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# Molecular Components of a Putative Membrane Androgen Receptor: Implications for Treating Glioma

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# MOLECULAR COMPONENTS OF A PUTATIVE MEMBRANE ANDROGEN RECEPTOR: IMPLICATIONS FOR TREATING GLIOMA

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ABSTRACT

Gliomas, which are tumors of any number of glial cell types in the brain or spinal cord, can be malignant or benign. They represent approximately 33% of all central nervous system tumors. Glioblastoma multiforme (GBM) is a fatal, grade 4 glioma, that accounts for nearly 14.5% of all central nervous system tumors. Current treatment options for GBM, however, remain ineffective, with the median survival period following diagnosis being only 15 months. Thus, there is an urgent and unmet need for the development of novel therapeutics for the treatment of GBM and other gliomas. Previously, our laboratory showed that testosterone, conjugated to bovine serum albumin (T-BSA), increased the sensitivity of rat C6 glioma cells to the mixed oxidative and metabolic insult, iodoacetic acid (IAA). Given that T-BSA does not readily cross the plasma membrane, we interpreted that this effect was a consequence of binding to a "receptor" on the plasma membrane. We thus referred to this entity as the putative membrane androgen receptor (mAR). Given that binding to this mAR increased cell death, we proposed that exploitation of this novel mechanism could enhance the efficacy of chemotherapeutics. However, a major limitation to translating our prior discovery to developing novel glioma therapeutics is the lack of understanding of the molecular identity of the mAR. Based on work conducted by our laboratory in evaluating the efficacy of sigma-2 receptor-active compounds, and the recent literature that hypothesizes the "molecular makeup" of the sigma-2 receptor, we hypothesized that the mAR consists of PGRMC1 and transmembrane protein 97 (TMEM97). Furthermore, our working model predicts that PGRMC1 and/or TMEM97 expression positively correlates with the efficacy of T-BSA in augmenting cytotoxicity, and

further, that reduced expression of either PGRMC1 or TMEM97 will reduce the efficacy of T-BSA. To test our hypothesis, we proposed two specific aims. First, we evaluated the expression of PGRMC1 and TMEM97 in the C6 and other cellular/in vivo models of glioma (including models of glioblastoma), in which we have evaluated the efficacy of T-BSA and determine if the expression of PGRMC1 and TMEM97 correlates with the efficacy of T-BSA. In Aim 2, determined whether siRNA-mediated knockdown of either or both of these genes diminishes the efficacy of T-BSA in enhancing the cytotoxicity of IAA. The data obtained from this study have provided insight into the identity of the putative membrane androgen receptor, and have also paved the way for future studies aiming at specifically targeting and promoting cell death in glioma cells. Accordingly, these data implicate a novel, and potentially druggable target to improve the efficacy of chemotherapy drugs used to treat such gliomas that include glioblastoma multiforme.

# CHAPTER ONE

# REVIEW OF THE RELEVANT LITERATURE

## Glioma

Gliomas are the most common central nervous system primary tumor, accounting for 30-40% of all intracranial tumors [1]. They also comprise 80% of primary malignant tumors. Gliomas are tumors of glial cells that include astrocytes, oligodendrocytes, and ependymal cells [2]. They most often occur during middle age, with incidences peaking between 40 to 65 years of age [1]. Astrocytomas (glial tumors of astrocytes), the focus of this thesis, account for nearly 80-85% of all gliomas, and the average age at diagnosis is 30-45 years [3]. Oligodendrogliomas, on the other hand, are found to be most common in adults between the ages of 35 and 44 but can also be found in children, usually between the ages of 6 and 12 [4]. Ependymomas account for roughly 5% of adult intracranial gliomas and 10% of childhood tumors. 50-70% of ependymomas are managed effectively with surgery and radiation, but many tumors recur [3].

Astrocytomas are classified into four grades. Grade 1 and grade 2 gliomas are termed low-grade gliomas because cells remain well-differentiated and can usually be resected [2]. Grade 1 gliomas are benign and total resection typically leads to full recovery. Grade 2 gliomas are low-grade malignancies. They cannot be resected entirely because of early diffuse infiltration into the surrounding brain areas [5]. Up to 70% of grade 2 gliomas progress to grade 3 and 4 astrocytomas within 5-10 years of diagnosis [3]. Grade 3 and 4 gliomas are deemed high-grade because the cells have become primarily undifferentiated, and the prognosis is usually much worse. Glioblastoma Multiforme (GBM) is a dominant subtype of a grade 4 glioma, accounting

for approximately 55% of gliomas. A diagnosis of GBM is essentially the kiss of death because the survival rate is very low, such that survival up to five years is noted only in 5% of patients diagnosed [2]. GBM can be broken down into two categories. Primary GBM, most found in elderly patients, presents *ex novo* with no previous symptom presentation or pre-existing low-grade glioma. Secondary GBMs are rarer and often occur in patients younger than 45. They usually result from the progression of a lower-grade astrocytoma [5].

**Clinical Presentation**

With tumor formation comes an onslaught of symptoms. The symptoms of gliomas vary, but they are often related to the grade and location of the tumor. The symptoms of low-grade gliomas often include a more subtle progression of neurological deficits. In contrast, high-grade gliomas often display more acute neurological symptoms in addition to other clinical manifestations [6]. Common symptoms seen in glioma patients include seizures, headaches, neurological deficits, and cognitive dysfunction [2]. The chances of showing cognitive dysfunction increase as age and tumor grade increase, and the effect seems to be more profound when the tumor is located/in the dominant hemisphere [6].

**Genetic Mutations in Glioblastoma Multiforme (GBM)**

With such severe symptoms and poor prognosis, it raises the question of what factors drive the formation and aggressiveness of gliomas. There are several acquired genetic mutations found in gliomas. For example, p53 and Rb (retinoblastoma tumor suppressor) pathways regulate normal cell cycle progression but are very susceptible to being mutated and/or inactivated in GBM [5]. The p53 tumor suppressor is critical for maintaining genomic stability, and it is frequently found to be inactivated in high-grade astrocytic gliomas. The compromised genetic stability can result in an increased number of mutations and a more malignant phenotype [7].

Disruptions in both p53 and Rb signal transduction pathways allow glioma cells to avoid apoptotic and growth-inhibitory signals [8]. PTEN, a lipid and protein phosphatase responsible for dephosphorylating phosphatidylinositol-3,4,5-trisphosphate (PIP3K), is another gene found to be mutated in glioma [9]. A loss of functional PTEN leads to continuous activation of PI3K, which in turn, leads to the accumulation of PIP3 and, thus, activation of the Akt pathway. The Akt pathway promotes cell survival [9], and thus promotes the resistance of the tumor from insults (e.g., chemotherapeutics) that could cause cell death.

Isocitrate dehydrogenase 1 (IDH1) is another gene whose mutations have been found in 5.6% of primary glioblastomas and more than 76% of secondary glioblastomas. IDH1 is a metabolic enzyme whose primary role is to convert isocitrate to α-ketoglutarate, while also producing NADPH as a byproduct. When mutated, IDH1 catalyzes that NADPH-dependent reduction of α-ketoglutarate to produce 2-hydroxyglutarate, which is an oncometabolite that promotes tumorigenesis [10].

Epidermal Growth Factor Receptor (EGFR) overexpression has been noted in many malignant gliomas, astrocytomas, oligodendrogliomas, and GBM. EGFR signaling pathways are involved in cell cycle progression, angiogenesis, inhibition of apoptosis, and tumor cell motility and metastasis. The EGFRvIII mutant, which has an exon 2-7 deletion, is the most common mutation that is present in 45% of EGFR-amplified GBM. This mutant does not recognize epithelial or transforming growth factors and remains constitutively activated, which leads to the continuous activation of the MAPK and PI3-K/Akt pathways [11, 12]. The dysregulation of the RAS-ERK pathway serves as a major stimulus in the development of many cancers, and hyperactivation of the ERK cascade, in particular, is evident in most cancers [13]. One commonality among most of the mutations noted in GBM is that they contribute to the

overactivation (or persistent activation) of those cell signaling pathways involved in the regulation of cellular survival and proliferation.

**Current Treatment Options for Glioma/Glioblastoma**

Patients diagnosed with high-grade gliomas typically undergo surgical resection, adjuvant chemoradiation, and six cycles of temozolomide (TMZ) [14]. TMZ is a chemotherapeutic drug used to treat newly diagnosed GBM. TMZ is a lipophilic alkylating agent that promotes cell cycle arrest and results in DNA methylation that in turn, leads to "nicks" in DNA, and apoptosis [15, 16]. Despite being the standard of care, it is not the most effective, as 55% of GBM patients are resistant to this treatment because of the expression of MGMT ($O^6$-methylguanine-DNA methyltransferase), a DNA repair enzyme that reverses the methylation elicitecd by TMZ. High doses of TMZ have been shown to induce toxicity [15]. In some cases, TMZ administration may cause mutations in surviving tumor cells that could result in the limited effectiveness of immunotherapy strategies [15]. If these mutations occur in critical pathways, they may also contribute to the adverse effects observed in TMZ chemotherapy [17].

Even with extensive therapeutic intervention, the recurrence of high-grade gliomas is often unavoidable. While surgery followed by radiation and chemotherapy is the standard practice for first-time tumor occurrence, there is no established treatment protocol after tumor recurrence [14]. Bevacizumab, an inhibitor against vascular endothelial growth factor-A (VEGF-A), inhibits angiogenesis and can be used in treating recurrent GBM; however, this approach is not very successful [18]. In an attempt to treat gliomas more effectively, targeted therapies are being developed. As discussed previously, there are many mutations that can occur in GBM, such as EGFR overexpression, IDH mutations, and PTEN loss; however, even attempts to target these mutations have not been successful [19].

Given the prevalence of IDH mutations, five distinct therapy classes are in preclinical and/or clinical development: direct mutant IDH inhibitors, bromodomain inhibitors, histone deacetylase inhibitors, nicotinamide phosphoribosyl transferase inhibitors, and poly-ADP ribose polymerase inhibitors. Because studies on these therapies have been done in small samples, it remains unclear how these might benefit patients diagnosed with GBM; however, based on initial findings, the approaches listed above may prove only modestly effective. The primary issues with most of these therapies have a small therapeutic window and have severe adverse effects [20]. Tumor-specific antigens, that serve as the basis for tumor vaccines, can also be used as a therapeutic tactic to elicit an immune response against GBM in patients. One such vaccine being tested is a vaccine against IDH1 (R132H), and termed IDH1-vac. In mice, this vaccine has been shown to induce specific therapeutic helper T-cell responses effective against tumors with specific IDH1 mutations [21]. Clinical trials have started in humans with some success, but there appears to be evidence of pseudoprogression associated with inflammatory responses to the vaccine [21].

