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Effects of the Indirubin Derivative E804 on Glioblastoma Associated Immunomodulation

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EFFECTS OF THE INDIRUBIN DERIVATIVE E804
ON GLIOBLASTOMA ASSOCIATED IMMUNOMODULATION

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ABSTRACT

Glioblastomas are the most common malignant brain tumor, with an annual incidence of 6 in 100,000 people. Conventional treatment modalities, including chemotherapy and radiation, are toxic and cause a decrease in quality of life as the disease progresses. Several investigators have turned to pharmacologically active derivatives of herbal remedies as less-toxic alternatives. Indirubin, a dark red isomer of indigo, is known to be effective in treating chronic inflammation and some forms of cancer, and the mechanism is, in part, due to activity as a ligand for the transcription factor aryl hydrocarbon receptor (AhR). As with most AhR ligands, indirubin induces the CYP1 family of metabolizing enzymes, alters cell cycling, and can be active through the GSK-3β pathway. In recent years, bioavailable derivatives have been synthesized, including indirubin-3'-(2,3 dihydroxy-propyl)-oximether (E804). Several lines of research show that E804 has anti-angiogenic and anti-proliferative properties, and we previously demonstrated anti-inflammatory properties of E804. In this study, T98G human glioblastoma cells were treated with E804 to determine the degree of AhR activation, the secretion of both immunosuppressive and pro-inflammatory cytokines by the tumor cells, and the effects of those tumor secretions on macrophage polarization. E804 is potent inducer of CYP1B1 in T98G cells, has modest effects on TGFβ2 secretion, and suppresses VEGF at high does, while enhancing VEGF secretion at low doses. The secretion of IL-6 by T98G cells was only modestly affected at the highest treatment levels. The effects of supernatants derived from E804-treated T98G cells on macrophage indicate that E804 may polarize macrophages to the pro-tumor M2 phenotype. Overall, the results of this study indicate

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that E804 concentrations would need to be carefully monitored in a clinical application.

Future studies should include an in vivo model of brain tumors to test the potential of

E804 as a standalone, or adjuvant treatment for glioblastoma.

ACKNOWLEDGEMENTS

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CHAPTER ONE

LITERATURE REVIEW

BRAIN TUMORS AND CURRENT TREATMENT MODALITIES

Brain tumors are among the most dreaded diagnosis in oncology. Brain and central nervous system cancers are the number one cause of cancer-related death in children 0-14, and the third leading cause in adolescents 15-19 according to the National Center for Health Statistics for 2014 (SEER, 2010). In general, prognosis is poor and there are few treatment options. Tumors that originate in the brain are similar to other cancers in many ways, but also provide their own unique challenges. All cancer cells possess certain key traits: sustained growth signals, evasion of growth suppressors, activation of invasion and metastasis, induction of angiogenesis, resistance to cell death, evasion of the immune system, and the dysregulation of cellular energetics (as reviewed by (Hanahan and Weinberg, 2011). However, brain tumors are especially difficult for patients and care providers to manage (Schwartzbaum et al., 2006). Depending on the location and size of the tumor, patients can experience personality and mood shifts, severe headaches, seizures, and loss of senses, memory, coordination or bodily control (SEER, 2010). These symptoms are compounded by the fact that the brain is enclosed in a hard shell, the skull. Growth of tissue in one area can cause pressure and swelling of other areas, pushing against the skull, causing severe pain and even brain damage (Figure 1). Tumors within the brain are also inherently more difficult to treat (Schwartzbaum et al., 2006). Surgery is not always an option due to the location of the tumor, but even if it is possible, there are many risks involved, including damage to other areas of the brain

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(Omuro, 2013; Schwartzbaum et al., 2006). Radiation treatment can also quickly damage healthy regions. Chemotherapeutic drugs must be able to access the tumor, which can be challenging if the blood-brain barrier is still intact (Schneider et al., 2004). Brain tumors also tend to be more chemo-resistant than other forms of cancer (Adams et al., 2012). In this way, brain tumors, particularly rapidly growing types, such as glioblastoma, have the ability to completely incapacitate an individual.

Glioblastoma (WHO grade IV) is a highly malignant, lethal form of brain cancer. Glioblastomas are the most common form of gliomas, which account for approximately 80% of malignant brain tumors (Schwartzbaum et al., 2006), but their precise causes remain unknown. Glioblastomas are composed of a mixture of astrocytes, supportive star-shaped cells in the central nervous system, and endothelial cells (Omuro, 2013). Secondary finger-like structures are usually found away from the primary tumor (Paw et al., 2015). Tumor cells are capable of traveling down myelinated nerves and following the basement membrane of blood vessels, particularly within Virchow-Robins spaces (Pedersen et al., 1995). Virchow-Robins spaces are part of the subarachnoid and sub-pial space below the meninges which surround blood vessels that enter the brain (Pedersen et al., 1995). In these areas, the hydrostatic pressure of cerebral spinal fluid allows cells to move quickly, spreading far beyond the primary tumor (Pedersen et al., 1995). This invasive growth pattern makes glioblastomas nearly impossible to completely remove surgically (Giese, 2003). However, current treatment modalities usually begin with surgical removal of the majority of the tumor to relieve pressure on the surrounding

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tissue, if the tumor is in an operable location. This also allows for more precise histological grading of the tumor (Omuro, 2013).

Following resection, first line treatments involve radiation therapy, chemotherapy, or a combination of the two. In glioblastoma and other progressed brain tumors, the blood brain barrier is often degraded, letting drugs more freely access the central nervous system (Schneider et al., 2004). Common chemotherapy agents include the DNA alkylating agents temozolomide (TMZ), carmustine, and lomustine (Omuro, 2013). After surgical removal of the majority of the tumor, carmustine-loaded chips called Gliadel® wafers can be implanted directly into the brain (Perry, 2007). These drugs, like all chemotherapy, are toxic and can have severe side effects, including impaired immune system function, nausea, vomiting, hypertension, and hematologic toxicity, such as anemia (Omuro, 2013; Perry, 2007). Newer treatments include a monoclonal antibody against vascular endothelial growth factor (VEGF) called bevacizumab (Avastin®) (Reardon et al., 2014). Bevacizumab inhibits angiogenesis, reducing the tumor's ability to grow new blood vessels, limiting its size (Reardon et al., 2014). However, this treatment could also lead to hypertension, wound healing delays, venous or arterial thrombosis, and hematologic toxicity, among other side effects (Omuro, 2013; Reardon et al., 2014). With treatment, median 2 year survival rate for glioblastoma patients remains between approximately 2-30%, depending on age at diagnosis (Schwartzbaum et al., 2006). Clearly, less toxic, more effective treatments are needed. For these alternatives, many researchers are looking to natural sources.
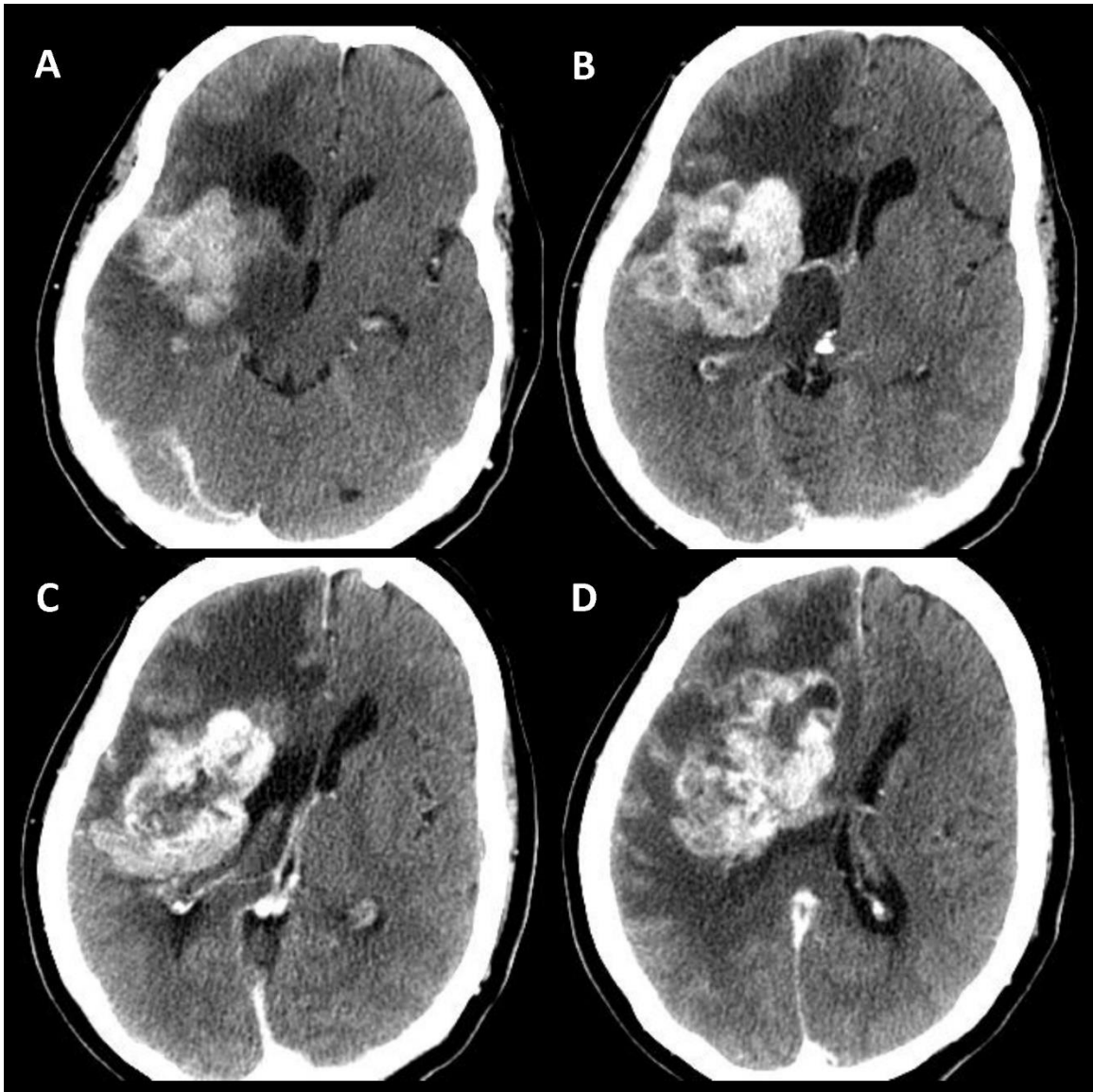
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Figure 1: WHO grade IV glioma, American Journal of Neuroradiology, submitted by Jason McKellop, Amir Paydar, Girish Fatterpekar, Alexander Baxter, NYU Langone Medical Center, New York, NY. http://www.ajnr.org/site/com/2012jun.xhtml

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INDIRUBINS AND THE ARYL HYDROCARBON RECEPTOR

Medicinal herbs and plants have been used for centuries to treat various ailments, including cancer. One such Chinese remedy is Danggui Longhui Wan, a mixture of 11 herbs used to treat chronic myelocytic leukemia and other inflammatory diseases (Hoessel et al., 1999). After extensive research, the active compound in Danggui Longhui Wan was found to be a red isomer of the blue dye indigo, called indirubin (Hoessel et al., 1999). Indirubin has been shown to slow or stop cell proliferation by inducing cell cycle arrest and apoptosis via cyclin dependent kinases (CDKs) (Hoessel et al., 1999) and glycogen synthase kinase-3β (GSK-3β) (Xie et al., 2004). Naturally occurring indirubin is not very bioavailable, so researchers have synthesized artificial indirubin derivatives (Moon et al., 2006). These derivatives vary in in the location and composition of their side chains, which alter their receptor binding affinity and, therefore, their downstream effects (Moon et al., 2006). One such derivative is indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804). E804 has been shown inhibit STAT3 and induce apoptosis in multiple human cancer cell lines (Nam et al., 2005b). E804 also has strong anti-inflammatory and anti-angiogenic properties, but the precise mechanism of this action remains unclear (Anderson, 2014; Chan et al., 2012). One known pathway is through interaction with the aryl hydrocarbon receptor.

