we explored whether this mechanism could be exploited in treating certain cancers. We focused on models of glioma since our earlier work was done in cellular models of astrocytomas. Accordingly, we evaluated the effect of T-BSA in C6 glioma cells, two models of glioblastoma (A172 and T98G cells), and primary astrocytes. The latter was important as we wanted to know if T-BSA could promote cell death, or vulnerability to a toxic insult, only in tumor models and not “normal”

astrocytes. If so, there would be a particularly valuable utility to developing this approach further as it would target the tumor cells while leaving normal, healthy cells unscathed.

In addition to evaluating the effects of T-BSA in different cell models, this project also explored whether the "damage-promoting" effect of ligand binding to the membrane AR is dependent on the mechanism of cytotoxicity. Therefore, we hypothesized that if the primary mechanism of the membrane AR is to reduce those defenses that mitigate metabolic dysfunction and/or oxidative stress, it is likely that T-BSA will have a significant effect in IAA-induced cytotoxicity; alternatively, if the major mechanism of T-BSA (in a given cell type) is to reduce DNA repair capacity (as previously discovered in C6 cells), then there may be more robust consequences to T-BSA treatment when applied in the presence of TMZ, an alkylating agent that leads to DNA damage.

Using C6 cells, the current data shows that T-BSA exacerbates cell vulnerability to cytotoxic insult induced by both IAA (Fig. 2) and TMZ (Fig. 4). C6 cells co-treated with 10uM T-BSA and 10uM IAA for 6 hours showed a 43.7% decrease in cell viability as compared to cells treated with 10uM IAA alone over the same amount of time (Fig. 2). C6 cells co-treated with 10uM T-BSA and 900uM TMZ for 48 hours showed a 22% decrease in cell viability as compared to cells treated with 900uM TMZ alone over the same time course. Based on the data utilizing C6 cells, it appears that the mechanism by which the membrane AR "promotes" cell vulnerability may broadly impact cell survival pathways, weakening cell viability signaling to render the cell vulnerable to either cytotoxic insult. However, the data also suggests that the membrane AR's capacity to induce these death-promoting effects may be dependent on cell type. Although both A172 and T98G cells show concentration-dependent responsiveness to both IAA and TMZ, the current data suggests that the presumed simultaneous presence of T-BSA with

either IAA or TMZ does not enhance cell sensitivity to either cytotoxic insult in A172 (Fig. 7, Fig. 14) or T98G (Fig. 8, Fig. 16) cells. Despite C6 cells being well-accepted as a model for glioma [48, 49], limitations to using a cell model derived from chemically-induced tumors from a non-human species remain. In particular, one literature review discussing chemically-induced tumor cell lines notes that cell lines passaged over long periods of time may undergo genetic drift *in vitro*, potentially resulting in overt sensitivity to certain experimental treatment and subsequent overestimation of treatment efficacy [50]. Alternatively, the A172 cell line was established from glioblastoma of a 53-year-old man, and the T98G cell line is derived from the T98 cell line obtained from glioblastoma of a 61-year-old man [51]. Given that the type of tumor a cell line is derived from may influence cell phenotype and genotype, thus contributing to cell sensitivity to experimental treatments, this could be one explanation as to why treatment of T-BSA with IAA or TMZ appears to have greater efficacy in C6 cells compared to A172 and T98G cells. Despite the difference, one inference that we argue is important is that the C6 cells express some cellular/molecular "feature" that renders the cell more responsive to the effects of T-BSA. Based on this, it would be interesting to further explore the identity of this mediator of T-BSA. The challenge here is compounded by the fact that the membrane AR is yet uncloned. However, preliminary data from the Singh lab suggests that progesterone receptor membrane component-1 (Pgrmc1) might be a component of the membrane AR. Accordingly, and as one possible hypothesis for future consideration, is that Pgrmc1 levels differ between the cell types, and higher Pgrmc1 levels predict not only T-BSA binding to the cell surface of cells, but also the extent to which T-BSA can promote the cytotoxicity of metabolic/oxidative insults.