Erlotinib and Gefitinib are small molecules that inhibit EGFR [22]. Gefitinib, the more studied drug, is a tyrosine kinase inhibitor that inhibits the EGFR pathway, resulting in cell cycle arrest in G1, and apoptosis. While studies show that this drug significantly inhibited constitutively activated EGFR and inhibited MAPK and AKT phosphorylation, that effect was not seen in all glioma models [12]. And while both of these drugs produce little toxicity, they show poor therapeutic efficacy in patients with recurrent or progressive malignant glioma [22].

**Role of Androgens in Glioma**

Given a male bias for gliomas, including GBM, androgens may be involved in glioma etiology and/or pathophysiology. Androgens are steroid hormones, structurally derived from

cholesterol, whose reproductive effects influence the development, differentiation, and growth of the male reproductive tract [23]. Androgens exert organizational and activational effects. Organizational effects are often permanent and occur during a critical window in development, typically associated with brain differentiation between males and females. Activational effects are more temporary and occur later in adulthood, primarily playing a role in stress responses, sexual behavior, emotions, etc. [24, 25].

Androgens, such as testosterone and its metabolite 5α-dihydrotestosterone (DHT), elicit their effects by binding to the androgen receptor. In fact, androgen receptors are expressed more in the tumor tissue of glioma patients relative to non-cancer patient tissue samples. The expression increases as the tumor grade progresses, irrespective of sex [26].

**The Classical Androgen Receptor**

The androgen receptor was first discovered and partially purified in 1969, and since then many discoveries have been made elucidating its properties and function [27]. The classical androgen receptor is a member of the nuclear receptor superfamily, and consists of three domains (or regions): the variable N-terminal transcriptional regulation domain, the conserved DNA-binding domain (DBD), and the ligand binding domain. A hinge region connects the DNA-binding domain and the ligand-binding domain [28]. The ligand binding domain is, as the name implies, the site where the androgen binds. In the absence of androgen, this part of the AR is bound by chaperone proteins, to include heat shock proteins. A ligand-independent signal sequence called AF-1 is on the N-terminal domain, which is necessary for maximal AR activity. On the ligand binding domain, there is a ligand-dependent signal sequence called AF-2, which is required to form the coregulator binding sites and mediate the interaction between the ligand binding domain and the N-terminal domain (**Figure 1**). A nuclear localization signal is located

between the DBD and the hinge region, and a nuclear export signal is in the ligand binding domain [29]. The nuclear localization sequence is responsible for nuclear import of the AR, while the nuclear export signal is responsible for export of the AR back into the cytoplasm.

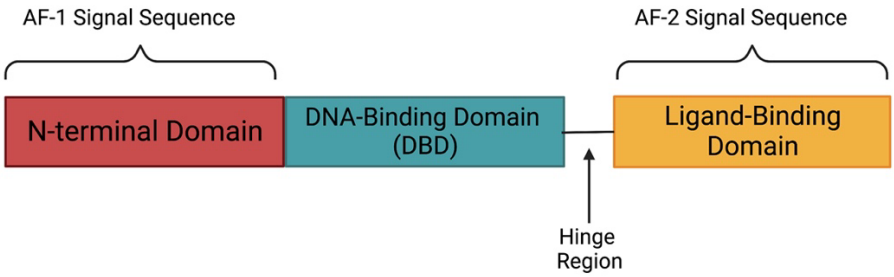


**Figure 1. Functional Domains of the Classical Androgen Receptor.** The classical androgen receptor contains three domains: the variable N-terminal domain, the highly conserved DNA-binding domain, and the ligand-binding domain.

The characteristics mentioned above are specific to the classical androgen receptor. This receptor is primarily located in the cytoplasm, interacting with cytoskeletal proteins, heat shock proteins, and other chaperones. Upon binding to testosterone or DHT, a conformational change occurs that dissociates the androgen receptor from the heat shock proteins, allowing it to interact with coregulators that bind to the nuclear localization signal. The androgen receptor then translocates to the nucleus, where it dimerizes and binds to androgen response elements on the promoter region of target genes. Once attached, the receptor recruits the transcriptional machinery, enzymes, and coregulators necessary to initiate transcription [30]. This represents the classical, or "genomic" action of androgens, but androgens have also been shown to exert their effects in a "non-genomic" fashion by activating second messenger pathways [31]. For example, in C6 cells, a rat glioma cell line, binding of DHT to the classical androgen receptor resulted in increased phosphorylation of ERK1/2 and Akt in minutes, a time course that is generally inconsistent with the "genomic" mechanism of steroid hormone action. ERK1/2 are key effectors of the cytoprotective MAPK pathway, and Akt is a vital component of the cell growth and

survival regulating PI3K pathway (**Figure 2**). Increased phosphorylation of these components facilitates cell survival, growth, and proliferation. While testosterone exerts a protective effect, higher levels have been shown to induce neural apoptosis [32]. The lack of neuroprotective effects at higher concentrations suggests that another mechanism may be involved in mediating androgen signaling.

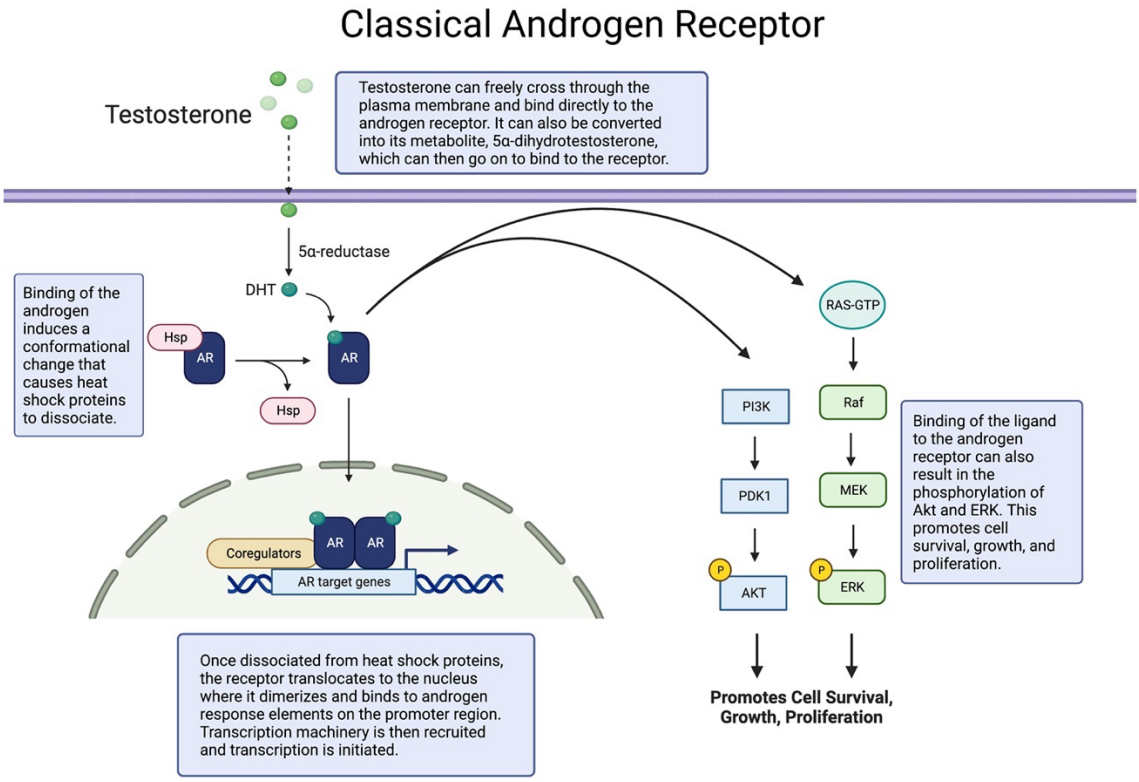


**Figure 2. Genomic and Non-Genomic Actions of the Classical Androgen Receptor.** The binding of androgens to the androgen receptor can elicit either genomic or non-genomic effects. The genomic pathway involves binding to the receptor, configuration changes, translocation to the nucleus, dimerization, binding to androgen response elements on the promoter region, and recruitment of transcription machinery, ultimately leading to the initiation of transcription. The non-genomic pathway involves phosphorylating downstream components, like ERK and Akt, of survival, growth, and proliferation promoting pathways.

**Membrane Androgen Receptors (mAR)**

For years, we have known that receptors for gonadal steroid hormones exist both intracellularly (cytoplasm and nucleus) and on the cell surface – i.e., membrane receptors. While the identity of some of these membrane receptors has been elucidated, properties of the membrane androgen receptor remain speculative. The presence of a putative membrane androgen receptor was first discovered in the Atlantic croaker by the laboratory of Peter Thomas [33]. Other presumptive membrane androgen receptors, based largely on findings from studies describing the efficacy of testosterone conjugated to bovine serum albumin (T-BSA), were also described in prostate cancer cells and breast cancer cells [34-36]. Our laboratory was the first to describe a putative membrane androgen receptor in glial cells [32, 37]. Since then, the membrane androgen receptor has been better characterized such that some are thought to consist of ZIP9, a zinc transporter, with another defined as a potential splice variant of the classical androgen receptor, termed AR45 [38, 39].

Rather than through the classical mechanism of action, involving the nuclear steroid hormone receptor and subsequent direct regulation of gene expression, membrane androgen receptors can elicit rapid effects, to include effects on intracellular signaling pathways. For example, binding of testosterone or DHT to the mAR results in the release of intracellular calcium, which affects protein kinases, such as MAPK (ERK) and Akt [30]. As noted above, the Singh laboratory has characterized one such putative mAR in astrocytes, showing that when DHT-BSA (DHT conjugated to bovine serum albumin) is bound to it, it elicits a decrease in the phosphorylation of ERK and Akt [32]. Further experiments from our laboratory showed that DHT-BSA binding to the mAR made cells more vulnerable to such mixed metabolic and oxidative insults as iodoacetic acid (IAA), a glycolytic inhibitor [40]. **These** results supported

our working model that the mAR is associated with an increased vulnerability of cells to a cytotoxic insult [41]. This membrane androgen receptor-mediated antiproliferative, proapoptotic effect is not only limited to the brain, as it has also been observed in prostate cancer cells. This led to our laboratory's working hypothesis that activation of intracellular signaling cascades elicited by binding to the mAR may enhance the cytotoxic effects of chemotherapeutics.

**Current Gap in Knowledge**

A fundamental limitation in our ability to exploit the mAR for the purposes of treating different cancers is that the molecular composition of the mAR remains unknown. While our understanding of the molecular mechanisms associated with the function of the classical androgen receptor is quite mature, the precise nature/composition and function of the mAR in the brain remains poorly understood. Progress toward a more complete understanding of the nature of the mAR will be critical to determining if this might indeed be a potentially druggable target for the treatment of various cancers, including GBM.