The aryl hydrocarbon receptor (AhR), a transcription factor of the family helix-loop-helix, has been heavily studied since its link to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) toxicity in the 1970's (Poland, 1976). The activation of AhR has been linked to several pathways, ranging from carcinogenesis (Andersson et al., 2002) to tumor

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therapies (Koliopanos et al., 2002), depending on the strength of the ligand binding. The stronger the binding of a ligand to the AhR, the more toxic and long lasting its effects seem to be. Indirubins have been shown to interact with AhR in varying degrees (Knockaert et al., 2004), but their interactions are brief due to the compounds' metabolism by cytochrome P450 enzymes like CYP1A1 and CYP1B1 (Spink et al., 2003). Thus, indirubins that bind strongly, but transiently, to AhR may have potent anti-inflammatory and anti-tumor properties without the side effects of more stable ligands like TCDD (Chan et al., 2012; Knockaert et al., 2004).

INFLAMMATION AND IMMUNE RESPONSE

Inflammation is a complex bodily response to tissue damage or pathogen intrusion. Local acute inflammation is beneficial in destroying these invaders, but a chronic or inappropriate inflammatory response can damage healthy tissue and cause severe pain (Coussens and Werb, 2002). Chronic inflammation has also been shown to contribute to many aspects of cancer initiation, promotion, and metastasis (Coussens and Werb, 2002). After an injury occurs, damaged cells release chemokines that attract monocytes, which differentiate into macrophages in tissue, and aid in wound repair (Coussens and Werb, 2002). Similarly, when a tumor begins to form, the transformed glial cells release chemokines that activate and attract immune cells from within the CNS, called microglia, and macrophages from outside the CNS (Bingle et al., 2002; Ransohoff et al., 2015). However, rather than destroying the cancerous cells, the macrophages'

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function is suppressed within the tumor microenvironment (Kennedy et al., 2013). These tumor-associated macrophages and microglia (TAMs) quickly accumulate, and can compose 30 to 40 percent of the tumor's volume (Kennedy et al., 2009). It has also been shown that the number of infiltrating macrophages positively correlates with the histological grade of the tumor (Nishie et al., 1999).

There are two main lineages of TAMs; M1, which are inflammatory and capable of killing cancer cells, and M2, which suppress immune function (Kennedy et al., 2013). The M1 versus M2 phenotype can be distinguished through genetic and cytokine secretion profiling (Kennedy et al., 2013). M2 type TAMs are thought to suppress inflammation though interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) secretion (Mantovani et al., 2002). Interleukin-6 (IL-6) has been shown to have both pro- and anti-inflammatory properties, as well as being implicated in increased angiogenesis and tumor metastasis (Liu et al., 2010). The M2 divergence also leads to increased VEGF release from tumor cells (Kennedy et al., 2013). During this progression, the tumor cells are releasing their own cytokines and growth factors, which can equal or exceed those produced by activated macrophages (Ulvestad et al., 1994). Interplay between the TAMs and tumor cells is also seen, which can feed each other's secretion in a paracrine and autocrine method (Kennedy et al., 2013). Other proteins can also effect M1 versus M2 polarization, such as arginase 1 and 2 (ARG1, ARG2), indoleamine 2,3-dioxygenase 1 (IDO1), cyclooxygenase-2 (COX-2) (Barksdale et al., 2004; Iachininoto et al., 2013; Ino et al., 2013; Mellor and Munn, 2004).

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The enzyme arginase catalyzes hydrolysis of L-arginine to L-ornithine and urea (Barksdale et al., 2004). There are two isomers of arginase, ARG1 and ARG2 (Bronte et al., 2003; Ino et al., 2013). Depletion of arginine within a given environment can reduce the expression of the CD3 ζ chain on cytotoxic (CD8+) T-cells, preventing the T-cell receptor from interacting with the major histocompatibility complex (MHC), resulting in T-cell anergy (Zea et al., 2005). Arginine is also necessary for nitric oxide (NO) synthesis, which is required for proper T-cell function (Barksdale et al., 2004). Increased ARG1 activity is one of the most well-defined markers of M2 polarization in mice, but ARG1 is only found in granulocytes in humans (Sica and Bronte). It has been shown that renal carcinoma granulocytes in humans may be acting through a similar mechanism as M2-polarized macrophages (Zea et al., 2005). ARG1 is also known to be induced by TGF-β, another cytokine implicated in immune suppression (Bodmer et al., 1989; Bronte et al., 2003). Alternatively, ARG2 is found in the mitochondria of most human cells, including monocytes. ARG2 is involved in the synthesis of polyamines and has been shown to be induced by IL-10 (Barksdale et al., 2004). Increased levels of ARG2 has been seen in macrophages isolated from patients with multiple sclerosis (MS) (Barksdale et al., 2004). However, linkage to cancer progression and severity depends on tissue type (Ino et al., 2013; Rotondo et al., 2008). Increased ARG2 expression has been found to correlate with poor prognosis in patients with pancreatic or prostate cancer, but not lung cancer (Ino et al., 2013; Rotondo et al., 2008).

The enzyme IDO1 is responsible for the breakdown of L-tryptophan to kynurenines (KYN) (Iachininoto et al., 2013). An increase in KYN within a

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microenvironment promotes FoxP3^{+} regulatory T-cell (T_{reg}) activity, suppressing T-cell and dendritic cell responses (Iachininoto et al., 2013; Mellor and Munn, 2004). High levels of IDO1 has also been correlated with poor prognosis in patients with acute myeloid leukemia and certain solid tumors (Chamuleau et al., 2008). COX-2 is one of the two isoforms of the cyclooxygenase enzyme responsible for prostaglandin synthesis (Iachininoto et al., 2013; Minghetti, 2004). COX-2 is also a main cause of inflammation-associated pain in the central nervous system, and the target of non-steroidal anti-inflammatory drugs (NSAIDs) (Minghetti, 2004; Samad et al., 2001; Wang and DuBois, 2009). Inhibition of COX-2 activity has been shown to suppress the activity T_{reg} cells, promoting the destruction of lung cancer cells (Sharma et al., 2005). Both IDO1 and COX-2 are produced by human macrophages, and induced by interferon gamma (IFN-γ), which has been implicated in both pro- and anti-tumor pathways (Iachininoto et al., 2013; Zaidi and Merlino, 2011).

The understanding of the cytokine and growth factor profile of a given tumor is critical to proper treatment, and can shape the approach taken, particularly when considering immunotherapy (Reardon et al., 2014; Zea et al., 2005). The particularly immunosuppressive nature of the glioblastoma microenvironment has been cited as the source of struggle in the development of successful immune therapies, but recent advances have shown promise (Reardon et al., 2014). There are currently many clinical trials being conducted on immunotherapeutic options, including combinations of traditional chemotherapies with monoclonal antibodies, such as ipilimumab and tremilimumab (Reardon et al., 2014).

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CHAPTER TWO

EFFECTS OF THE INDIRUBIN DERIVATIVE E804 ON GLIOBLASTOMA

ASSOCIATED IMMUNOMODULATION

INTRODUCTION

Because of their location and quick growth, brain and central nervous system tumors are among the most difficult to treat and lethal types of cancer (SEER, 2010). The most common malignant brain tumor, glioblastoma, is highly invasive, rapidly growing, and impossible to completely remove by surgery alone (Giese, 2003; Holland, 2000; Omuro, 2013; SEER, 2010). As a result, these tumors must be treated in other ways, such as radiation and chemotherapy (Holland, 2000; Perry, 2007; Schwartzbaum et al., 2006). However, commonly used chemotherapeutic drugs like the DNA alkylating agents carmustine (BCNU), lomustine (CCNU), and temozolomide (TMZ) have severe side effects, including impaired immune system function, nausea, vomiting, hypertension, and hematologic toxicity, such as anemia (Newlands et al., 1997; Omuro, 2013; Ransohoff et al., 2015; Shinkaruk et al., 2003; Xiao and Link, 1998).

To improve anti-tumor treatment options, scientists are exploring natural alternatives as treatments or adjuvants (Blažević et al., 2015; Chan et al., 2012; Choi et al., 2010; Heshmati et al., 2013a; Ichimaru et al., 2015). One natural compound, indirubin, is the main active ingredient in Danggui Longhui Wan, a Chinese herbal remedy used to treat chronic myelocytic leukemia and other inflammatory diseases (Blažević et al., 2015; Hoessel et al., 1999; Jakobs et al., 2005; Xiao et al., 2002). Natural

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indirubin is isolated from the indigo plant, and is not very bioavailable because of its low solubility and absorption (Blažević et al., 2015; Chan et al., 2012; Hoessel et al., 1999). To improve bioavailability, synthetic indirubin derivatives have been synthesized (Blažević et al., 2015; Choi et al., 2010; Heshmati et al., 2013a; Kim et al., 2011; Polychronopoulos et al., 2004; Ribas et al., 2006). Most indirubins are thought to act mechanistically through the aryl hydrocarbon receptor (AhR), a ligand activated transcription factor and member of the basic-helix-basic loop pers-arnt-sims superfamily of proteins involved in environmental sensing, cell cycle regulation, circadian rhythms, and xenobiotic metabolism. One of the most characterized activities, and a sensitive marker of the AhR activation, is the inductionof CYP1 family of xenobiotic metabolizing enzyme systems associated with Phase I, II, and III processing of environmental contaminants and endogenous ligands (Nebert et al., 2000). AhR activation has been linked to a variety of effects, ranging from carcinogenic to chemotherapeutic, depending on the binding strength of the ligand (Andersson et al., 2002; Koliopanos et al., 2002).

All cancer cells possess key traits in common: sustained growth signals, evasion of growth suppressors, activation of invasion and metastasis, induction of angiogenesis, resistance to cell death, evasion of the immune system, and the dysregulation of cellular energetics (as reviewed by (Hanahan and Weinberg, 2011). One of the most important aspects for the persistence of solid tumors, like glioblastoma, is the induction of angiogenesis to promote neo-vascularization to allow blood and nutrient flow to the otherwise hypoxic tumor center (Byrne et al., 2005; Carmeliet, 2005; Omuro, 2013). Immune cells and their secreted products play a large role in angiogenesis in tumors,

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much like the process seen in wound healing (Carmeliet, 2005; Coussens and Werb, 2002).

Glioblastoma patients are known to be immunocompromised in several ways (Bodmer et al., 1989; Chamuleau et al., 2008; Ino et al., 2013; Kennedy et al., 2009; Nishie et al., 1999; Paw et al., 2015; Ransohoff et al., 2015; Roy et al., 2014; Ryuto et al., 1996; Sharma et al., 2005; Sica and Bronte; Ulvestad et al., 1994; Xiao and Link, 1998). Immunosuppression due to increased regulatory T cells, anti-inflammatory cytokine production, and decreased cytotoxic T cell activity in the tumor microenvironment has been well documented, and is known to contribute to tumor growth and metastasis (Bodmer et al., 1989; Letterio, 1998; Ransohoff et al., 2015; Reardon et al., 2014). Because of this, immunotherapeutic treatment targets are currently being researched (Kennedy et al., 2009; Mantovani et al., 2002; Nishie et al., 1999; Ransohoff et al., 2015; Reardon et al., 2014; Sharma et al., 2005; Sica and Bronte; Ulvestad et al., 1994). One area of interest is the polarization of macrophages and microglia, macrophage-like cells in the CNS, to M1 or M2 phenotypes within the tumor (Mantovani et al., 2002; Nakanishi et al., 2011; Nishie et al., 1999; Zea et al., 2005). M1 macrophages are classical inflammatory immune cells that can actively suppress tumor cell growth through secretion of pro-inflammatory cytokines, while M2 macrophages support tumor growth by secreting anti-inflammatory cytokines and hindering cytotoxic T cell activity by increasing regulatory T cell presence and downregulating antigen presentation (Kennedy et al., 2013; Mantovani et al., 2002; Sica and Bronte; Wu et al., 2010).