The second aim of this research was to determine if presumed ligand binding to the membrane AR sensitized A172 and T98G cells to treatment with TMZ. While treatment with

TMZ is considered standard treatment for glioblastoma, the regimen often involves prolonged systemic exposure and harsh side-effects, which can have negative impacts on patient quality of life. Improvements to current TMZ treatment should aim to reduce systemic toxicity to, in turn, reduce side effects, and improve patient quality of life. As previously discussed, ligand binding to the membrane AR using T-BSA did not enhance cell sensitivity to cytotoxic insult under conditions of co-treatment of T-BSA with TMZ. As an alternative approach to simultaneous introduction of T-BSA and TMZ, we wanted to determine if pre-treatment with T-BSA prior to treatment with TMZ would differentially influence cell viability, as compared to co-treatment. The rationale for this approach was to consider that reduction in the levels of MGMT, the DNA repair enzyme previously noted to be decreased in response to T-BSA, might take some time. As such, the system would "benefit" from pre-treatment so as to reduce DNA repair defenses prior to the administration of the chemotherapeutic. Alternatively, given that the membrane AR remains uncloned and thus, not fully characterized, the presumed binding dynamics of T-BSA to the membrane AR remain undefined as well. Accordingly, it is possible that the optimal binding of T-BSA to the membrane AR occur over time such that effects of T-BSA might not be observable through co-application of T-BSA and the "insult". To explore this pre-treatment model, A172 and T98G cells received an initial treatment with T-BSA for 24 hours before the existing media was spiked with various concentrations of TMZ. While our paradigm of treatment in the co-application experiments (i.e., when T-BSA and TMZ were administered concomitantly) was to treat for 48 hours with TMZ, we also wanted to look at the response to a shorter duration of treatment with TMZ (i.e., 24 hr) in the pre-treatment paradigm where T-BSA was applied 24 hr prior to TMZ. In A172 cells, TMZ did not effectively induce cell death after 24 hours, supporting our assertion that 48 hours is the more ideal timeframe to evaluate the cytotoxic

effects of TMZ. Additionally, pre-treatment with T-BSA for 24 hours prior to spiking media with various concentrations of TMZ for an additional 24 hours did not induce any significant decreases in cell viability (Fig. 9). We interpret that under conditions where no obvious signs of cell dysfunction are noted (e.g., some, even modest, level of cell death), T-BSA in and of itself does not have death-promoting effects. This is consistent with that noted in many of the figures presented.

What was somewhat unexpected is that, in A172 cells, treatment with T-BSA for 24 hours prior to spiking media with various concentrations of TMZ for an additional 48 hours also did not enhance the cytotoxic effects of TMZ (Fig. 10). We did, however, identify what we conclude as a "statistical anomaly" in Figure 10, where A172 cells pre-treated with 1uM of T-BSA for 24 hours prior to treatment with 200uM TMZ for an additional 48 hours showed a paradoxical increase in cell viability compared to that noted with cells treated with 200uM TMZ alone. However, there was not a statistical difference between cells treated with 200uM TMZ alone and the vehicle control, which means that there was no effect of the presumptive toxin (TMZ). As such, we don't place much weight on the statistically significant difference between the 1uM T-BSA+200 uM TMZ and the 200 uM TMZ alone groups.

While the effect of TMZ on T98G cells was relatively smaller, relative to that seen in A172 cells, a result that is expected and based on reports that T98G cells are generally TMZ-resistant, we did notice a small but significant effect of 500uM TMZ on cell viability after both 24 hours and 48 hours. Pre-treatment with T-BSA for 24 hours prior to spiking media with various concentrations of TMZ for an additional 24 hours did show an enhanced reduction in cell viability in T98G cells relative to TMZ alone (Fig. 11). However, this enhanced cell vulnerability was no longer seen in cells pre-treated with T-BSA for 24 hours prior to spiking

media with TMZ for an additional 48 hours (Fig. 12). These findings may suggest that in T98G cells, there may be an optimal time window in which T-BSA is capable of effectively engaging the membrane AR while in the presence of TMZ, rendering cells more sensitive to TMZ for a certain amount of time. Nevertheless, the data are encouraging from the standpoint that at least one time point revealed a positive, death-enhancing effect of T-BSA, in the chemotherapeutic-resistant T98G cell line. With respect to the A172 cells, the lack of observable enhancement of TMZ-induced cell death by T-BSA may reflect more on the experimental tool used, recognizing the caveat of not having a complete understanding of the binding dynamics of T-BSA (or the stability of T-BSA in solution) in the various cell types studied. Future studies in the Singh lab are aimed at addressing this, including using a new membrane AR – targeting ligand currently under development.