The insight we have to date into the potential "makeup" of the mAR include preliminary data from our laboratory, showing that Progesterone Receptor Membrane Component 1 (PGRMC1) may be a constituent. PGRMC1 is a heme-binding protein that appears to be involved in various cell and tissue functions, including heme homeostasis, cancer, cytochrome P450 activity, protein quality control, and female reproduction. It has a predicted single-pass transmembrane domain and has been found to localize in the endoplasmic reticulum [42] and plasma membrane. PGRMC1 is overexpressed in many tumors, thus representing a crucial biomarker for cancer progression and a potential therapeutic target for inhibiting tumorigenesis [43].

In our laboratory, we used a bovine serum albumin-conjugated testosterone, T-BSA, as our mAR targeting compound, and we found that it augmented the cell death-inducing effect of the metabolic and oxidative insult iodoacetic acid (IAA). This cell death-promoting effect was inhibited upon the coadministration of a PGRMC1 receptor antagonist, AG205 (**Figure 3**).

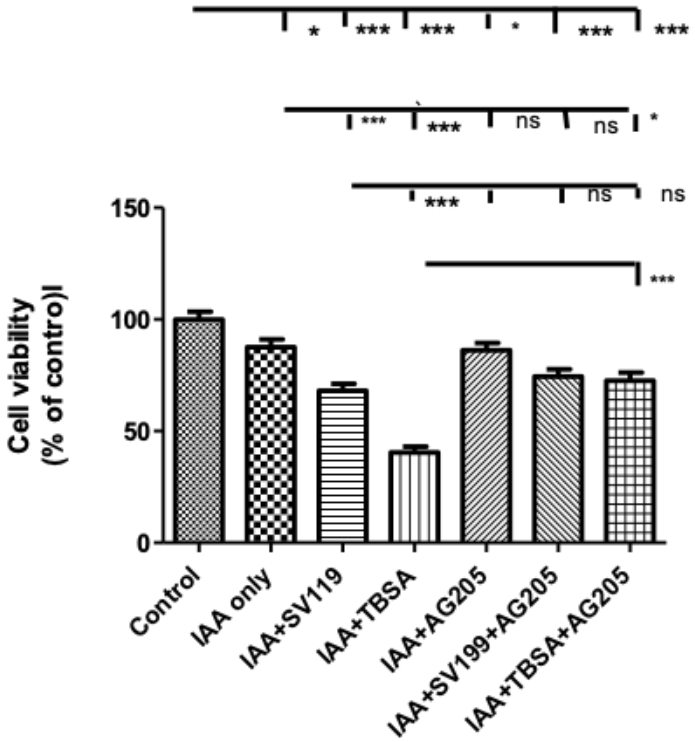


**Figure 3. The Sigma 2 Receptor Ligand, SV119, Elicits Effects Similar to T-BSA and the PGRMC1 Antagonist, AG205, Inhibits the Effects of T-BSA.** C6 Cells were treated with either vehicle control, 7 μM of IAA alone, 7 μM of IAA co-treated with 15 μM of the sigma-2 ligand SV119, 7 μM of IAA co-treated with 10 μM of T-BSA, 7 μM of IAA cotreated with AG205, 7 μM of IAA co-treated with 15 μM of SV119 and AG205, or 7 μM of IAA co-treated with 10 μM of TBSA and AG205. IAA alone presented a modestly significant decline in cell viability. SV119 co-administration significantly augmented the cytotoxic effect of IAA but not to the same degree as seen in the IAA and T-BSA treated group. Co-treating IAA with TBSA and AG205 nullified the cytotoxicity-enhancing effects of T-BSA. The data were normalized to the control which is set at 100%, and a one-way ANOVA was done followed by Tukey's post hoc analysis (*, ** or ***: $p<0.05$). Unpublished data from the Singh Laboratory.

Furthermore, a preliminary flow cytometry experiment showed that the surface binding of FITC (a fluorophore)- labeled T-BSA on astrocytes is displaced by AG205 (**Figure 4**). Given only a

partial reduction of T-BSA-FITC labeling of the cell surface, we hypothesized that the mAR may also be comprised of other constituents.

Other clues regarding the molecular composition of the mAR were derived from the sigma-2 receptor field, and serves as an important foundation for the hypothesis of my thesis project.

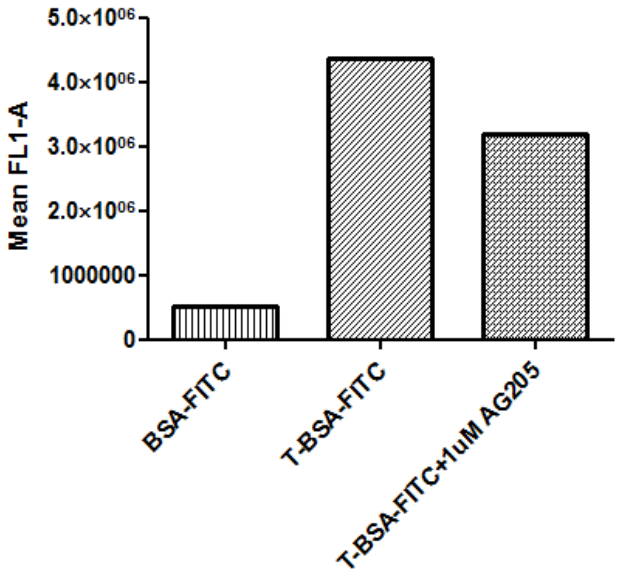


**Figure 4. AG205 Displaces T-BSA Binding to the Surface of C6 Cells.** The relative fluorescence of C6 cells incubated with 10 μM TBSA-FITC and T-BSA-FITC+AG205 were measured and compared to the fluorescence of C6 cells incubated with 10 μM BSA-FITC. The signal obtained from the use of BSA-FITC served as "background" due to nonspecific binding. The mean fluorescent intensity detected when cells were incubated with TBSA-FITC was at least partially displaced from the mAR when the PGRMC1 antagonist, AG205, was co-administered. Unpublished data from the Singh Laboratory.

**The Sigma-2 Receptor**

Sigma-2 receptor expression is a biomarker for tumor cell proliferation, as it is more prevalent in proliferative tumor cells than quiescent tumor cells [44]. The molecular composition of the sigma-2 receptor, like the mAR, remained unknown for a long time. Mass spectrometry sequence analysis and photoaffinity labeling experiments conducted by Xu et al., suggested that PGRMC1 was a strong candidate for the sigma-2 receptor. PGRMC1 and the sigma-2 receptor

had similar subcellular localizations. Furthermore, sigma-2 receptor ligand binding decreased when PGRMC1 siRNA was administered, and the binding increased when PGRMC1 was overexpressed. This supported the idea that the sigma-2 receptor ligands were binding to the PGRMC1 protein complex [43]. While these data do not prove that PGRMC1 is the sigma-2 receptor, it provides evidence that manipulating PGRMC1 expression levels alters the pharmacological properties of molecules interacting with the sigma-2 receptor [45], and further, was consistent with our data showing that the binding of T-BSA-FITC to a putative mAR was displaced by an inhibitor to PGRMC1. More recent studies have provided complementary information to suggest that transmembrane protein 97 (TMEM97) may represent an additional component of the sigma-2 receptor [45-47].

TMEM97 is a membrane-bound protein that is localized to lipid rafts. It can be found in lysosomes, endoplasmic reticulum, and the plasma membrane. TMEM97 is thought to play a role in human malignancies because it is upregulated in some tumors and downregulated in others [48]. Its overexpression has been seen in various cancers, including ovarian, breast, and lung, and in some cancers, the expression of TMEM97 has been correlated with worse prognosis and metastasis [47]. It also plays a critical role in cholesterol homeostasis and is a binding partner for NPC1, a lysosomal cholesterol transporter [47]. PGRMC1 has also been implicated in cholesterol trafficking, so TMEM97/sigma-2 and PGRMC1 may play a role in the same biochemical pathways within the cell [45]. Confocal microscopy studies show a similar distribution of PGRMC1, TMEM97, and LDLR (low-density lipoprotein receptor) on the plasma membrane, suggesting they form a protein complex [45].

There is additional evidence to suggest that PGRMC1 may be a component of both the mAR and sigma-2 receptor. Specifically, findings from our laboratory suggest that binding to

both receptors result in similar effects on cell viability. That is, co-administration of a sigma-2 receptor ligand, SV119, with iodoacetic acid (IAA), augmented IAA-induced cytotoxicity, similar to the effects of T-BSA seen in our lab (**Figure 3**). We interpreted this to suggest that both receptors may share common constituents.

**Hypothesis**

Taken together, I hypothesized that the putative membrane androgen receptor consists of a complex containing PGRMC1 and TMEM97. ***My working hypothesis was that PGRMC1 and/or TMEM97 expression positively correlated with the efficacy of the mAR ligand, T-BSA, in promoting IAA-induced cytotoxicity of a glioma model.***

# CHAPTER TWO

# MATERIALS AND METHODS

## Cell Culture

C6 cells (C6; ATCC, Manassas, VA) are a rat glioma cell line that was derived from a tumor induced by N-nitromethylurea [57]. Cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) without sodium pyruvate media (Gibco Laboratories, Montgomery County, MD) that was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. U118 cells (U118; ATCC), a glioblastoma cell line, were isolated from a male, 50-year-old patient [58]. The cells were grown and maintained in DMEM without sodium pyruvate media (Gibco) that was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. T98g cells (T98g; ATCC), a fibroblast-like glioblastoma cell line, were isolated from the brain tissue of a male, 61-year-old patient [59]. The cells were grown and maintained in Eagle's Minimum Essential Medium (EMEM) (ATCC) media that was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. A172 cells (A172; ATCC), a human glioblastoma cell line, were isolated from the brain tissue of a male, 53-year-old patient [60]. The cells were grown and maintained in DMEM without sodium pyruvate media (Gibco) that was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All cell lines were incubated at 37°C in a humidified incubator containing 5% carbon dioxide.

## Expression Profile Analysis

To evaluate the expression of PGRMC1 and TMEM97 mRNA in each of the cell lines, 2,000,000 cells were plated in a 100 mm petri dish (Falcon; FisherScientific, Pittsburgh, PA). After 48 hours, the cells were scraped into a lysis buffer, and total RNA was isolated using the Qiagen RNeasy Mini Kit, according to the manufacturer's instructions. Once the RNA was isolated, a Nanodrop was used to calculate the concentration of RNA obtained, through the measurement of the optical density of the sample at a wavelength of 260nm. A 260/280 ratio was then calculated to help determine that the RNA was of good quality (target ratio was 1.8). The RNA was converted to cDNA using MultiScribe Reverse Transcriptase (ThermoFisher, Waltham, MA), and real-time PCR was conducted to evaluate the expression of PGRMC1 and TMEM97 (Taqman Probes, ThermoFisher, Waltham, MA). The measurement of GAPDH (Thermo) mRNA was assessed from the same samples and used as a control/housekeeping gene. It served as the basis for normalizing the expression of the transcript of interest (e.g., PGRMC1) to GAPDH, and allowed us to account for potential variation in the amount of starting template loaded into the rt-PCR process. The real-time PCR data were analyzed by using $2^{-\Delta\Delta Ct}$ method developed by Livak and Schmittgen [61].