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Polarization of macrophages to M1 versus M2 can be measured in their gene expression (Mantovani et al., 2002). Prior studies have identified increased ARG2 and IL-6 as M1 macrophages markers, and ARG1, IDO1, and IL-10 as M2 macrophage markers (Bronte et al., 2003; Ino et al., 2013; Mantovani et al., 2002; Nakanishi et al., 2011; Sharma et al., 2005; Zea et al., 2005). COX-2 has been suggested as a marker for both M1 and M2 macrophages, but the M2 polarization seems to dominate in tumors (Iachininoto et al., 2013; Minghetti, 2004; Nakanishi et al., 2011; Sharma et al., 2005; Tjiu et al., 2009). Macrophage recruitment and polarization in the tumor microenvironment has been shown to be impacted directly by glioblastoma cells through the STAT3 pathway (Wu et al., 2010). The STAT3 pathway is known to be impaired by indirubin, a natural compound isolated from the indigo plant, and synthetic indirubin derivatives to varying degrees (Li et al., 2011; Nam et al., 2005b; Zhang et al., 2011; Zhang et al., 2015; Zhou et al., 2009).

Indirubin and indirubin derivatives have been shown to reduce angiogenesis, inflammation, and tumor progression (Blažević et al., 2015; Chan et al., 2012; Heshmati et al., 2013b; Ichimaru et al., 2015; Jakobs et al., 2005; Kim et al., 2011; Nam et al., 2005a; Zhang et al., 2011; Zhang et al., 2015). Previous studies have shown the indirubin derivative indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804) to possess immunomodulatory and anti-angiogenic properties (Anderson, 2014; Babcock et al., 2013; Chan et al., 2012; Ichimaru et al., 2015; Nam et al., 2005a; Nam et al., 2012; Zhang et al., 2015). E804 has also been shown to block STAT3 signaling (Nam et al., 2005b; Shin and Kim, 2012; Zhang et al., 2015). However, the effects of E804 on

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angiogenesis and immunomodulation associated with glioblastoma have yet to be evaluated. Here, I show that E804 potent inducer of CYP1B1 in T98G cells, has modest effects on TGFβ2 secretion, and suppresses VEGF at high does, while enhancing VEGF secretion at low doses. However, E804 could also polarize macrophages to the pro-tumor M2 phenotype. The results of this study provide concerns with utilizing E804 as a treatment. The anti-angiogenic effects of E804 are promising, but the concentration would be difficult to achieve *in vivo*, and would need to be carefully monitored. However, if E804 enhances immune suppression within the tumor microenvironment, any anti-angiogenic effects may be lost or proven null. Further studies, particularly *in vivo* experiments, are needed, but use of E804 to reduce angiogenesis and tumor microenvironment immunosuppression in glioblastoma patients could be a viable option.

MATERIALS AND METHODS

Cells and Cell Culturing

The human glioblastoma cell line T98-G (American Type Culture Collection, Manassas, Va.) was grown and maintained at 37°C with 5% CO_2 in Corning 75 cm^2 culture flasks, using Dulbecco's Modified Eagle Medium (DMEM, Cellgro) supplemented with 10% Fetal Calf Serum (FCS), 20 mM HEPES, 10 mM L-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, 110 µg/ml sodium pyruvate, 1% non-essential amino acids (100x stock), and 4.5 g/L glucose. Previously published studies indicate an active AhR signaling pathway (Gramatzki et al., 2009a).

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Human monocyte THP-1 (American Type Culture Collection, Manassas, Va.) cells were grown and maintained at 37°C with 5% CO_2 in Corning 75 cm^2 culture flasks using DMEM (Cellgro) supplemented with 10% FCS (Atlas; Fort Collins CO), 20 mM HEPES, 10 mM L-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, 110 µg/ml sodium pyruvate, 1% non-essential amino acids (100x stock), 4.5 g/L glucose, and 1.5 g/L of $NaCO_3$. To differentiate THP-1 cells to a macrophage phenotype, cells were pulsed with 0.1 µM phorbol myristate acetate (PMA, Alexis Chemicals) for 72 hr (Daigneault et al., 2010; Schwende et al., 1996).

Mouse Hepa-1c1c7 cells are an AhR-active line commonly used for AhR activation and characterization studies (Bernhard et al., 1973; Hankinson, 1995; Hankinson et al., 1991). Cells were obtained from ATCC and maintained at 37°C with 5% CO_2 in Corning 75 cm^2 culture flasks using DMEM (Cellgro) supplemented with 10% FCS (Atlas; Fort Collins CO), 20 mM HEPES, 10 mM L-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, 110 µg/ml sodium pyruvate, 1% non-essential amino acids (100x stock), 4.5 g/L glucose, and 1.5 g/L of $NaCO_3$.

Chemicals

The chemical structures of natural indirubin, E804, and TMZ are shown below in Figure 2. Indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804; Alexis Biochemical, San Diego CA) was solubilized in DMSO (Sigma Aldrich) to a stock solution of 10^{-2} M and stored at -20° C. Working dilutions were made with DMEM (supplemented as previously described) to final concentrations of 10 µM, 1 µM, and 0.1 µM. 10 µM Temozolomide

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(TMZ; Tokyo Chemical Industry), the most common chemotherapeutic agent for glioblastoma, was also solubilized in DMSO, and brought to working concentration in DMEM, was used as a comparison to the activity of E804.
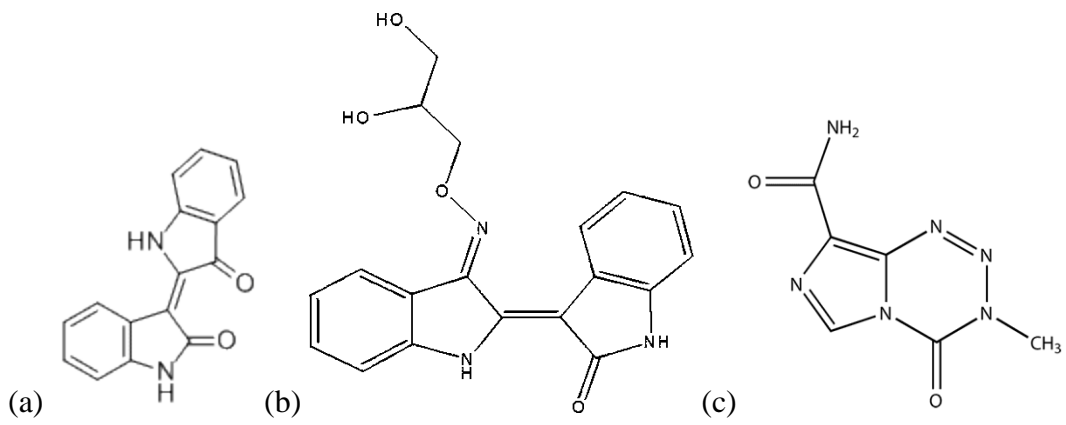
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Figure 2: Chemical structures of (a) natural indirubin, (b) E804, (c) TMZ.

Cytotoxicity Assay

Viability of the cells was quantified as a function of their succinate dehydrogenase activity, a measure of cellular respiration, by performing an MTT (3-[4,5-28 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Two treatment time points were analyzed: 6 and 24 hours. Each cell line was treated with 0.1, 1, or 10 μM E804 and a vehicle control of DMSO. Four hours prior to the end time point, 20 μl of a 5 mg/ml MTT solution in PBS was added to each well. At the end of the time point, wells were aspirated and 100 μl of acidified isopropanol (4 mM HCl, 0.1% Nondet P-40 (NP40) in isopropyl alcohol) were added to each well (Babcock et al., 2013). The plates were then shaken for 10 minutes to disrupt cells and dissolve any remaining formazan. The optical density of each well was then quantified at a wavelength of 550 λ. A positive control of tributyltin (TBT) was also tested (Kergosien and Rice, 1998). Data were recorded as optical densities and used to calculate cytotoxicity as a percentage of control treatments.

Ki-67 Labeling Index

As a secondary method to assess cell toxicity, the cellular proliferation marker Ki-67 was used as a means to determine labeling index (Louis et al.; Montine et al., 1994). T98-G cells were seeded in 12-well plates, and incubated for 24 hours to allow adherence. Cells were then treated, in triplicate, with 0.1, 1, and 10 μM E804 and a vehicle control of DMSO. At 12 and 24-hours post-treatment, media was aspirated from each well and the cells were fixed in 0.3% formalin in PBS buffer for 15 minutes at room temperature. The fixative was then aspirated and PBS buffer added to the wells. Cells were permeabilized

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using 0.1% Triton-X in PBS for 30 minutes at room temperature. The wells were then

incubated with non-specific antibody blocking solution (10% FBS in PBS), washed x 3 in

PBS with 0.5% tween-20 (PBS-T20), washed again x 3 with PBS-T20, then incubated

mouse anti-Ki67 (mAb 4A1, 1:1000 in PBS) primary antibody (Thermo Fisher), and then

goat anti-mouse secondary antibody conjugated with alkaline phosphatase (1:1000,

Southern Biotech), each for 1 hour. Alkaline phosphatase activity was detected using 5-

bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) in alkaline

phosphatase buffer. The wells were then imaged and the number of stained cells out of

the total number of cells were counted to establish a labeling index.

CYP1B1 induction by E804 in T98G Cells

Quantitative real time PCR was then performed to measure the expression levels of

CYP1B1 following treatment with either 0.1, 1, or 10 μM E804, 10 μM DMSO carrier,

or 10 μM Temozolomide (TMZ). T98-G cells were seeded into 6-well plates and grown

to near confluence, then exposed for 6, 12, and 24-hours. Overlying media from each

well was aspirated, and cells were collected using 1 mL of RNAzol® (Molecular

Research Center, Inc.). RNA was then isolated per the manufacturer's instructions.

cDNA was synthesized using qScript cDNA Supermix (Quanta) according to the

manufacturer's instructions. Using primer sets listed in Table 1, qRT-PCR was

performed using the SensiFAST™ SYBR® & Fluorescein kit (BioLine) according to the

manufacturer's instructions. Each reaction was performed in triplicate. Plates were run

and data was collected using the BioRad IQ5 detection system. Data were expressed as

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fold increase in expression compared to GAPDH house-keeping gene expression using the Pfaffl method (Pfaffl, 2001).

Effects of E804 on Growth Factor and Cytokine Secretion

T98-G cells were grown to near confluence in T-150 flasks, then treated with either 0.1, 1, or 10 µM E804, 10 µM DMSO carrier, or 10 µM Temozolomide (TMZ). Supernatants and cells were collected at 24 and 48 hours post treatment, then frozen at -20ºC until ready for use. Supernatants were then analyzed by commercially available human Cytokine Enzyme-linked Immunosorbent Assays (ELISA) kits (Biolegend, Thermo Fisher) for IL-6, TGF-β2, VEGF, and IL-10 per the manufacturer's instructions.