In an effort to assess if the mechanism of the membrane AR is dependent on the type of cytotoxic insult in human glioblastoma cells, we also looked at whether treatment with T-BSA enhanced the cytotoxic effect of IAA in A172 and T98G cells. While A172 cells were responsive to IAA (Fig. 13), co-treatment with T-BSA and IAA did not significantly enhance the cytotoxic effect of IAA, compared to cells treated with IAA alone (Fig. 14). It is possible in this case that treatment with 75uM IAA resulted in a "floor effect" in A172 cells, even though we chose the EC_{50} value determined from a concentration-response analysis (see Figure 13). In this scenario, we could argue that cell death associated with ligand binding to the membrane AR cannot be differentiated from the cytotoxic effect of IAA alone.

Due to variable response of T98G cells to IAA, we were not able to discern whether T-BSA alters IAA-induced cytotoxicity. As such, we believe the differences we noted between the IAA group and the IAA + T-BSA groups are also a "statistical anomaly". That is, even though

co-treatment with T-BSA and 75uM IAA showed significant increases in cell viability relative to treatment with 75uM IAA alone, it is important to note that 75uM IAA alone did not effectively induce cell death in these experiments (i.e., no statistical difference relative to vehicle control). Interestingly, there is literature which suggests that introducing hypoxic conditions, which results in an increase in oxidative stress (which also occurs in response to treatment with IAA), may stimulate tumor cells to increase exosome release which is thought to be beneficial to tumor cell survival and homeostasis [52, 53, 60]. Several studies have suggested that altered exosomal activity in tumor cells may help these cells more effectively eliminate chemotherapeutic and/or cytotoxic drugs, potentially contributing to treatment resistance and drug-resistant phenotypes [61, 62]. Although the current research did not further explore the potential changes in exosome release in response to treatment with IAA, this is one area of research that could be extended in the future.

The final aim of this research was to determine if the suggested "death-promoting" effect associated with binding to the membrane AR enhances cell death in "normal" astrocytes, or if this phenomenon was specific to cancer cells. Ideally, primary astrocytes, a cell model not derived from tumor cells, do not respond to ligand binding to the membrane AR in a damaging manner; the benefit here being selective targeting of cancer cells, as most current chemotherapies result in cell death in both the target cell (i.e., the cancer cell) as well as non-cancer cells. Based on the current data, primary astrocytes were seen to be similarly responsive to treatment with TMZ (Fig. 17) compared to human glioblastoma cell lines. In primary astrocytes, co-treatment with 1uM T-BSA and 200uM TMZ led to a statistically significant decrease in cell viability compared to cells treated with 200uM TMZ alone, although the effect was relatively modest (13.3% difference) (Fig. 18). Again, we wanted to explore whether the effect of pre-treatment

with T-BSA differentially impacted cell viability compared to co-treatment with T-BSA and TMZ. Pre-treatment with T-BSA for 24 hours prior to treatment with TMZ at various concentrations for either 24 or 48 hours did not lead to any significant changes in cell viability. Similar to the results seen with A172 cells, treatment with TMZ alone did not effectively induce cell death in primary astrocytes after 24 hours (Fig. 19), whereas treatment with TMZ alone for 48 hours did significantly reduce cell viability at a higher TMZ concentration, yet this effect was not enhanced by pre-treatment with TMZ prior to spiking the existing media with TMZ (Fig. 20). Although the current data does not fully support that ligand binding to the membrane AR enhances tumor cell sensitivity to TMZ, it does support the idea that this phenomenon is not seen in "normal", non-cancer cells. Since our lab has previously found evidence that primary astrocytes do express the membrane AR, this difference in responsiveness to ligand binding is perhaps due lower expression of the membrane AR in primary astrocytes, compared to tumor cells.

Again, to assess if the mechanism of the membrane AR is dependent on the type of cytotoxic insult, we looked at the effect of co-treating primary astrocytes with T-BSA and IAA. While primary astrocytes are responsive to IAA (Fig. 21), co-treatment with T-BSA and IAA did not significantly enhance the cytotoxic effect of IAA as compared to cells treated with IAA alone, further suggesting that presumed ligand binding to the membrane AR does not promote cell death in primary astrocytes. However, these findings were contrary to what we expected; as mentioned, our lab has previously found evidence that treatment with DHT-BSA, as a presumed ligand to the membrane AR, does enhance the cytotoxic effects of IAA in primary astrocytes [20]. Perhaps this difference in membrane AR effect can be attributed to the use of different presumed ligands (DHT-BSA versus T-BSA). This difference may be important when

considering using the membrane AR as a therapeutic tool; in the case of using IAA as a cytotoxic insult, it may be beneficial to use T-BSA rather than DHT-BSA in order to avoid inflicting enhanced vulnerability in non-tumor primary astrocytes.