**IAA Concentration Curve**

24 hours before plating the cells in a white, opaque 96-well plate (ThermoFisher, Waltham, MA), the media was changed to DMEM without sodium pyruvate (Gibco), 10% charcoal-stripped fetal bovine serum (CFBS), 1% and penicillin-streptomycin. The switch to CFBS is done to minimize the presence of the steroid hormones. The next day, 10,000 C6 cells were plated in each well of the 96-well plate (100 uL total volume) and incubated for 24 hours, so they could attach to the plate. The following day, the cells were treated with 0.1 μM, 1 μM, 5 μM, 10 μM, 20 μM or 100 μM of Iodoacetic Acid (IAA; Sigma-Aldrich, St. Louid, MO). 6 hours

after treatment, the plate was equilibrated to room temperature and treated with CellTiter-Glo (Promega, Madison, WI). CellTiter-Glo is a luminescent viability assay that measures intracellular ATP production and is a surrogate measure of cell health/viability [49]. The plate was covered and shaken for 2 minutes and then allowed to sit at room temperature for an additional 10 minutes before readings were taken. The absolute luminescence was then measured using a FlexStation 3 plate reader (Molecular Devices, San Jose, CA). Based on these raw values, percent viability was calculated and an $EC_{50}$ value was determined using GraphPad Prism (Boston, MA)

**T-BSA Concentration Curve**

Once the $EC_{50}$ of IAA was determined, we evaluated the influence of increasing concentrations of testosterone conjugated to bovine serum albumin (1 μM, 5 μM, or 10 μM) (T-BSA; Sigma-Aldrich, St. Louid, MO), on IAA-induced cytotoxicity. This range of concentrations was based on those used in prior published studies [32, 41]. The plating density of 10,000 cells per well was used, as described above. 6 hours after treatment, the plate was equilibrated to room temperature and treated with CellTiter-Glo. The plate was covered and shaken for 2 minutes and then allowed to sit at room temperature for an additional 10 minutes before readings were taken. The absolute luminescence was measured using a FlexStation 3 plate reader. Based on these raw values, percent viability, relative to control, was calculated.

**siRNA Concentration Curve**

100,000 C6 cells were plated in 60 mm wells in 6-well plates. 2 mL of CFBS-containing media was added to each well. 24 hours after plating, the media was aspirated and replaced with 1 mL of CFBS-containing media. A 20uM stock of siRNA (Qiagen, Germantown, MD), was made for both PGRMC1 and TMEM97. The plated cells were then transfected using the

HiPerfect reagent (Qiagen, Germantown, MD) with various concentrations (0 nM, 25 nM, 50 nM, 75 nM, or 100 nM) of PGRMC1 or TMEM97 siRNA. For the 0 nM concentration of siRNA (serving as the control), 100 µL of serum-free media, 12 µL of HiPerfect reagent, and 25 µL of RNAse-free water were added to each well. For the 25 nM concentration, 100 µL of serum-free media, 12 µL of HiPerfect reagent, and 6.25 µL of siRNA were added to each well. For the 50 nM concentration, 100 µL of serum-free media, 12 µL of HiPerfect reagent, and 12.5 µL of siRNA were added to each well. For the 75 nM concentration, 100 µL of serum-free media, 12 µL of HiPerfect reagent, and 18.75 µL of siRNA were added to each well. For the 100 nM concentration, 100 µL of serum-free media, 12 µL of HiPerfect reagent, and 25 µL of siRNA were added to each well. The plates were then rocked for 20 seconds and incubated. 48 hours after transfection, the RNA was scraped, isolated, and converted to cDNA. Real-time PCR was conducted to measure the expression levels of PGRMC1 and TMEM97, as described above.

**T-BSA/IAA Co-treatment in Cells With Reduced Expression of PGRMC1 and TMEM97 Knockdowns**

Once the optimal knockdown siRNA concentrations for PGRMC1 and TMEM97 were determined, 2,000,000 cells were plated in 100 mm dishes (Falcon) and transfected with the respective PGRMC1 and TMEM97 siRNA concentrations identified from the siRNA concentration curve experiment. 48 hours after transfection, 10,000 cells per 100 µL of media per well were plated in a white, opaque 96-well plate. 24 hours after plating, the cells were treated with either a vehicle control, IAA alone, or co-treated with the IAA and T-BSA concentrations that previously produced the most cytotoxicity. A T-BSA alone treated group and a 100uM IAA alone group served as controls, with the latter serving as the positive control, resulting in maximal cell death. 6 hours after treatment, the plate was equilibrated to room temperature and

treated with CellTiter-Glo. The plate was covered and shaken for 2 minutes and then allowed to sit at room temperature for an additional 10 minutes before readings were taken. The absolute luminescence was measured using a FlexStation 3 plate reader. Based on these raw values, percent viability, relative to control, was calculated.

**Statistical Analysis**

"Percent of Control" was calculated by normalizing the raw, luminescence values to that of the control levels. The data were the result of at least three independent experiments and analysis for statistical significance was performed using an analysis of variance (ANOVA), followed by a Tukey's *post hoc* analysis to measure group differences. Using GraphPad Software, the data has been presented in bar graphs (average +/- standard error of the mean (SEM)).

CHAPTER THREE

RESULTS

## C6 Cells Have the Highest Expression of PGRMC1 and TMEM97

Our focus on the C6 cells for my project was based on an analysis of whether expression of PGRMC1 and/or TMEM97 was associated with the efficacy of T-BSA in enhancing the cytotoxicity IAA in the various glioma cell lines. Analysis of PGRMC1 mRNA and TMEM97 mRNA levels revealed that C6 cells had the highest expression levels, while U118 cells, T98g cells, and A172 cells showed similar, albeit lower expression levels, than that observed in C6 cells. Interestingly, the efficacy of T-BSA in augmenting IAA-induced cytotoxicity was greatest in C6 cells. We appreciate that expression of protein levels of PGRMC1 and TMEM97 would have been a nice complement to the mRNA analysis presented, but unfortunately, the quality of the antibodies we obtained were suboptimal, and prevented us from also assessing protein expression.

**Table 1. Relationship between PGRMC1 and TMEM97 Expression and the efficacy of T-BSA Efficacy in Glioblastoma Cell Lines**

| Cell Line | C6 | U118 | T98g | A172 |
|---|---|---|---|---|
| PGRMC1 | +++ | ++ | ++ | ++ |
| TMEM97 | +++ | ++ | ++ | ++ |
| Efficacy of T-BSA in Augmenting Cytotoxicity | Yes | n.s. | n.s. | n.s. |

*+++: $C_t$ value < 26
*++: $C_t$ value of 26.1-30
*+: $C_t$ value 30.1-34
*-: $C_t$ value > 35

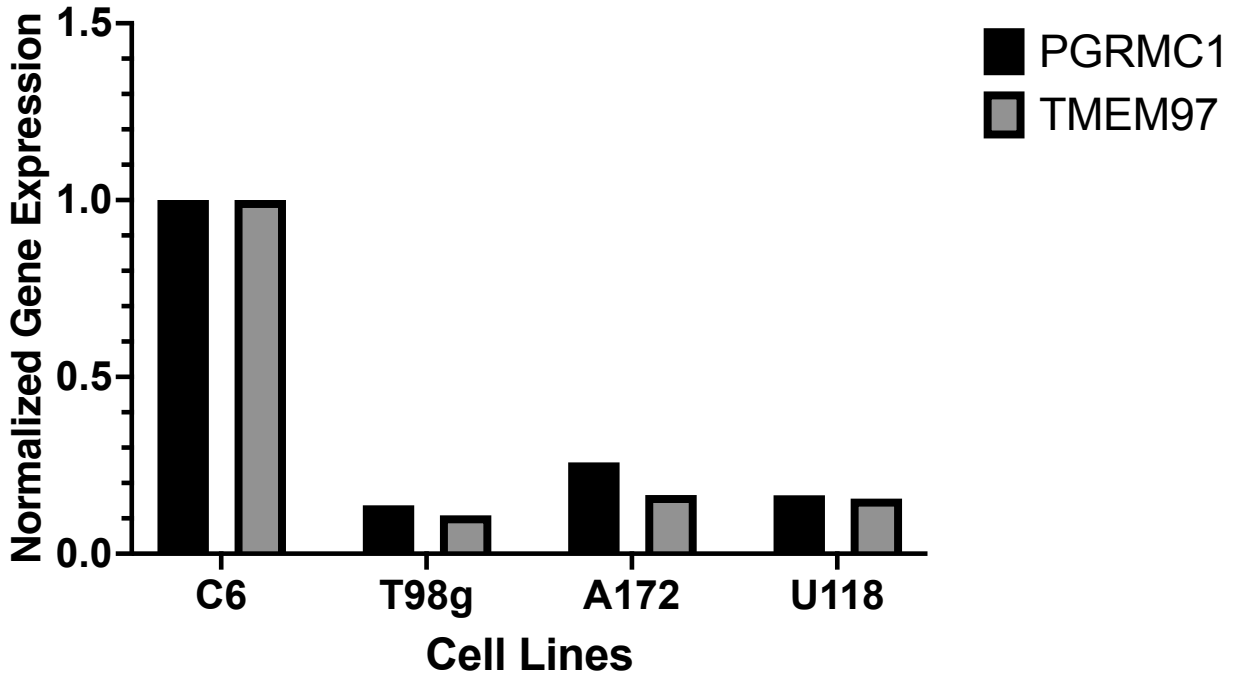


**Figure 5. The Expression of PGRMC1 and TMEM97 mRNA in Different Models of Glioma.** PGRMC1 and TMEM97 expression in the human glioblastoma cell lines (T98g, A172, and U118 cells) were relatively lower than that measured in the rat C6 glioma cells. Data were expressed relative to that in C6 cells, which is set at 1 (n=3).

**IAA Induces Cytotoxicity in C6 Cells**

After using data presented above to select our cell model, our next step was to determine the concentration of IAA that resulted in 50% cell death (which we defined as the $EC_{50}$). We used CellTiter-Glo to measure cell viability and created a concentration-response curve (**Figure 6**). Based on preliminary data, we used a cell density of 10,000 cells per well and a treatment incubation time of 6 hours. The data from the concentration-response curve showed that 10 μM of IAA resulted in approximately 50% cell death and was subsequently used as our $EC_{50}$ for IAA.