Effects of E804 on IDO1, TDO2, CYP1B1, and GAPDH Protein Expression

At the time of supernatant collection described above, cells were lysed in 1 ml of RIPA lysis buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing EDTA and protease inhibitor cocktail (Pierce) and transferred to snap cap tubes on ice for 30 minutes. Afterward, the tubes were vortexed again, then centrifuged at 12,000g for 10 minutes. The supernatant was then transferred to a new 1.5 ml tube, and its protein concentration quantified. Protein samples were diluted to a common concentration so that equal concentrations were then run on a (4-20% SDS-PAGE) acrylamide gel, then transferred to PVDF blotting membrane for 1 hour on ice. The PVDF blot was then incubated with non-specific antibody blocking solution overnight at 4ºC. The following day, the blocking solution was removed, and the PVDF blot was incubated with a targeted primary antibody for 1

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hour with gentle shaking. Primary antibodies used were goat-anti-TDO2, rabbit-anti-IDO1, rabbit-anti-CYP1B1, and goat-anti-GAPDH, each diluted 1:000 in PBS. The blot was the washed using PBS-T20 (0.05% Tween-20 in PBS buffer) 3 times for 5 minutes with vigorous shaking. The blot was then incubated with either goat anti-rabbit IgG-AP (1:000 in PBS, Southern Biotech) or rabbit anti-goat IgG-AP, depending on the origin species of the primary antibody. The blot was washed again as previously described, and alkaline phosphatase activity visualized with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) in alkaline phosphatase buffer. Once fully developed, blots were rinsed with water and dried. Blots where then scanned and analyzed via ImageJ.

Effects of Supernatants from Treated T98G Cells on Polarization of THP-1 Cells

$1x10^6$ THP-1 cells were seeded into 6-well plates and incubated for 72 hours with 0.1 μM tetradecanoyl phorbol acetate (PMA) to differentiate them into a macrophage lineage (Chanput et al., 2014; García et al., 1999). Following differentiation and adherence, media was removed and cells were incubated for 24 hours with 50% dilution of supernatants from previously treated T98-G cells and fresh DMEM. Each experiment was performed in triplicate. After incubation, cells were collected with 500 ul RNAzol and processed for RNA and cDNA for qRT-PCR as described above. Genes of interest were IL-6, IL-10, Arginase1, Arginase2, IDO1, and COX-2, all of which were normalized against GAPDH. See Table 1 for primers utilized.

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Determining if T98G Supernatants Following Treatment Contain Residual AhR ligand Activity

Hepa-1c1c7 cells were seeded into 6-well plates and grown to confluence. Cells were then incubated for 24 hours with a 1:1 mixture of supernatants from previously treated T98-G cells and fresh DMEM. Cells were lysed and protein concentrations were quantified as previously described. Protein levels of CYP1B1 and GAPDH were then measured by immunoblot as previously described.

Statistical Analysis

Comparisons of replicates were performed by ANOVA followed by Bonferroni's multiple contrast post-hoc tests using GraphPad Prism's statistical software. An α value of 0.05 was established as statistically significant prior to experimentation (Fisher, 1946).

RESULTS

MTT Assay

The T98-G cells showed no significant decrease in cellular respiration as a result of treatment with up to 25 μM E804 for 6 or 24 hours (Figures 3 and 4). Therefore, all studies continued to use E804 at concentrations of 0.1, 1, and 10 μM.
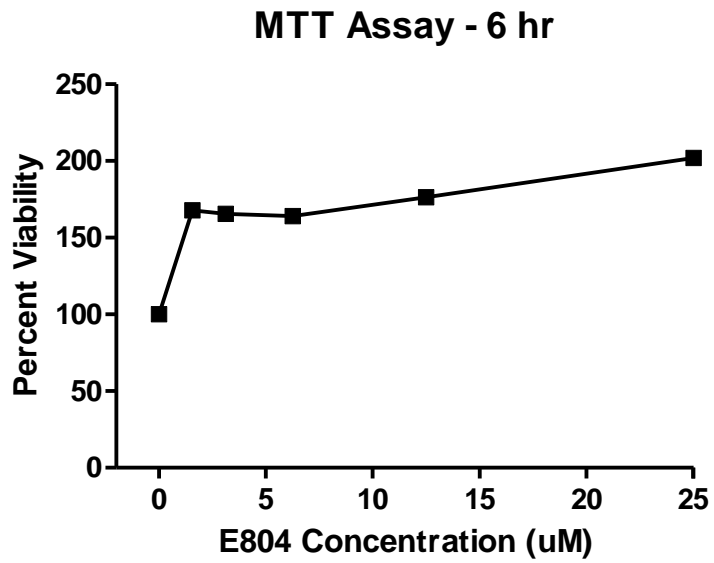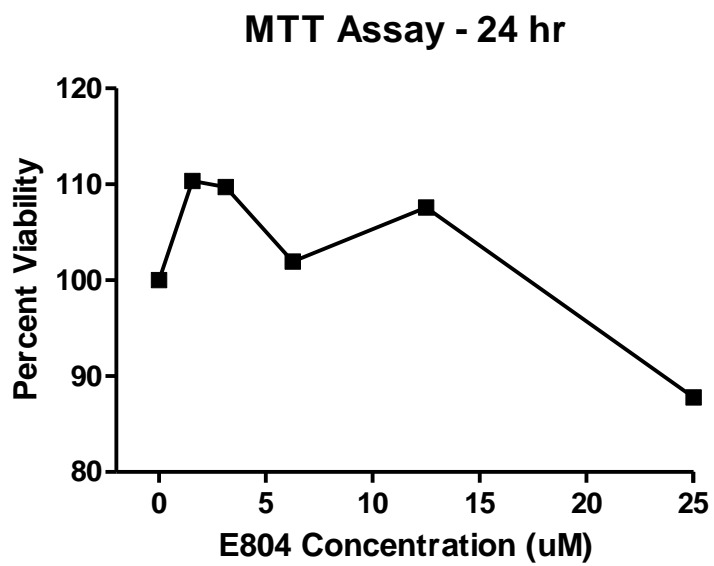
Figure 3: MTT Assay, 6-hour incubation

Figure 4: MTT Assay, 24-hour incubation

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Ki-67 Labeling Index

The protein Ki-67 is used as a marker of cellular proliferation. No significant difference was seen in the number of Ki-67 positive T98-G cells between the treatments of 0.1, 1, and 10 μM E804 and vehicle control of DMSO for 12 or 24 hours. Representative images of each time point are shown below (Fig 5). Ki-67 positive cells are dark purple, while negative cells appear clear.
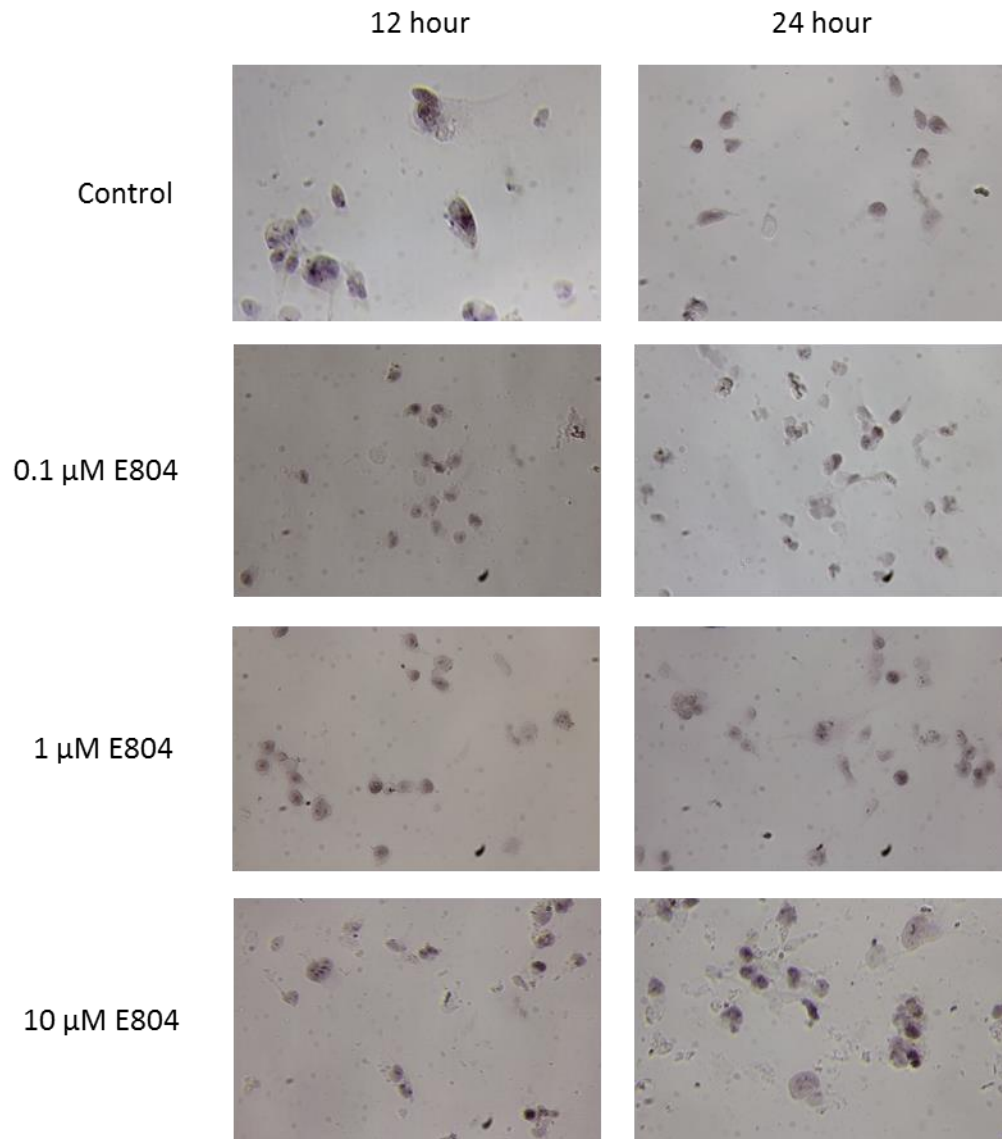
25