Overall, the current research aimed to further explore and understand the mechanisms by which ligand binding to the putative membrane AR may influence cell vulnerability in response to cytotoxic insults in different glioma cell models. The data here suggests that the "death-promoting" effect of the membrane AR vary across cell types as a result of differential cellular phenotypes and genotypes. This difference in effectively enhancing cell vulnerability can be beneficial when considering the lack of effect seen in "normal" primary astrocytes. Mechanistically, we view one logical future direction as one that determines whether a correlation exists between the effectiveness of T-BSA as a cell death sensitizing/promoting agent and the binding of T-BSA to the cell surface. It is possible that cell types, such as C6 cells have higher levels of (surface) expression of the membrane AR (as assessed by flow cytometry, or quantitative immunocytochemistry), allowing these cells to be more readily influenced by the presence of a presumed ligand. However, assessing differences in membrane AR expression remains a difficult at the present time since the membrane AR is yet uncloned, even though it has been characterized pharmacologically [20,21].

When considering the exploitation of the membrane AR as a therapeutic tool to improve the efficacy of chemotherapeutics, it is beneficial that non-cancer cells (i.e. primary astrocytes) are not responsive to T-BSA in a way that would render cells more vulnerable to TMZ. Looking at cell models for human glioblastoma, the data does not present strong findings to suggest that ligand binding to the membrane AR significantly improves the efficacy of the chemotherapeutic TMZ. One potential way to expand this research and better understand how engaging the

membrane AR may influence cell viability would be to include additional time points at which cell viability is assessed, in order to determine if there is a distinct time window in which treatment with T-BSA and TMZ is the most optimal. As seen in the T98G cells, it appears that there is a timepoint of about 24 hours in which T-BSA effectively influences cell viability. It is possible that the effective timepoint for T-BSA in A172 falls outside of the 24- or 48-hour mark, which could explain why there was no significant effects seen in the current data.

There are also clear limitations of using T-BSA (or its related analog, DHT-BSA) as the sole experimental tool to evaluate the functional consequence of binding to the putative membrane AR. First, it is a large and bulky compound (due, in particular, to the BSA moiety) and would likely pose significant challenges as a clinically-used therapeutic. Our lab is currently addressing potentially novel membrane AR ligands that may be more readily translatable. We also recognize that it is not clear whether T-BSA is an agonist or antagonist (or inverse agonist), and as such, we consistently define T-BSA as a "ligand" to the membrane AR.

Though we recognize that there remain questions surrounding the biology of the membrane androgen receptor in various glial cell models, the current research does contribute significantly to our current knowledge surrounding the membrane AR. Most notably, the data generated as part of this Thesis project supports the conclusion that engaging the membrane AR in C6 cells enhances the cytotoxic effects of both IAA and TMZ, rendering cells more sensitive to distinct types of insult. This research also suggests that various glioma cell models are differentially responsive to presumed ligand binding to the membrane AR, possibly attributed to relative differences in the abundance of functional membrane AR. Future studies to better define optimal conditions/methods for engaging the membrane AR may serve as a step forward in improving chemotherapeutic treatment with TMZ by increasing cell vulnerability, specifically in

cancer cells. Lastly, the current research supports the idea that the mechanism by which the membrane AR promotes damaging effects can be used to intentionally target tumor cells, as primary astrocytes were not shown to be negatively impacted by presumed engagement of the membrane AR. This was a significant finding, in contrast to the experiments conducted with the tumor models. This current research therefore provides further information that should be expanded upon with the aim of improving current therapeutic tools used in the chemotherapeutic treatment of glioblastoma, a disease for which the prognosis is exceedingly poor.

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VITA

The author, Emma Yuen, was born in Oak Bluffs, Massachusetts on December 25, 1995 to Leigh Ann and Richard Yuen. She attended Saint Michael's College in Colchester, Vermont where she earned a Bachelor of Science in Neuroscience with a minor in Chemistry in May 2018. After graduation, Emma matriculated into the Loyola University Chicago Graduate School Biomedical Science program and began her graduate education in the Neuroscience program under the mentorship of Dr. Meharvan Singh.