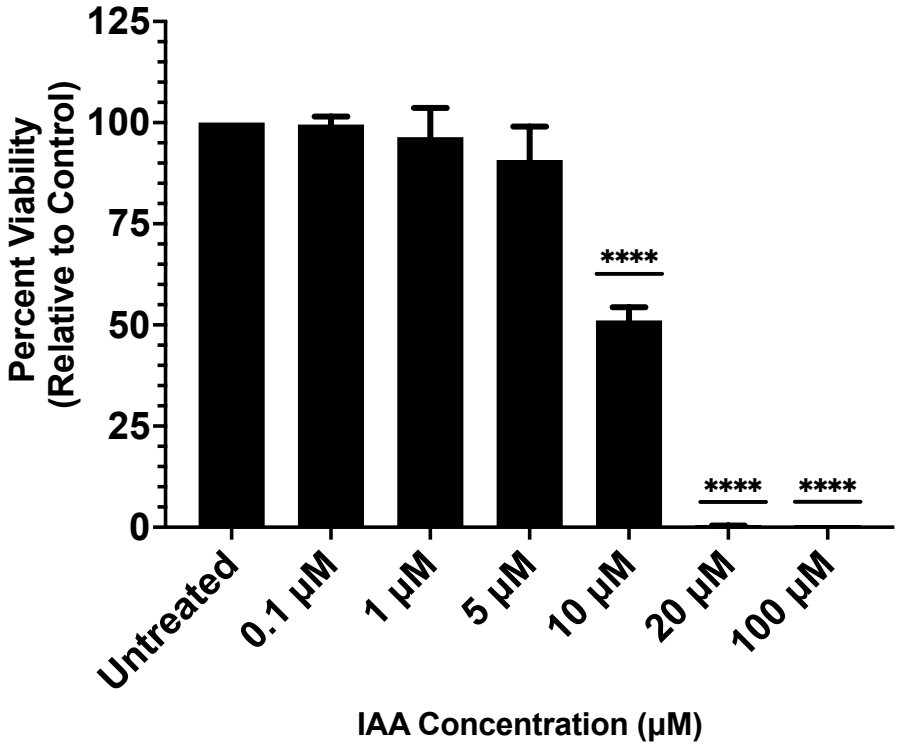


**Figure 6. Concentration-Response Curve for for Iodoacetic Acid (IAA)-Induced Cytotoxicity in C6 Cells.** 24 hours before IAA treatment, 10,000 C6 cells per well were plated in a white, opaque 96-well plate. Various concentrations of IAA were administered and incubated for 6 hours. The IAA concentration-response curve showed that 10 µM was the half-maximal effective concentration ($EC_{50} \pm$ S.E.M. from replicate experiments) needed to elicit significant cytotoxicity. CellTiter-Glo was used to assess cell viability. The data were normalized to the untreated control condition which is set at 100% (n=3), and a one-way ANOVA was conducted to analyze the results. (****: $p < 0.0001$)

**T-BSA Augments IAA-Induced Cytotoxicity**

Once an $EC_{50}$ for IAA was determined, the next step was determining the concentration at which T-BSA could augment the IAA-induced cytotoxicity. In this experiment, IAA, alone reduced cell viability to 63.15% of control. 1uM, 5uM, and 10uM of T-BSA were co-administered with 10uM of IAA to determine the most effective concentration to further augment cell death (**Figure 7**). The data showed that 1uM of T-BSA co-administered with IAA had no significant effect on cell viability relative to the vehicle control. We started to see significant augmentation when 5uM of T-BSA was co-administered (total cell death was approximately

47.13%). The most significant cytotoxicity augmentation was seen when 10uM of T-BSA was co-administered with 10uM of IAA (total cell death was about 51.51%). 1uM, 5uM, and 10uM of T-BSA were tested alone as a control and showed no significant effect on cell viability relative to the vehicle control. BSA, both alone and co-administered with IAA, was administered as a control for T-BSA, and it also had no significant effect on cell viability relative to the control.

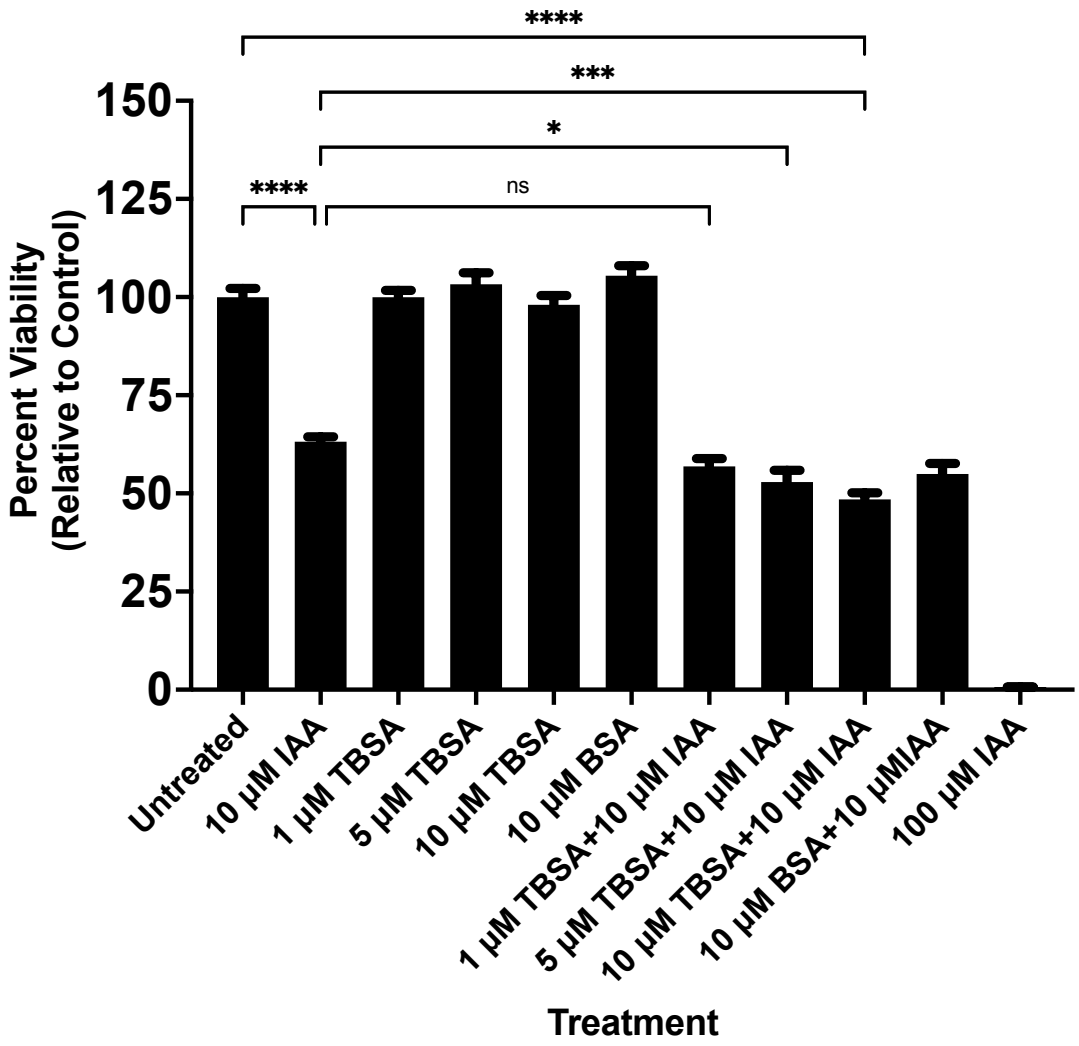


**Figure 7. Concentration-Response Curve Representing Efficacy of T- induced enhancement of Iodoacetic Acid (IAA)-Induced Cytotoxicity in C6 Cells.** 24 hours before treatment, 10,000 C6 cells per well were plated in a white, opaque 96-well plate. The cells were treated with either the vehicle control, 10 µM IAA alone, T-BSA alone [1 µM, 5 µM, or 10 µM], 10 µM BSA alone, 10 µM IAA co-administered with T-BSA [1 µM, 5 µM, or 10 µM], 10 µM IAA co-administered the 10µM BSA, or 100 µM IAA alone and left to incubate for 6 hours. Treatment with 10 µM IAA alone significantly reduced cell viability compared to the vehicle control. T-BSA and BSA treatments alone had no significant effect on cell viability relative to the vehicle control. No significant cell death augmentation was seen when 1 µM of T-BSA was co-administered with 10 µM of IAA. A modest, but statistically significant, augmentation in cell death was seen when 5 µM of T-BSA was co-administered with 10 µM of IAA. However, with 10 µM of T-BSA co-administered with 10 µM of IAA, there was greater cytotoxicity noted. 10 µM of BSA, serving as a control for T-BSA, co-administered with 10 µM of IAA had no significant effect on cell viability relative to the vehicle control. 100 µM of IAA was used as a positive control for maximum cell death. Cell viability was measured using a CellTiter-Glo assay. The data were normalized to the untreated control condition which is set at 100% (n=3). The bars represent mean $\pm$ S.E.M. A one-way ANOVA was conducted to analyze the results. (*,***, or ****: $p < 0.05$).

**siRNA Against PGRMC1 and TMEM97 Reduces Their Expression in a Concentration-Dependent Manner**

Given that at physiological PGRMC1 and TMEM97 expression levels T-BSA was able to augment IAA-induced cytotoxicity, our next set of experiments addressed whether the efficacy of T-BSA would change when PGRCM1 and TMEM97 expression is knocked down. To do this, we first had to determine the optimal concentration of the siRNA to use. We transfected C6 cells with various concentrations of PGRMC1 siRNA and isolated the RNA 48 hours after transfection. The RNA was reverse-transcribed to cDNA, and real-time PCR was performed to measure the level of knockdown in expression at each siRNA concentration (**Figure 8**). The data showed that 50nM of PGRMC1 siRNA consistently knocked down the expression of PGRMC1 by approximately 70%. We used the same protocol to evaluate the efficacy of the siRNA to TMEM97, and identify the ideal concentration to use for subsequent experiments (**Figure 9**). The data showed that 50nM of TMEM97 siRNA also consistently knocked down expression of TMEM97 by approximately 70%.

We also conducted experiments to ensure that the knockdown of one presumptive constituent of the mAR did not affect the other. A GAPDH, PGRMC1, and TMEM97 primer was used for each knockdown condition. The data showed that in the PGRMC1 knockdown condition, PGRMC1 expression was significantly decreased, but there was no effect on TMEM97 expression (**Figure 10**). Likewise, in the TMEM97 knockdown condition, TMEM97 expression was reduced considerably, but there was no effect on PGRMC1 expression (**Figure 11**).