Figure 5: T98-G cells labeled with Ki-67

CYP1B1 mRNA induction as a marker for AhR activity

To determine the strength of interaction of E804 with the aryl hydrocarbon receptor, expression of CYP1B1, a downstream gene product of AhR activation, was measured via qRT-PCR. CYP1B1 expression was normalized against GAPDH. A dose and time-dependent response was demonstrated. The highest CYP1B1 expression difference was seen in the 1 µM E804 treated cells, with a 5 to 10-fold change with respect to the DMSO vehicle control (Figure 6).
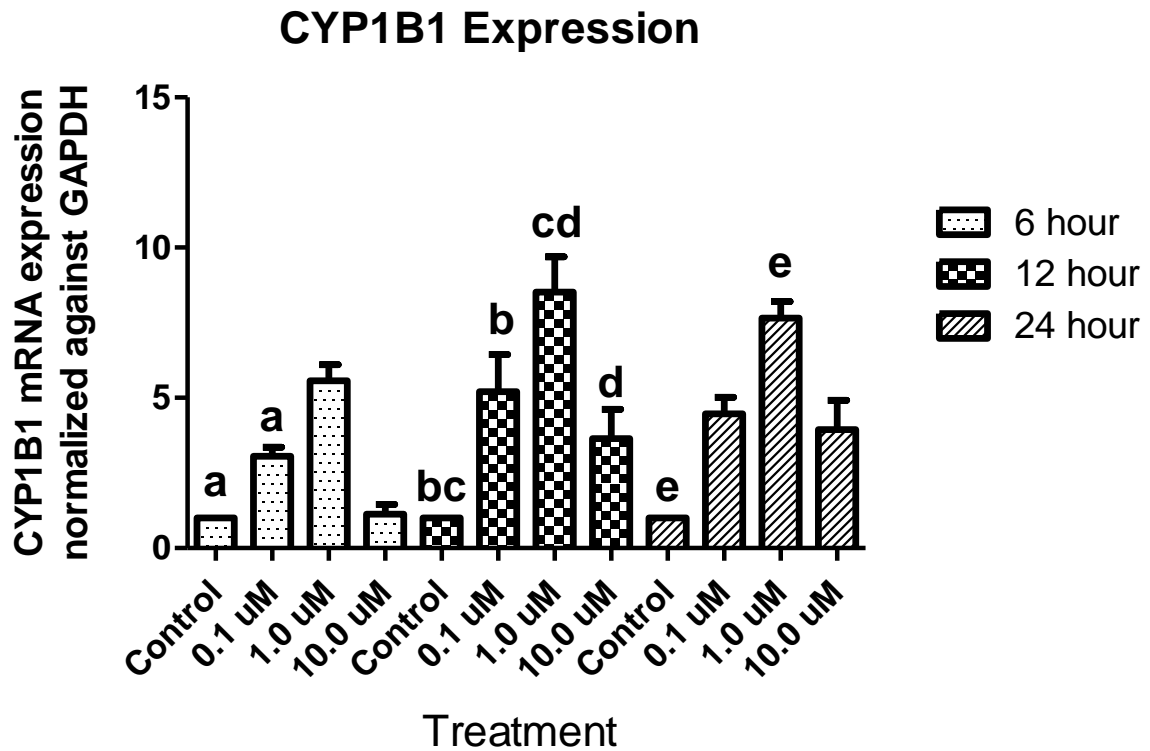
27

CYP1B1 Expression

Figure 6: CYP1B1 mRNA expression in T98-G cells. Cells were treated with either DMSO vehicle control, 0.1 μM, 1 μM, or 10 μM E804 for 6, 12, or 24-hours. Corresponding letters above bars indicate a significant difference. (a) $P \leq 0.01$, (b) $P \leq 0.05$, (c) $P \leq 0.0001$, (d) $P \leq 0.01$, (e) $P \leq 0.0001$.

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Cytokine ELISAs using T98-G Supernatants

After treatment of T98-G cells with E804 and TMZ at 24 and 48 hours, the supernatants of T98-G cells were collected. ELISAs were performed using these supernatants to measure secretion of IL-6, TGF-β, VEGF, and IL-10. IL-6 secretion was significantly decreased as a result of 10 µM E804 at both 24 and 48 hours (Figure 7). TGF-β secretion was decreased at 24 hours, but increased at 48 hours at lower concentrations (Figure 8). VEGF secretion was increased at all concentrations of E804 except 10 µM, which significantly decreased expression (Figure 9). IL-10 secretion was not significantly impacted (Figure 10). Corresponding letters above bars indicate significance.

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Figure 7: IL-6 secretion from T98-G cells. Cells were treated with DMSO vehicle control, 0.1 µM, 1 µM, or 10 µM E804, or TMZ for 24- or 48-hours. Corresponding letters above bars indicate a significant difference. (a) $P \le 0.05$, (b) $P \le 0.01$, (c) $P \le 0.01$, (d) $P \le 0.05$, (e) $P \le 0.001$, (f) $P \le 0.001$, (g) $P \le 0.001$, (h) $P \le 0.01$.

TGF-Beta Secretion

Figure 8: TGF-Beta secretion from T98-G cells. Cells were treated with DMSO vehicle control, 0.1 μM, 1 μM, or 10 μM E804, or TMZ for 24- or 48-hours. Corresponding letters above bars indicate a significant difference. (a) P ≤ 0.05.
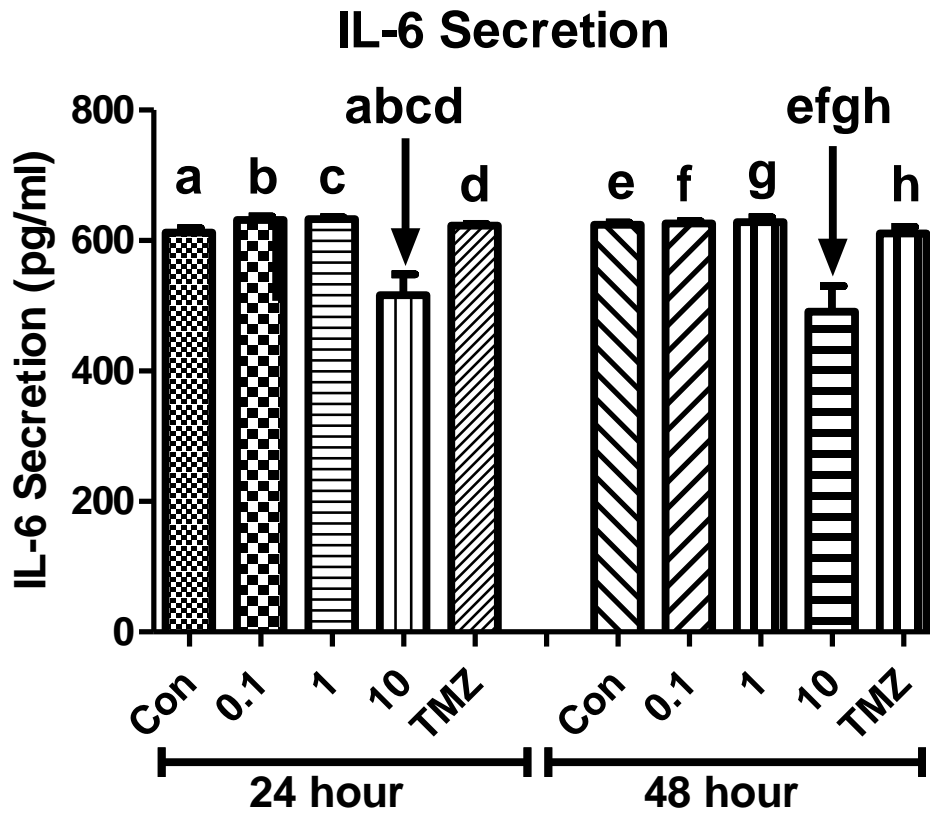
Figure 9: VEGF secretion from T98-G cells. Cells were treated with DMSO vehicle control, 0.1 μM, 1 μM, or 10 μM E804, or TMZ for 24- or 48-hours. Corresponding letters above bars indicate a significant difference. (a) $P \leq 0.05$, (b) $P \leq 0.01$, (c) $P \leq 0.0001$, (d) $P \leq 0.05$, (e) $P \leq 0.0001$, (f) $P \leq 0.0001$, (g) $P \leq 0.05$, (h) $P \leq 0.05$, (i) $P \leq 0.0001$, (j) $P \leq 0.0001$, (k) $P \leq 0.0001$, (l) $P \leq 0.0001$, (m) $P \leq 0.001$.

IL-10 Secretion
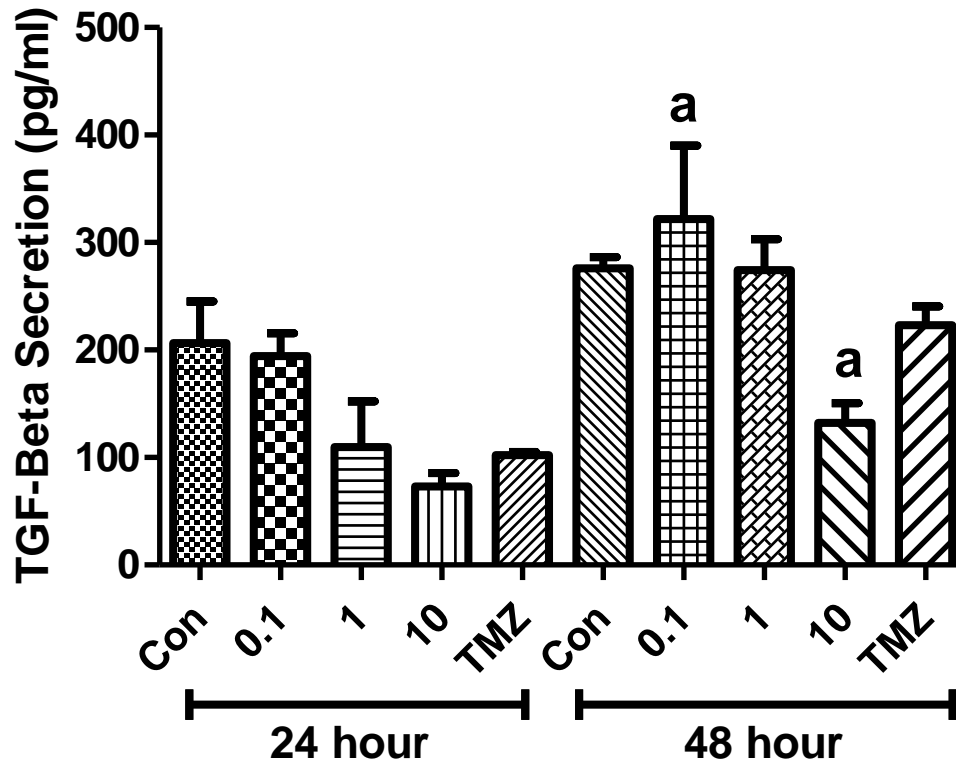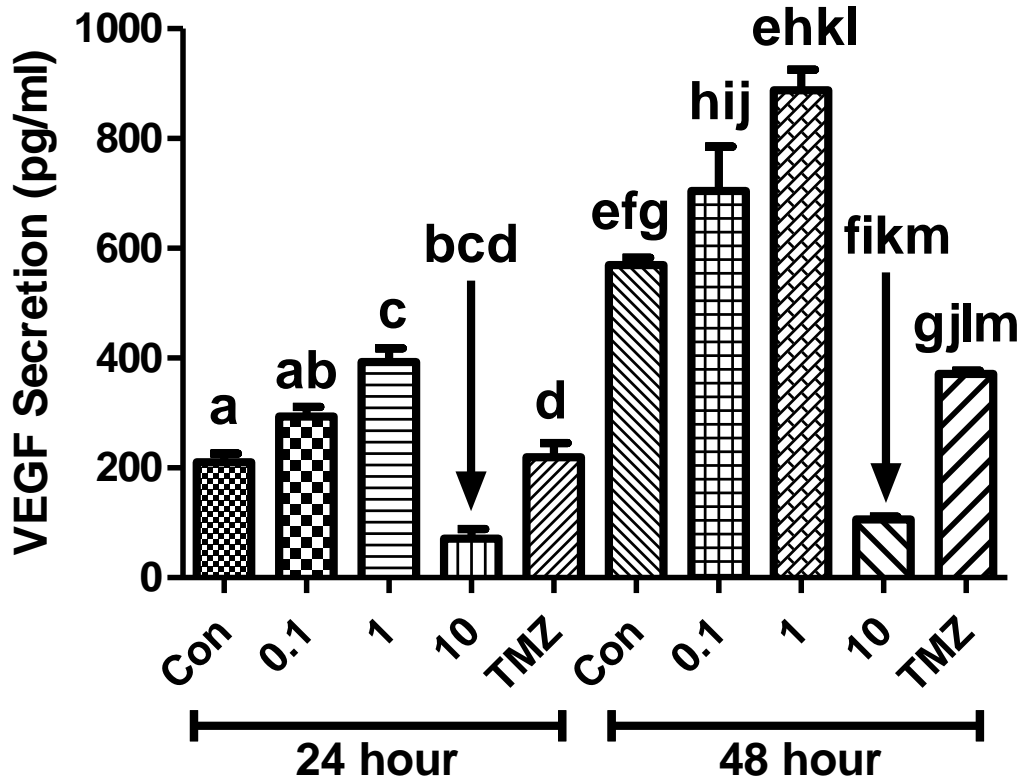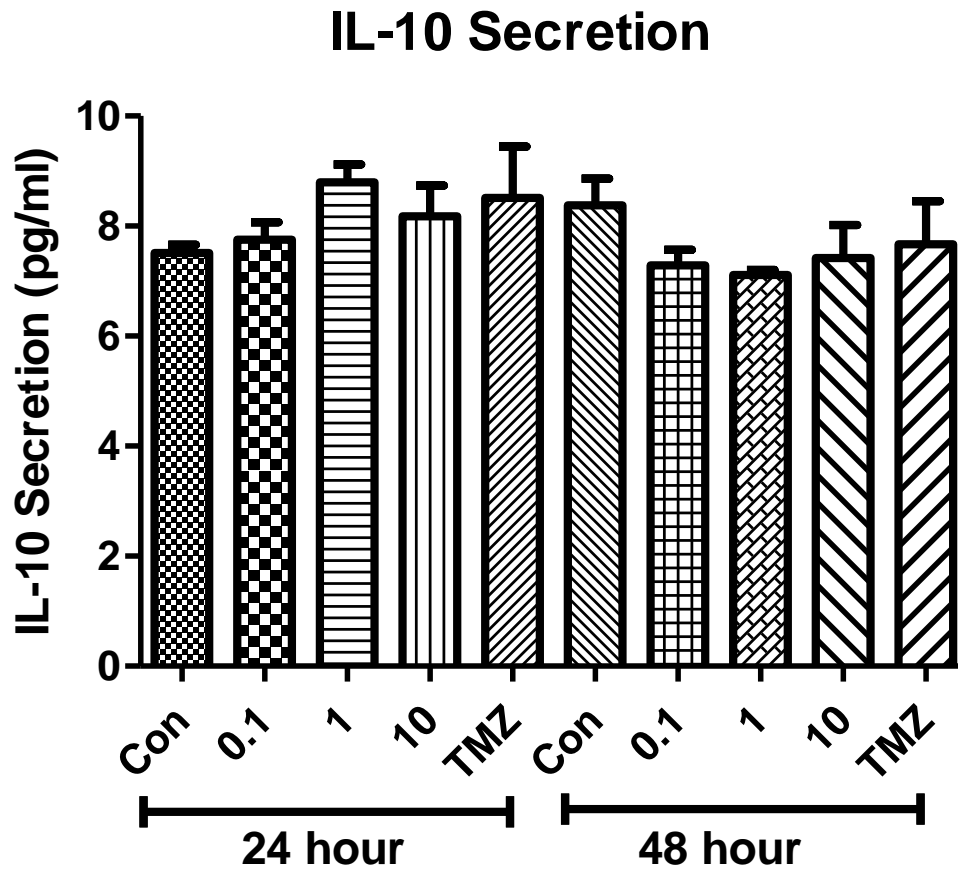
Figure 10: IL-10 secretion from T98-G cells. Cells were treated with DMSO vehicle control, 0.1 μM, 1 μM, or 10 μM E804, or TMZ for 24- or 48-hours. No significant differences were seen.