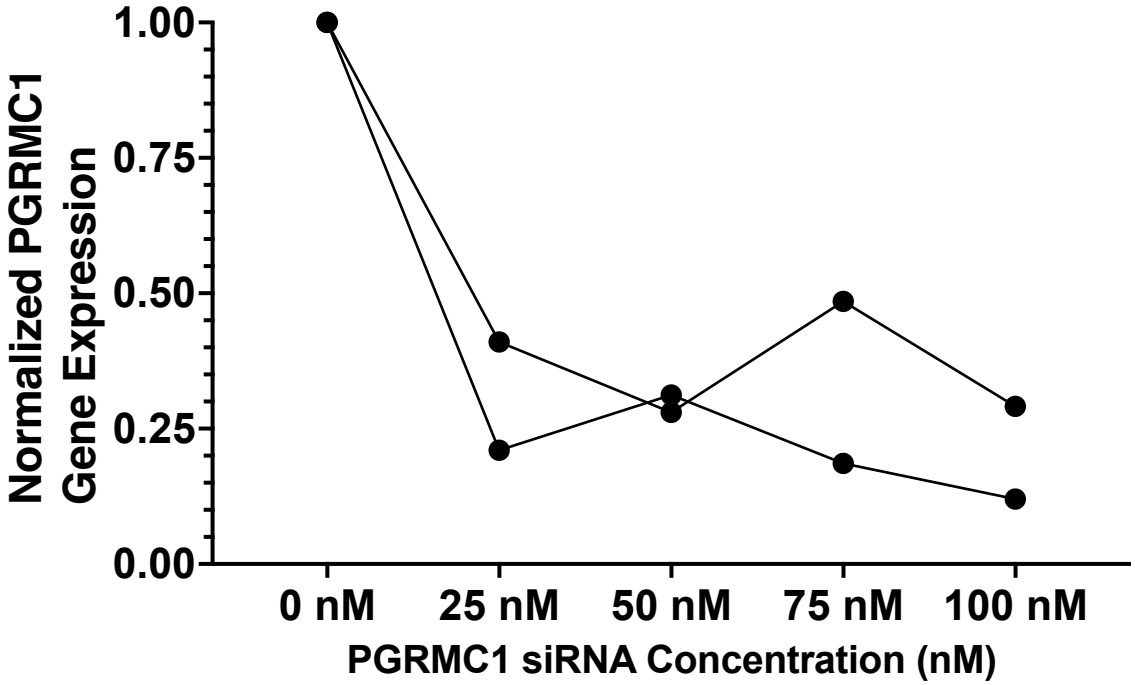


**Figure 8. Evaluation of the Optimal Concentration of siRNA to Elicit Knockdown of PGRMC1 Expression.** C6 cells were plated and transfected with either 0 nM, 25 nM, 50 nM, 75 nM, or 100 nM of PGRMC1 siRNA. Forty-eight hours after transfection, the RNA was isolated, converted to cDNA, and real-time PCR was done to measure the level of genetic knockdown achieved at each siRNA concentration. The most consistent knockdown was seen at the 50 nM concentration, where there was an approximately 70% knockdown in expression. The data were normalized to the 0 nM condition which is set at 1 (n=2).

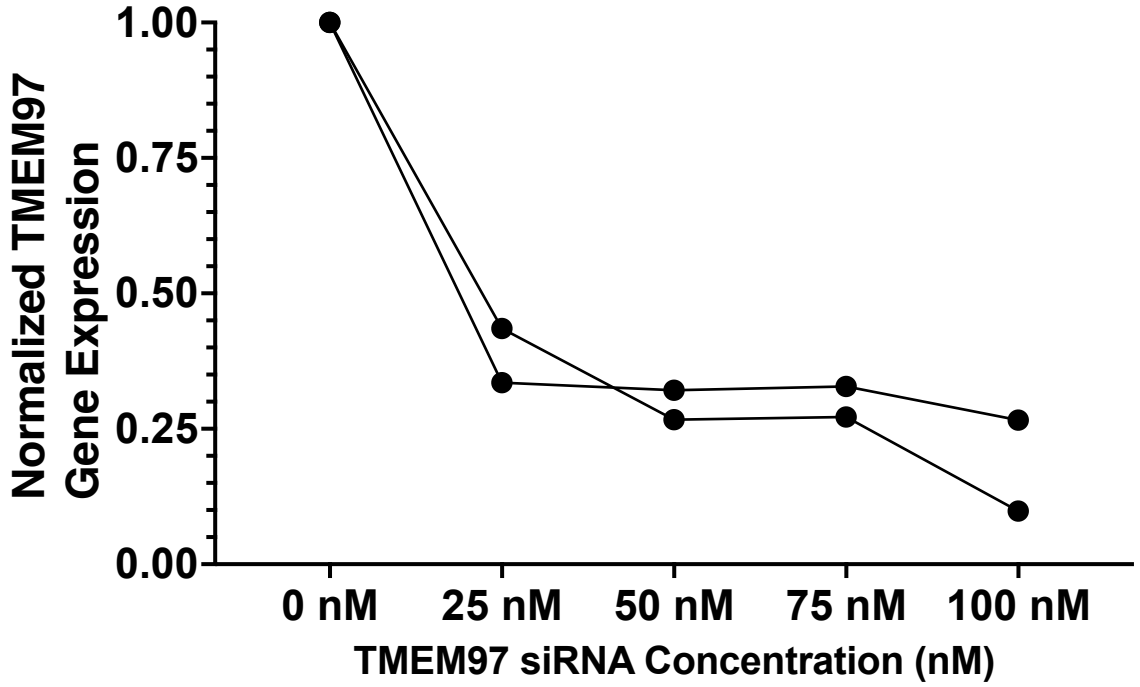


**Figure 9. Evaluation of the Optimal Concentration of siRNA to Elicit Knockdown of TMEM97 Expression.** C6 cells were plated and transfected with either 0 nM, 25 nM, 50 nM, 75 nM, or 100 nM of TMEM97 siRNA. Forty-eight hours after transfection, the RNA was isolated, converted to cDNA, and real-time PCR was done to measure the level of genetic knockdown achieved at each siRNA concentration. The most consistent knockdown was seen at the 50 nM concentration, where there was an approximately 70% knockdown in expression. The data were normalized to the 0 nM condition which is set at 1 (n=2).

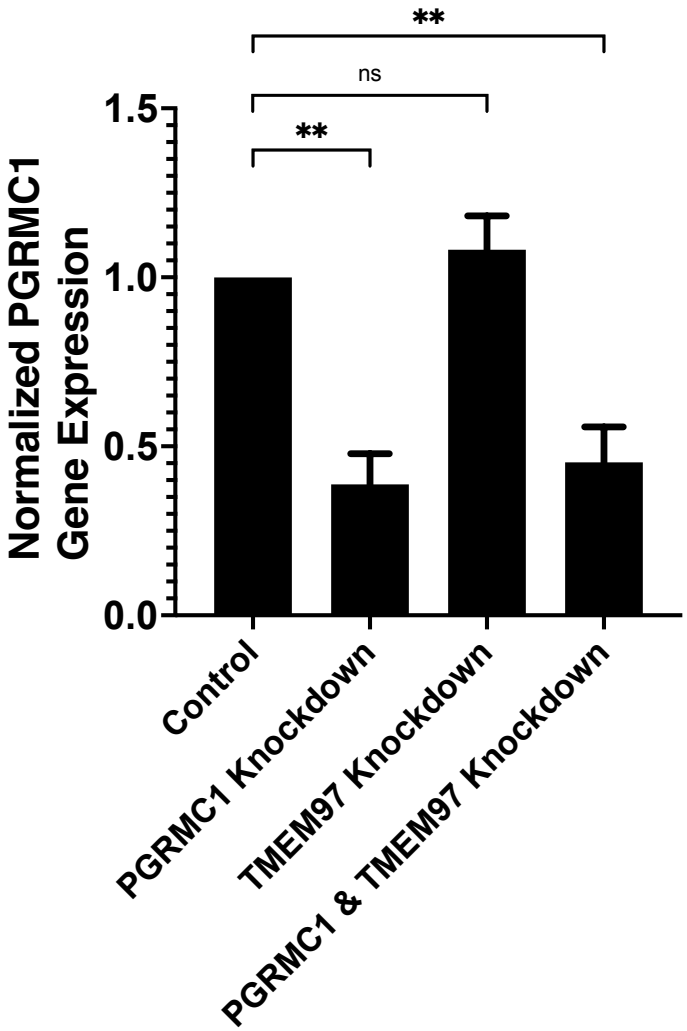


**Figure 10. The Effect of PGRMC1 Knockdown on TMEM97 mRNA Expression.** 2,000,000 C6 cells were plated in a 10 cm dish and transfected with 50 nM of PGRMC1 siRNA. After 48 hours, the RNA was isolated, converted to cDNA, and real-time PCR was done using a GAPDH, PGRMC1, and TMEM97 primers. The siRNA transfection resulted in a 61.23% reduction in PGRMC1 expression, but there was no significant effect on TMEM97 expression. The data were normalized to the untreated control condition which is set at 1 (n=3). The bars represent mean $\pm$ S.E.M. A one-way ANOVA was conducted to analyze the results. (**: $p < 0.005$).

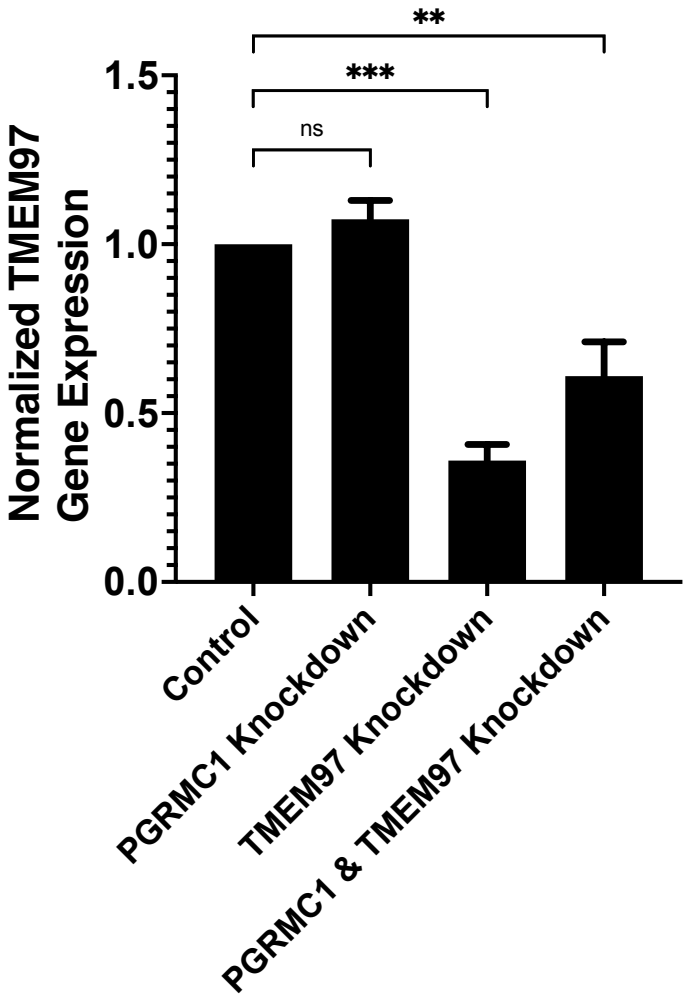


**Figure 11. The Effect of TMEM97 Knockdown on PGRMC1 mRNA Expression.** 2,000,000 C6 cells were plated in a 10 cm dish and transfected with 50 nM of TMEM97 siRNA. After 48 hours, the RNA was isolated, converted to cDNA, and real-time PCR was done using a GAPDH, PGRMC1, and TMEM97 primer. The siRNA transfection resulted in a 64.07% reduction in TMEM97 expression, but there was no significant effect on PGRMC1 expression. The data were normalized to the untreated control condition which is set at 1 (n=3). The bars represent mean $\pm$ S.E.M. A one-way ANOVA was conducted to analyze the results. (** or ***: $p < 0.005$).