Immunoblotting using T98-G Lysates

T98-G cellular lysates were collected after E804 and TMZ treatment. Western blots were performed probing for protein expression of IDO1, TDO2, CYP1B1, and GAPDH. No induction was seen relative to the control for any of the proteins (data not shown).

Expression of mRNAs indicative of M1 vs. M2 macrophage polarization

T98-G cellular supernatants were collected after treatment as previously described, then applied to macrophage-like differentiated THP-1 cells for 24 hours. In this experiment, the 24 and 48 hour groups are with respect to the treatments of T98-G cells with E804. The mRNA expression of IL-6, Arginase 1 (ARG1), Arginase 2 (ARG2), IDO1, COX-2, and IL-10 were quantified and normalized against GAPDH. See Table 1 for primer sets utilized. IL-6 expression decreased in response to the 24-hour treated supernatants with respect to a media-only control, but significantly increased in response to the 48-hour supernatants treated with 0.1 µM E804. ARG1 expression decreased with respect to the media-only control in response to both the 24- and 48-hour treated supernatants. Alternatively, ARG2 expression generally increased in response to both groups. IL-10 expression initially increased in response to the 24-hour treated supernatants, particularly the 10 µM, but decreased in response to the 48-hour treated supernatants. IDO1 expression also generally increased in response to both groups. COX-2 expression decreased in response to the 24 hour DMSO vehicle control, 0.1, and 1 µM E804 treated supernatants and the 48 hour 1 µM E804 treated supernatant. However, COX-2 expression increased in response to the other treated supernatants. IL-10 expression

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increased significantly in response to the 24- and 48-hour treated 10 µM E804

supernatants.

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Gene	Primer Sequence (5' to 3')	Anneal Temp (°C)	Product Size (bp)
GAPDH	F: AGCCTCAAGATCATCAGCAATGCC	57	105
	R: TGTGGTCATGAGTCCTTCCACGAT		
COX-2	F: TACTGGAAGCCAAGCACTTT	52	92
	R: GGACAGCCCTTCACGTTATT		
IL-6	F: TCAATGAGGAGACTTGCCTGGTGA	58	123
	R: TACTCATCTGCACAGCTCTGGCTT		
IL-10	F: TCCTTGCTGGAGGACTTTAAGGGT	57	109
	R: TGTCTGGGTCTTGGTTCTCAGCTT		
ARG1	F: CAGGGCTACTCTCAGGATTAG	52	115
	R: CCGAAACAAGCCAAGGTTATT		
ARG2	F: CATGGACAGCCAGTTTCATTTC	52	127
	R: CCACGTCTCTCAGACCAATATAC		
IDO1	F: CACTTTGCTAAAGGCGCTGTTGGA	57	140
	R: GGTTGCCTTTCCAGCCAGACAAAT		

Table 1: qRT-PCR primer sets.

IL-6 mRNA Expression

Figure 11: IL-6 mRNA expression in THP-1 cells. Cells were treated with DMEM (Med Con), or supernatants from T98-G cells treated with DMSO vehicle control, 0.1 μM, 1 μM, or 10 μM E804, or TMZ for 24- or 48-hours. Corresponding letters above bars indicate a significant difference. (a) $P \leq 0.01$, (b) $P \leq 0.05$, (c) $P \leq 0.05$, (d) $P \leq 0.001$, (e) $P \leq 0.01$.

ARG1 mRNA Expression

Figure 12: ARG1 mRNA expression in THP-1 cells. Cells were treated with DMEM (Med Con), or supernatants from T98-G cells treated with DMSO vehicle control, 0.1 µM, 1 µM, or 10 µM E804, or TMZ for 24- or 48-hours. Corresponding letters above bars indicate a significant difference. (a) P ≤ 0.05.

Figure 13: ARG2 mRNA expression in THP-1 cells. Cells were treated with DMEM (Med Con), or supernatants from T98-G cells treated with DMSO vehicle control, 0.1 μM, 1 μM, or 10 μM E804, or TMZ for 24- or 48-hours. Corresponding letters above bars indicate a significant difference. (a) $P \leq 0.01$, (b) $P \leq 0.05$, (c) $P \leq 0.05$.
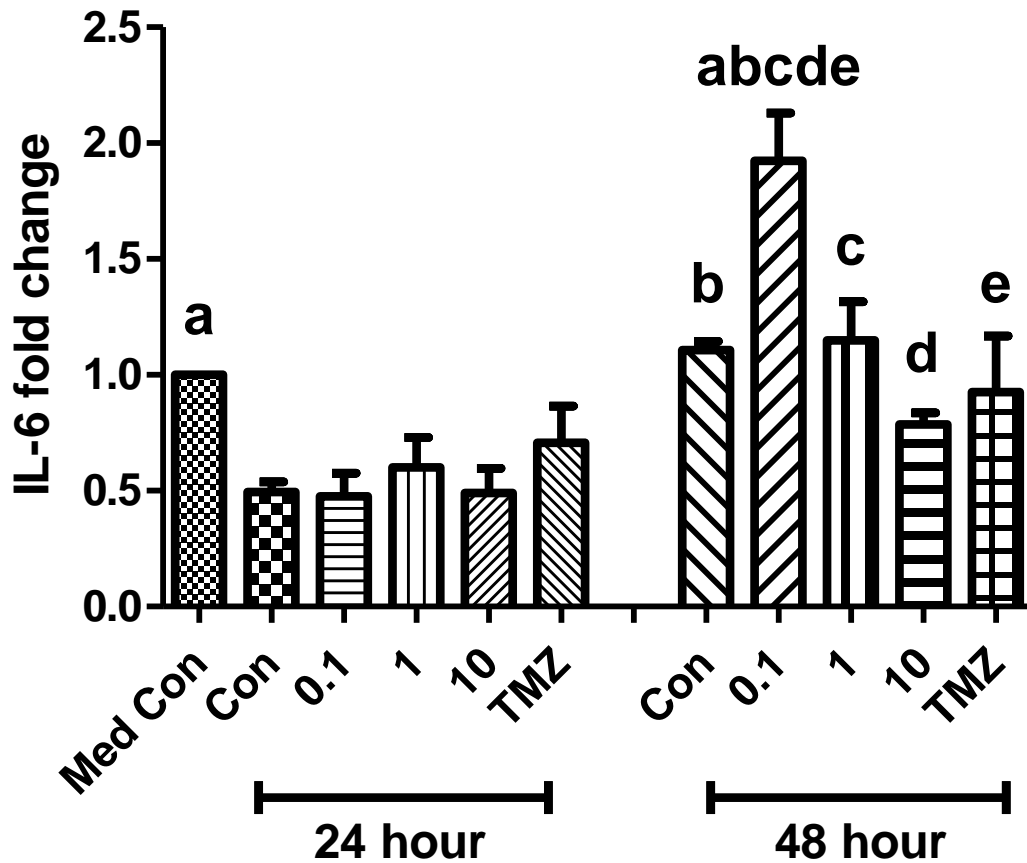
IDO1 mRNA Expression

Figure 14: IDO1 mRNA expression in THP-1 cells. Cells were treated with DMEM (Med Con), or supernatants from T98-G cells treated with DMSO vehicle control, 0.1 μM, 1 μM, or 10 μM E804, or TMZ for 24- or 48-hours. Corresponding letters above bars indicate a significant difference. (a) $P \leq 0.05$.