**Knocking Down PGRMC1 and TMEM97 Alters the Efficacy of T-BSA**

Once we determined the PGRMC1 and TMEM97 siRNA concentrations that significantly knocked down their expression, we tested the effect of T-BSA in augmenting IAA-induced cytotoxicity in cells with reduced expression of PGRMC1, TMEM97, or reduced expression of both PGRMC1 and TMEM97. In the control condition, in which the cells were administered the transfection reagents without the siRNA, IAA alone reduced cell viability to 65.92% compared

to the vehicle control (**Figure 12**). As seen previously, administering T-BSA and IAA at the same time resulted in the augmentation of cell death and reduced total cell viability to 55.56%. While knockdown of PGRMC1 expression did not alter the efficacy of IAA, it did prevent the ability of T-BSA to augment IAA-induced cytotoxicity (**Figure 13**). Similarly, in cells transfected with TMEM97 siRNA, T-BSA did not augment IAA-induced cytotoxicity (**Figure 14**). Finally, we also assessed the consequence of dual knockdown (of both PGRMC1 and TMEM97). Similar to the individual gene knockdown experiments, T-BSA did not augment IAA-induced cytotoxicity (**Figure 15**).

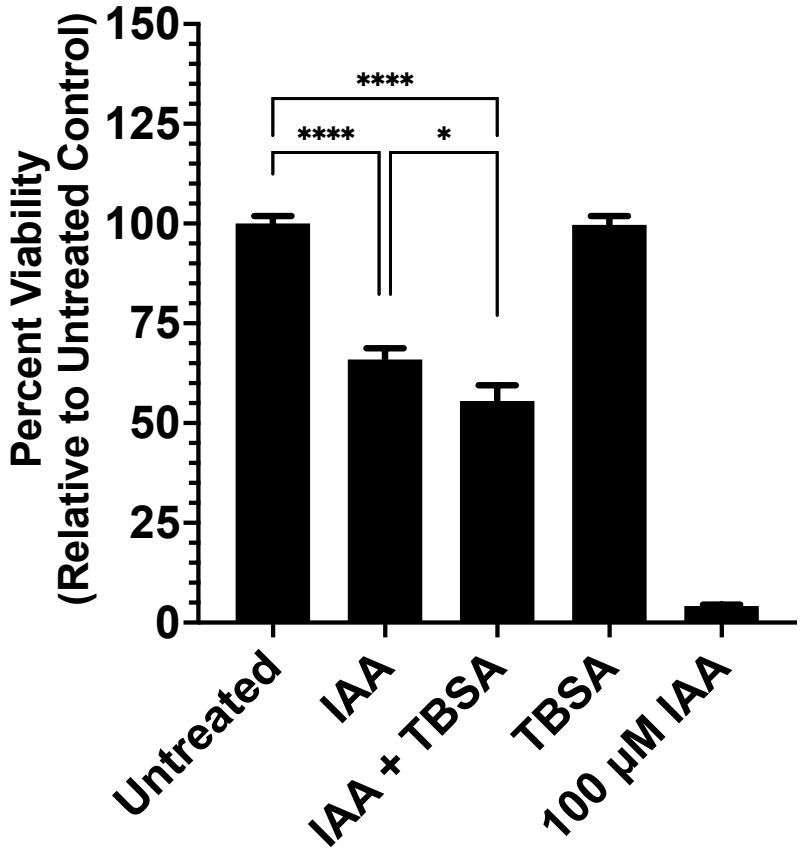


**Figure 12. T-BSA Enhances IAA-Induced Cytotoxicity in Cells Expressing Normal PGRMC1 and TMEM97.** IAA alone reduced cell viability to 65.92% compared to vehicle control. T-BSA augmented IAA-induced cytotoxicity by reducing cell viability to 55.56%. compared to vehicle control. T-BSA alone had no significant effect on cell viability compared to vehicle control. T-BSA and 100 µM of IAA were used as controls. The data were normalized to the untreated control condition which is set at 100% (n=3). The bars represent mean $\pm$ S.E.M. A one-way ANOVA was conducted to analyze the results. (* or ****: $p < 0.05$).

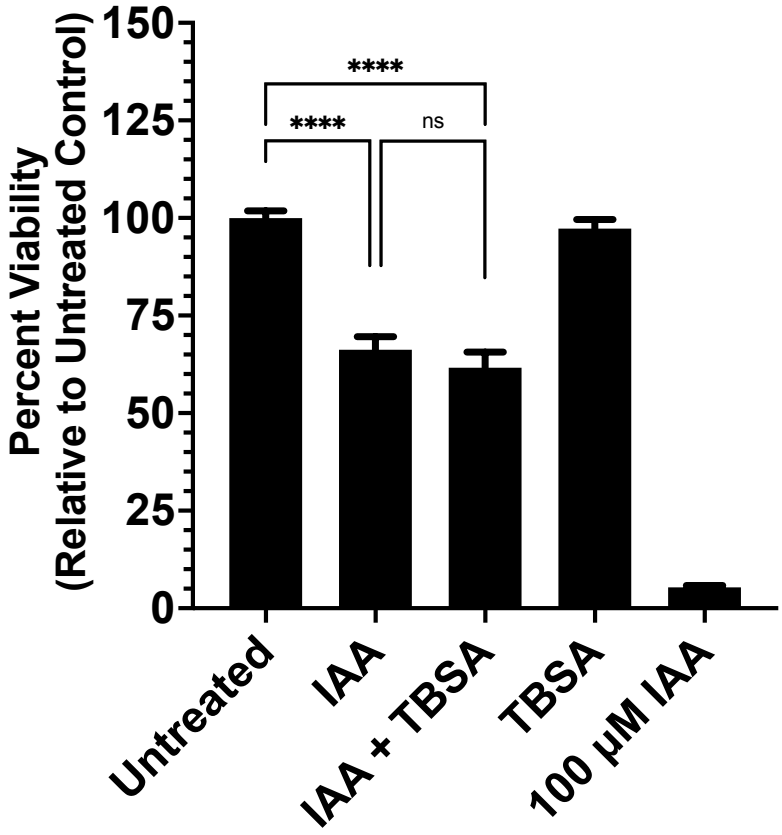


**Figure 13. Evaluation of T-BSA on IAA-Induced Cytotoxicity in Cells with Reduced Expression of PGRMC1.** IAA alone reduced cell viability to 66.25% compared to vehicle control. T-BSA did not augment IAA-induced cytotoxicity. T-BSA alone had no significant effect on cell viability compared to vehicle control. T-BSA and 100 µM of IAA were used as controls. The data were normalized to the untreated control condition which is set at 100% (n=3). The bars represent mean $\pm$ S.E.M. A one-way ANOVA was conducted to analyze the results (****: $p < 0.0001$).

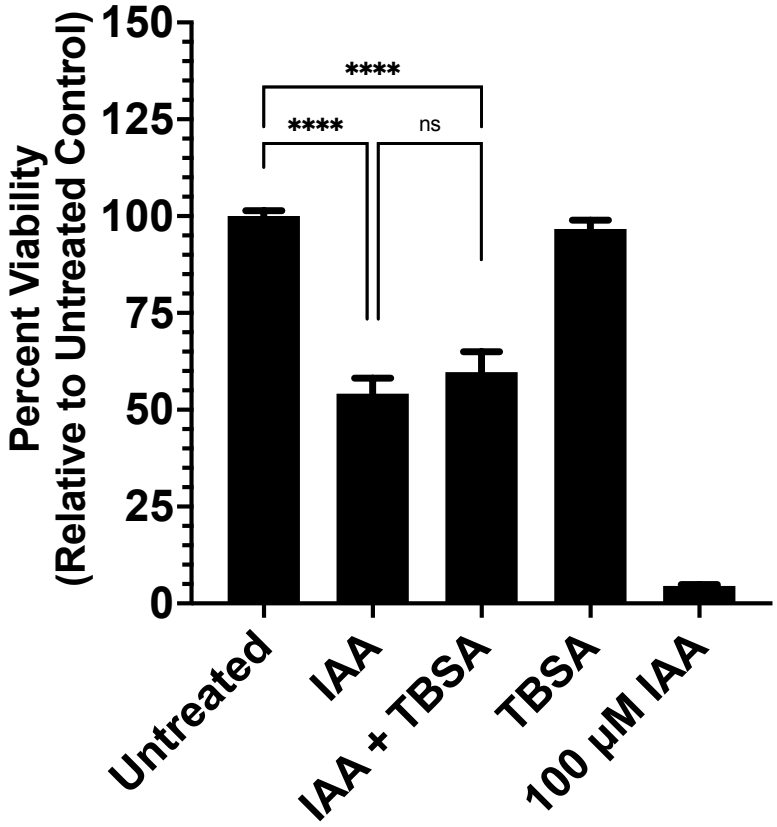


**Figure 14. Evaluation of T-BSA on IAA-Induced Cytotoxicity in Cells With Reduced Expression of TMEM97.** IAA alone reduced cell viability to 54.14 % compared to vehicle control. T-BSA did not augment IAA-induced cytotoxicity. T-BSA alone had no significant effect on cell viability compared to vehicle control. T-BSA and 100 μM of IAA were used as controls. The data were normalized to the untreated control condition which is set at 100% (n=3). The bars represent mean ± S.E.M. A one-way ANOVA was conducted to analyze the results (****: $p < 0.0001$).