COX2 mRNA Expression

Figure 15: COX-2 mRNA expression in THP-1 cells. Cells were treated with DMEM (Med Con), or supernatants from T98-G cells treated with DMSO vehicle control, 0.1 μM, 1 μM, or 10 μM E804, or TMZ for 24- or 48-hours. Corresponding letters above bars indicate a significant difference. (a) P ≤ 0.05.
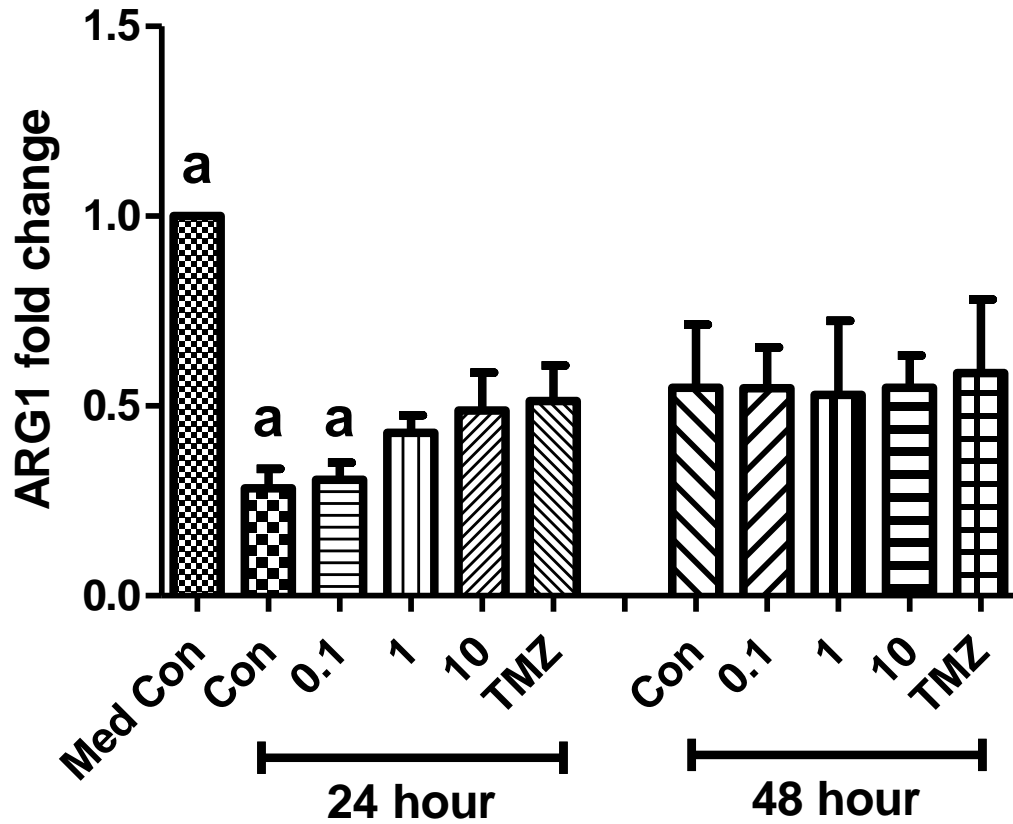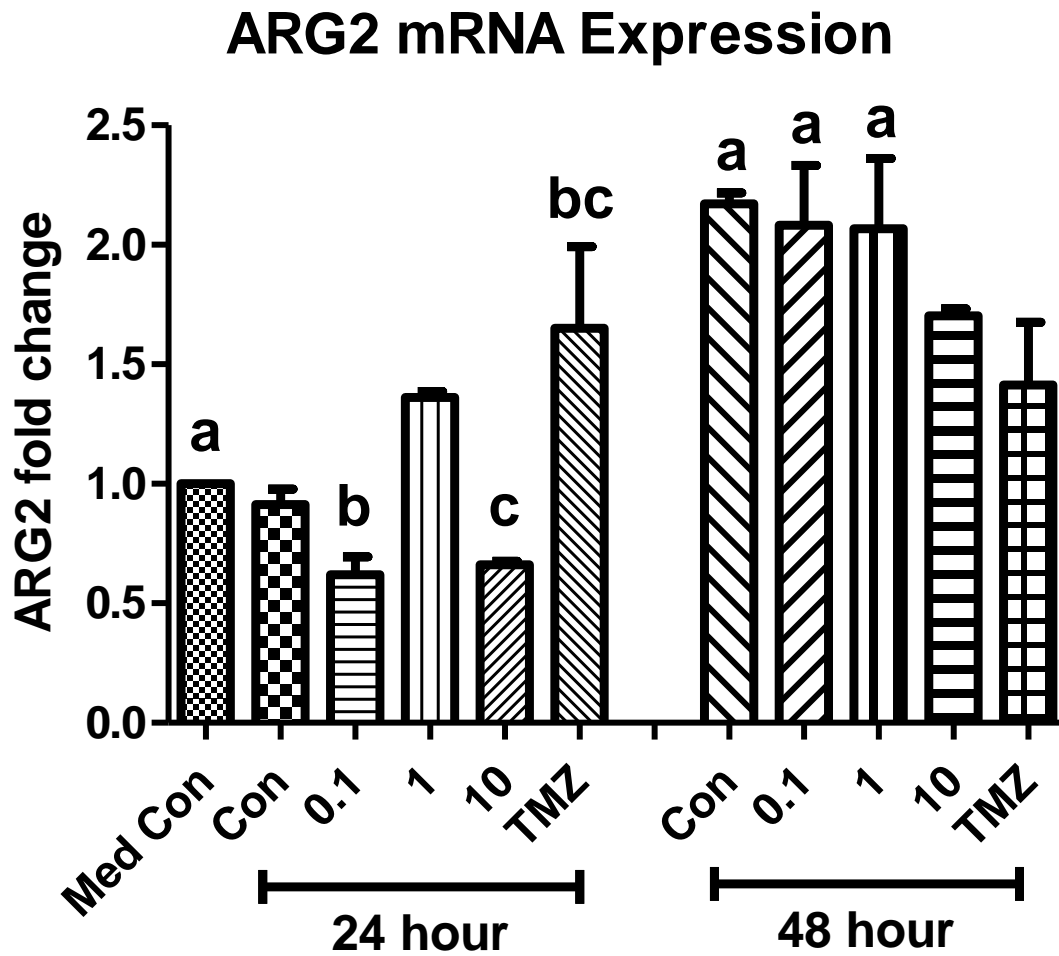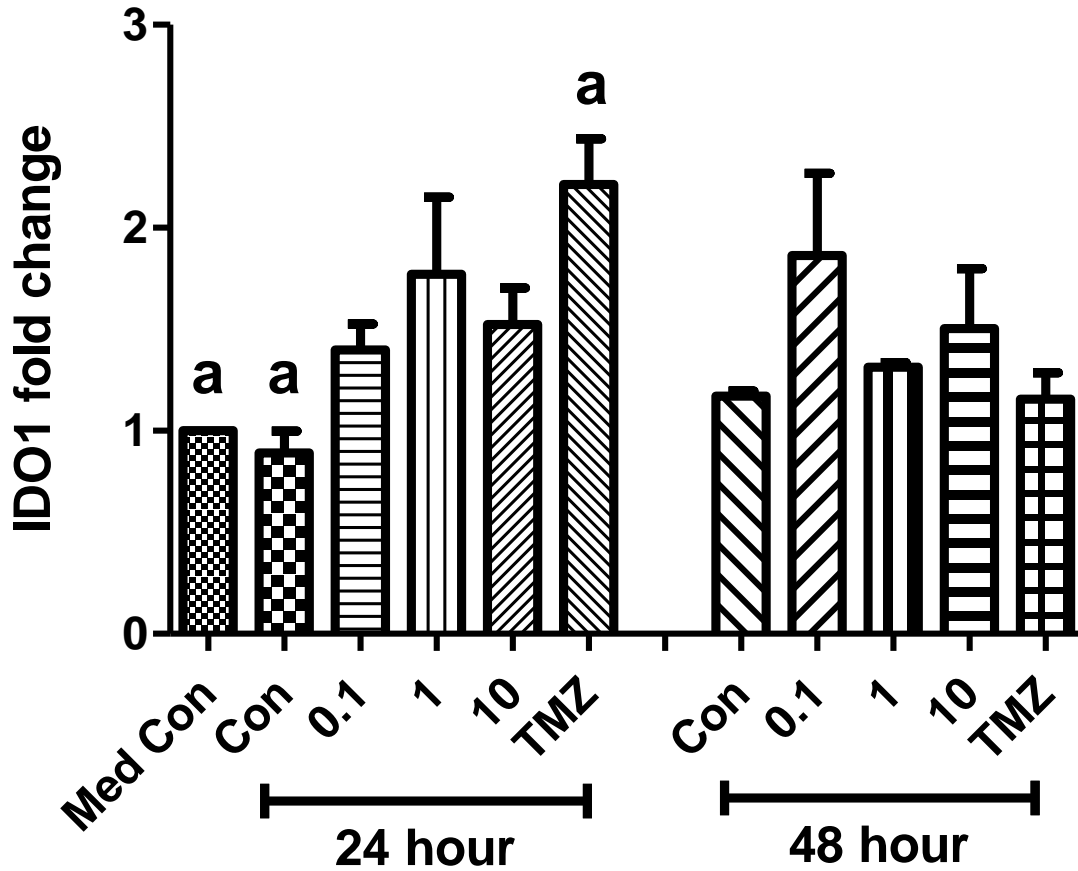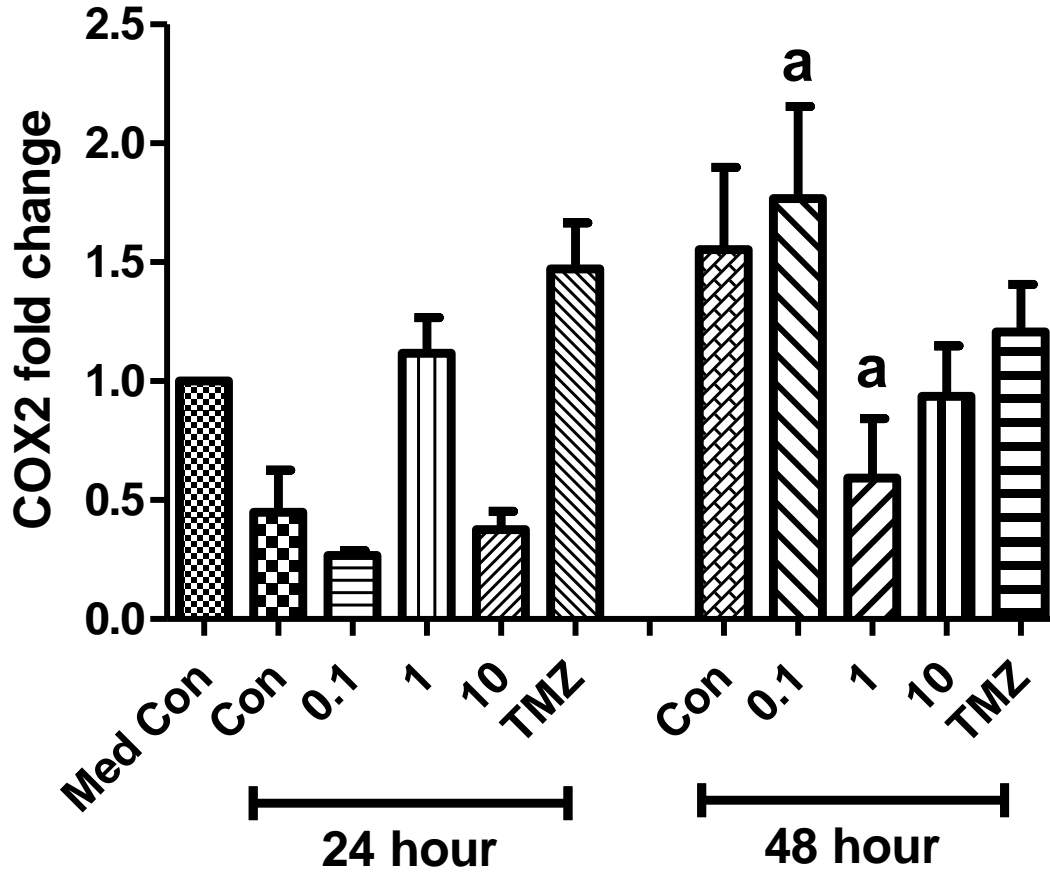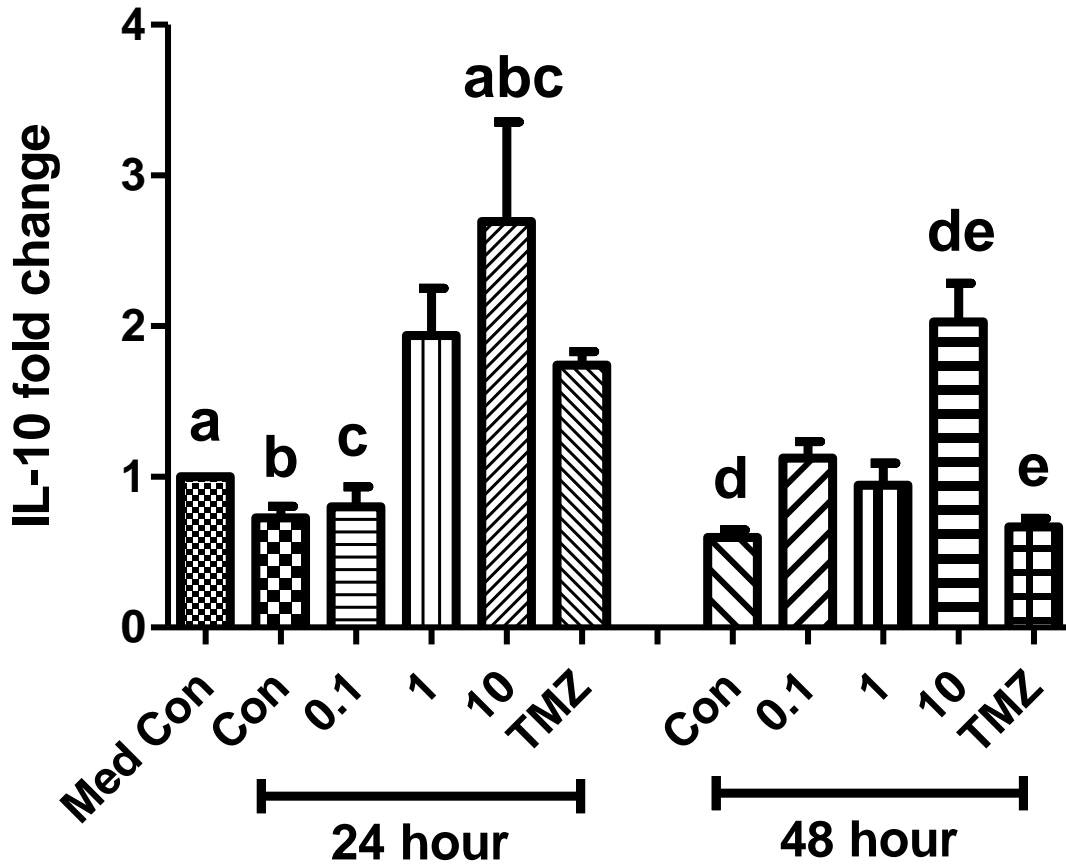
Figure 16: IL-10 mRNA expression in THP-1 cells. Cells were treated with DMEM (Med Con), or supernatants from T98-G cells treated with DMSO vehicle control, 0.1 μM, 1 μM, or 10 μM E804, or TMZ for 24- or 48-hours. Corresponding letters above bars indicate a significant difference. (a) $P \leq 0.01$, (b) $P \leq 0.001$, (c) $P \leq 0.01$, (d) $P \leq 0.05$, (e) $P \leq 0.05$.

Immunoblotting for CYP1B1 expression using Hepa-1c1c7 reporter cells

T98-G cellular supernatants were collected after E804 and TMZ treatment, and then applied to Hepa-1c1c7 cells, which were incubated for 24 hours. Hepa-1c1c7 cellular lysates were then collected, and Western blots performed probing for CYP1B1 and GAPDH protein expression. No induction of CYP1B1 was seen (data not shown).

DISCUSSION

In this study, I show that basal expression of IL-6, TGF-β, and VEGF T98G cells is extremely high, further supporting the notion that treating glioblastomas is difficult when they are already influencing local immune responses and angiogenesis at the time of diagnosis and first treatment. The secretion of IL-6, TGF-β, and VEGF could be reduced by 10 μM E804, and this reduction in cytokine and growth factor secretion could possibly lead to reduced angiogenesis. I also demonstrate that lower doses of E804 enhanced the secretion of angiogenesis-inducing VEGF, which causes concern for even administering high doses of E804 because this indirubins in general are quickly metabolized (Gillam et al., 2000), thus lowering local levels to the point of more VEGF secretion. Furthermore, E804 treatment increased the mRNA expression levels IL-6, IDO1, COX-2, and IL-10 in THP-1 macrophages, indicating an M2 polarization. Thus, E804 may enhance immunosuppression in the tumor microenvironment. More studies are needed, but with careful monitoring, E804 could be a viable option to reduce angiogenesis and immunosuppression in glioblastoma patients.

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Initial cytotoxicity studies using MTT showed that E804 treatment is not affecting cellular respiration, which is an indirect measurement of cell viability. Cells were also labeled with Ki-67, a marker of cellular proliferation (Louis et al.; Montine et al., 1994). After treatment, T98-G cells continued to proliferate, confirming the MTT data. Therefore, the E804 concentrations used experimentally were not cytotoxic. Previous studies from our lab have also shown E804's lack of cytotoxicity on RAW264.7 murine macrophages (Anderson, 2014).

To measure if E804 is interacting with the aryl hydrocarbon receptor (AhR), I quantified CYP1B1 mRNA expression using qRT-PCR. The fold expression of CYP1B1 increased in a time and dose-dependent manner with E804 treatment. This suggests that E804 is indeed interacting with the AhR and is being metabolized in the cell by the cytochrome p450 enzyme CYP1B1 (Denison and Nagy, 2003; Spink et al., 2003). Future studies should consider the possibility of incorporating AhR antagonist when using chemotherapeutic agents.

Using the T98-G supernatants, ELISAs were performed to quantify IL-6, TGF-β, VEGF, and IL-10 secretion. Previous work from our lab has shown that LPS-stimulated RAW 264.7 macrophages secrete approximately 400 pg/ml of IL-6, so the fact that T98-G glioma cells are producing around 600 pg/ml of IL-6 is considerable (Anderson, 2014). Heightened levels of IL-6 have been implicated in increased angiogenesis and tumor metastasis (Liu et al., 2010; Mauer et al., 2015; Taniguchi and Karin, 2014). This agrees with other studies that have shown the anti-angiogenic properties of E804 in human umbilical vein endothelial cells (Ali et al., 2005; Chan et al., 2012; Shin and Kim, 2012).

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TGF-β secretion increased at 48 hours in response to lower concentrations of E804 and a slight reduction in TGF-β was seen in response to 10 µM E804 at both time points. TGF-β has been shown to suppress T-cell function in tumor cells, and increase angiogenesis (Bodmer et al., 1989; Constam et al., 1992; Gramatzki et al., 2009b; Letterio, 1998; Wu et al., 2010). VEGF secretion was increased at all concentrations of E804 except 10 µM, which is cause for concern because VEGF is the main signal protein that initiates neovascularization, which is key for solid tumors to sustain growth (Ali et al., 2005; Byrne et al., 2005; Cabebe and Wakelee, 2007; Carmeliet, 2005; Kiselyov et al., 2007; Shibuya, 2006; Shin and Kim, 2012; Shinkaruk et al., 2003; Zhang et al., 2011). If high levels of E804 could be sustained, then local angiogenesis may be reduced.

The collected T98-G supernatants were also applied to differentiated macrophage-like THP-1 cells. Overall, the treated T98-G supernatants incubated with the THP-1 cells for 24 hours generally decreased IL-6 and ARG1 expression, and increased IDO1 expression as compared to the media-only control. ARG2 and COX-2 expression did not significantly change. For the 48-hour group, there was a general decrease in ARG1 expression, and an increase in IL-6, IDO1, ARG2, and COX-2 expression. An increase in IL-10 expression was seen as a result of the 10 µM E804 treated T98-G supernatants at both time points. Prior studies have identified increased ARG2 and IL-6 as M1 macrophages markers, and ARG1, IDO1, and IL-10 as M2 macrophage markers (Bronte et al., 2003; Mantovani et al., 2002; Zea et al., 2005). COX-2 has been suggested as a marker for both M1 and M2 macrophages, but the M2 polarization seems to dominate in tumors (Nakanishi et al., 2011; Tjiu et al., 2009). It is difficult to tease apart individual

effects, since there is no doubt a balancing act involved in the expression of each gene, but with an increase in IL-6, IDO1, COX-2, and IL-10, the mRNA expression profile of THP-1 cells treated with T98-G supernatants suggests that there is a slight M2 polarization occurring.

To confirm that there was no biologically active E804 remaining in the treated T98-G supernatants, heap-1c1c cells were incubated with the supernatants and CYP1B1 protein levels were measured via immunoblotting. No induction of CYP1B1 was seen, therefore, the supernatant's effects seen on gene expression in the THP-1 qRT-PCR results are from factors secreted into the media by the T98-G cells, and not the presence of biologically active E804. This observation bodes well for using potent, but labile AhR ligands as therapeutic agents in a variety of disease applications.

The results of this study suggest the E804 could have anti-angiogenic properties at a concentration of 10 µM. However, it would be difficult to reach a concentration that high *in vivo*, except perhaps with a wafer implant, such as currently used with the chemotherapeutic drug carmustine (Gliadel®) (Perry, 2007). It is also worrisome that lower concentrations of E804 could potentially have the opposite effects, increasing cytokine production and angiogenesis. Another concern would be the slight M2 polarization of macrophages seen in response to E804-treated T98-G supernatants. M2 macrophages are pro-tumor and help suppress immune response the surrounding microenvironment (Kennedy et al., 2009; Kennedy et al., 2013; Mantovani et al., 2002; Wu et al., 2010). As a result, E804 may increase the immunosuppressive tumor environment, enhancing tumor survival and growth. Further characterization of the

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effects of E804 is needed, particularly *in vivo*, but with controlled release or precise concentration monitoring, E804 has potential as a treatment option to reduce angiogenesis and immunosuppression in glioblastoma patients.

FUTURE EXPERIMENTS

Moving forward, there are several *in vitro* experiments that would help clarify the mechanism by which E804 causes its effects. First, an experiment to knock-down or knock-out the aryl hydrocarbon receptor would definitively prove if E804's action was mediated by this pathway. There are many ways to approach this type of experiment, but the most precise method would be to use the CRISPR/Cas9 system. Next, I would explore the nature of the immunomodulatory mechanism by which the T98-G supernatants act on the THP-1 differentiated macrophages. It would be interesting to see if the T98-G cells are releasing something other than cytokines and growth factors into the media, such as extracellular vesicles. To explore this question, I would compare the application of supernatants collected from T98-G cells to THP-1 cells to co-culturing T98-G cells with THP-1 differentiated macrophages. Another method would be to acquire bone-marrow derived macrophages, and use them in place of the differentiated THP-1 cells (Weischenfeldt and Porse, 2008). Finally, I would like to explore the effect of T98-G cells on the growth and migration of endothelial cells, such as human umbilical vein endothelial cells. For this experiment, I would also like to compare application of T98-G supernatants to endothelial cells with co-culturing of these two cell types.

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After further *in vitro* characterization, I would move into an *in vivo* mouse model to compare to the *in vitro* results, in order to see if E804 could perform in a similar way in a live organism. To do this, I would want to first use an immunocompromised mouse model with a xenograft human glioma, such as U251 or U1242 MG, to look at angiogenesis, growth, and metastasis (Chen et al., 2013; Pontén, 1975; Zhao et al., 2010). To see the effect of E804 on immunomodulation *in vivo*, I would use adult mice or rats with an active immune system in which the formation of glioma-like tumors were initiated using a retrovirus, such as described in other studies (Assanah et al., 2006; Kamiyama et al., 2005; Kennedy et al., 2009; Uhrbom et al., 1998).

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