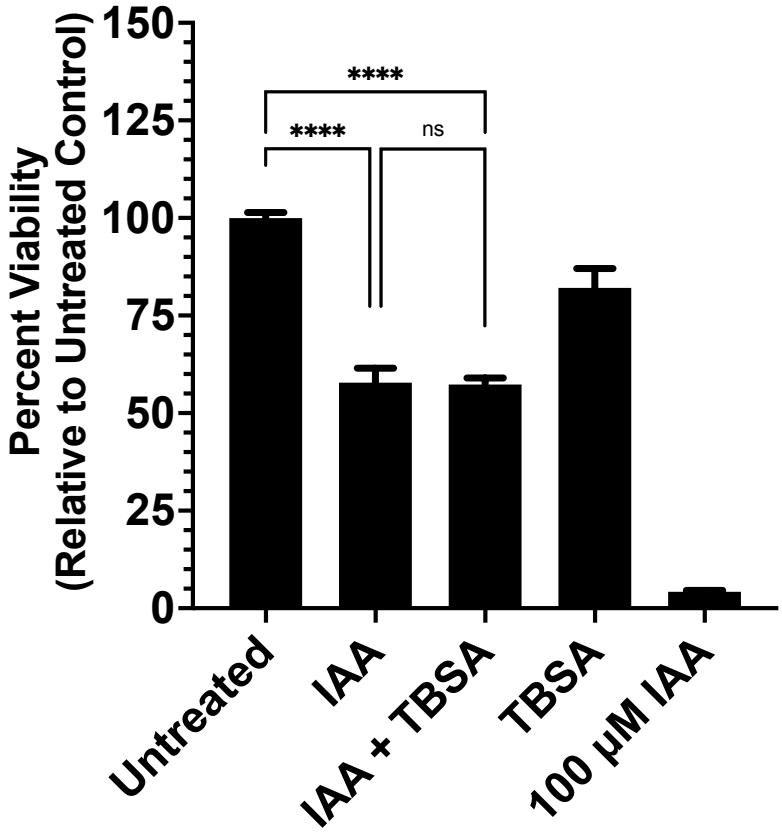


**Figure 15. Evaluation of T-BSA on IAA-Induced Cytotoxicity in Cells With Reduced Expression of Both PGRMC1 and TMEM97.** IAA alone reduced cell viability to 57.81% compared to vehicle control. T-BSA did not augment IAA-induced cytotoxicity. T-BSA alone had no significant effect on cell viability compared to vehicle control. T-BSA and 100 µM of IAA were used as controls. The data were normalized to the untreated control condition which is set at 100% (n=3). The bars represent mean $\pm$ S.E.M. A one-way ANOVA was conducted to analyze the results (****: $p < 0.0001$).

# CHAPTER FOUR

# DISCUSSION

The data I present in this thesis supports the conclusion that PGRMC1 and TMEM97 are two key components of the mAR. While additional studies are needed to further evaluate the nature of the interaction/complex, my work represents an important step from which novel approaches to target the mAR can be developed. These studies serve as the framework by which the mAR could be a novel, druggable target for the treatment of the devastating disorder, GBM.

This putative membrane androgen receptor represents a potential novel target that can be exploited to better treat gliomas; however, its molecular composition remains unknown, thus making its exploitation a challenge. On the other hand, other membrane androgen receptors have been characterized as containing ZIP9 or being a product of a splice variant of the classical androgen receptor (AR45).

ZIP9 is a zinc transporter protein that plays an important role in zinc homeostasis. Its cDNA was initially identified as the mAR in the ovary of the Atlantic croaker, as mentioned above, and it is noteworthy that binding of testosterone to this mAR induced apoptosis, an effect similar to that seen in the mAR identified by Gatson and the Singh laboratory [32, 41, 53]. While this may lend support to the idea that these two membrane androgen receptors are the same, previous data from our laboratory showed low expression levels of ZIP9 in glioma cell models. And though early studies from the Singh lab suggest that ZIP9 is not a target for DHT-BSA, additional studies are required to validate this.

AR45, is another candidate for a membrane-associated androgen receptor. AR45 is splice variant of the classical androgen receptor that inhibits transcriptional activity [54]. Since our cell model (the C6 cells) was devoid of the classical androgen receptor, and T-BSA was still able to exert an effect, we do not think AR45 is a component of the mAR. It was when we started to see similarities in the effects of the sigma-2 ligands and the effects of TBSA did we start wondering whether the astrocyte mAR might be similar in molecular composition to the sigma-2 receptor.

Prior work from the Singh laboratory showed that both the sigma-2 ligand, SV119, and the mAR ligand, T-BSA, exhibited similar effects in augmenting IAA-induced cytotoxicity. The expression of both the mAR and the sigma-2 receptor have been suggested to be a biomarker for cancer, and data from our laboratory and the literature provide evidence that PGRMC1 may be a component of both. Based on these facts, the hypothesis of this project was that the mAR consists of PGRMC1 and TMEM97, and PGRMC1 and/or TMEM97 expression positively correlates with the efficacy of T-BSA in augmenting IAA-induced cytotoxicity.

In testing our hypothesis, the first aim was to measure the effects of IAA and T-BSA under baseline PGRMC1 and TMEM97 expression levels. T-BSA augmented IAA-induced cytotoxicity. The second aim was to observe how this effect changed when PGRMC1 and/or TMEM97 expression was knocked down. The data showed that knocking down PGRMC1 expression nullified the cell-death augmenting effect induced by T-BSA, and the same result was seen in the TMEM97 knockdown condition and the dual knockdown condition. This data supports our hypothesis that PGRMC1 and TMEM97 may be constituents of this putative membrane androgen receptor.

One observation of note is that under PGRMC1 and TMEM97 expression levels, co-treating C6 cells with IAA and T-BSA produced very significant cell-death augmentation;

however, in the knockdown groups, the control condition only showed a slightly significant augmentation of cell death. Our data showed that 50 nM of both PGRMC1 and TMEM97 siRNA reduced genetic expression by approximately 70%, and interpret that is 70% knockdown across the cell population, not in each individual cell. C6 cells are not homogenous, and as such, we recognize that an important consideration for future studies would be to use a lentivirus-mediated delivery of the siRNA, to more robustly knockdown expression. Under such conditions, we may be able to further bolster our current data, implicating PGRMC1 and TMEM97 as key components of the mAR.

This small augmenting effect could also be attributed to the poor efficacy of T-BSA as a ligand. T-BSA, by its nature, is a very bulky compound. Multiple testosterone molecules are conjugated to one big BSA molecule. Conjugating testosterone to this compound allows us to infer that any effect we are seeing is being initiated at the level of the plasma membrane; however, the structure of the compound can lead to decreased/partial binding, thus causing poor efficacy. Using/developing an alternative, membrane-impermeant testosterone model may help us better understand how its therapeutic efficacy can change under different PGRMC1 and TMEM97 expression levels.

While the sigma-2 receptor and the mAR do appear to share possible common constituents, they are not identical. This is supported by the fact that co-treatment of SV119 did not produce the same degree of cytotoxicity augmentation as when IAA was co-treated with T-BSA, suggesting that other receptor components may be involved; however, this may also be attributed to different affinities of SV119 and T-BSA for its presumptive target. Though we acknowledge that this might be partially explained by different pharmacological profiles for SV119 and T-BSA (e.g., affinities for the receptor, whether they represent partial/full agonists,

etc.), the other piece of evidence was that the administration of the sigma-2 receptor antagonist (RHM-1, provided by Dr. Robert Mach, University of Pennsylvania), also did not block the effect of T-BSA, unlike the antagonist for PGRMC1, AG205. Despite these differences, looking at the sigma-2 receptor brought us one step closer to uncovering the identity of the mAR and improving the treatment efficacy for glioma.

Treatment efficacy is significantly compromised by a lack of sufficient targets on glioma cells and by the limited penetration capability of drug delivery systems [55]. The blood-brain barrier (BBB) is an integral factor that prevents treatment regimens from showing significant success against gliomas, as it hinders the optimum delivery of drugs to the tumor site [18]. While Temozolomide (TMZ), the previously mentioned “gold standard” drug, can penetrate the BBB and does not require hepatic metabolism for activation, many clinical trials have shown lower efficacy rates, bringing attention to the notion that TMZ efficacy can possibly be improved by combining it with other agents [56].

Previous data from our laboratory showed that administering TMZ with T-BSA augmented the efficacy of the chemotherapy drug, in a similar fashion to what we saw with IAA in this thesis project. These effects were mediated via the mAR, and with a better understanding of the molecular nature/constituents of the mAR, we could develop specific compounds to target/exploit this receptor to enhance the efficacy of chemotherapeutics for gliomas and potentially other tumors/cancers found to express the mAR. If successful, we will have set the foundation for a novel approach by which we can better treat glioma and improve the prognosis of people suffering from glioblastoma multiforme.

Much work still needs to be done before we can achieve this goal. Firstly, the findings of this project were determined using C6 cells. Experimentally, the use of C6 cells is advantageous

in that it offers a “clean” system to investigate the nature of the mAR, noting that C6 cells do not express the classical AR. There are four glioma cell lines that the Singh laboratory has available: A172, U118, T98g, and C6. Unlike the first three, the C6 cells are a non-human, N-nitrosomethylurea - induced tumor line. The initial approach was to focus on C6 cells, which expressed the highest levels of PGRMC1 and TMEM97. Accordingly, we inferred that there may be a critical threshold of PGRMC1 and/or TMEM97 expression that allows T-BSA to elicit its cell-death promoting effect. Further supporting the critical role of PGRMC1 and TMEM97, our data validated our hypothesis that knockdown of either PGRMC1 and/or TMEM97 reduced the efficacy of T-BSA.

The expression profile analysis done in this project supports these observations, but one thing to note is that the exact mRNA and protein expression of PGRMC1 and TMEM97 in each cell line is unknown. The other human cell lines mentioned in this project seemed to contain very low PGRMC1 and TMEM97 mRNA levels relative to C6 cells, but that does not mean there is less protein. One severe limitation in this field is that there are very poor antibodies currently available, so determining protein expression poses a significant challenge. It would be beneficial to overexpress both PGRMC1 and TMEM97 in various glioma and GBM cell lines to determine if increasing mRNA levels, and by extension, protein levels, improves the efficacy of T-BSA.

The clinical significance of this line of research may be to reveal a method of defining pharmacological treatment following pathological analysis of the resected tumor. That is to say, an abundance of PGRMC1 and/or TMEM97 may suggest that the tumor could be better managed by the co-application of a mAR ligand along with TMZ. This would be analogous to the assessment of estrogen receptors, progesterone receptors and HER2 in breast cancer, where the

abundance of these molecules may not only predict outcome but also guide the pharmacological management of this cancer.

Future studies that expand our analysis to other (human) glioma cell models will help validate the translatability of these observations. Moreover, it would be worthwhile to determine if this (mAR) mechanism is also active/expressed in “normal” (non-cancerous) astrocytes. In the ideal, the mAR should be expressed in abundance in the cancer cell (as the literature appears to support – i.e., that the sigma2 receptor and/or the mAR appear to be particularly highly expressed in cancer cells, thus making them markers of such tumors). Not having the mAR expressed in “normal” astrocytes, may predict that the treatment strategy envisioned may target the cancer cells, while sparing the normal/healthy astrocytes from the combined chemotherapeutic strategy proposed. This, in turn, might lead to fewer side effects. Taken together, the data shown in this document provide important new insight into the molecular identity of a potentially novel membrane-associated androgen receptor, that when bound, enhances the cytotoxicity of chemotherapy. The additional steps outlined may then help facilitate the discovery of new and important ways by which we treat devastating brain cancers that include glioma/glioblastoma.

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# VITA

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