2012

EFFECTS OF 1α,25-DIHYDROXYVITAMIN D3 (CALCITRIOL) ON CULTURED GLIOBLASTOMA MULTIFORME CELLS

Justine M. Pinskey
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EFFECTS OF 1α,25-DIHYDROXYVITAMIN D3 (CALCITRIOL) ON CULTURED GLIOBLASTOMA MULTIFORME CELLS

By

Justine M. Pinskey

THESIS

Submitted to
Northern Michigan University
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE, BIOLOGY

Graduate Studies Office

2012
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Justine M. Pinskey, Born December 18, 1986
ABSTRACT

EFFECTS OF 1α,25-DIHYDROXYVITAMIN D3 (CALCITRIOL) ON CULTURED GLIOBLASTOMA MULTIFORME CELLS

By

Justine M. Pinskey

Glioblastoma multiforme (GBM) is an aggressive, incurable type of brain tumor. Brain tumor stem cells (BTSCs) make up a small subset of GBM cells. BTSCs resist standard treatments, initiate recurrence, and pose a significant challenge for GBM treatment. This study examined gene expression and proliferation of GBM cells in vitro to evaluate the effects of 1α,25-dihydroxyvitamin D3 (vitamin D3) treatment. Vitamin D3 is a safe, natural inhibitor of the hedgehog signaling pathway—a mechanism essential to BTSC function. The data in this study demonstrate that the hedgehog signaling pathway is active in both differentiated GBM cell lines and GBM-derived BTSCs. Vitamin D3 reduced GBM cell proliferation, especially in BTSC lines, although the dose at which vitamin D3 was effective varied within the studies. Vitamin D3 also prevented neurosphere formation from a single cell suspension and induced apoptosis in BTSCs, indicating its potential as a therapeutic agent. Because of active vitamin D3’s environmental instability, an in vivo model might provide a more complete indication of its anti-tumor effects. Ultimately, vitamin D3 may enhance standard GBM treatments by disabling BTSCs, but further research is needed to determine vitamin D3’s role in modulating the growth of GBM cells.
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This thesis is dedicated to those who are suffering or have suffered from Glioblastoma Multiforme, and to those who work hard every day to improve GBM treatments.
ACKNOWLEDGEMENTS

The completion of this thesis would not have been possible without financial support from the Upper Michigan Brain Tumor Center, the Ronald E. McNair Postbaccalaureate Achievement Program, the Excellence in Education Grant, and the Charles C. Spooner Research grant from Northern Michigan University.

In addition, I would like to extend my sincere gratitude to my advisor, Dr. Robert J. Winn, and my committee members, Dr. Erich N. Ottem and Dr. Richard A Rovin for their guidance and support, Dr. John Rebers for his technical assistance, and the members of the Upper Michigan Brain Tumor Center for their various contributions, especially Danny LeBert, Vanessa Thibado, Keith Sabin, and Justin Segula. I would also like to thank the Hermelin Brain Tumor Center at Henry Ford Hospital for their collaboration and generosity.

Last but not least, I am incredibly grateful for the love, patience, and support I received from my parents: Rob and Darlene Pinskey, my sisters: Melissa and Nicole Pinskey; and my significant other Andrew Blumenthal during the ups and downs of the research process.
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BTSCs</td>
<td>Brain tumor stem cells</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phénylindole</td>
</tr>
<tr>
<td>Dhh</td>
<td>Gene encoding desert hedgehog protein</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>EMEM</td>
<td>Eagle’s Minimum Essential Medium</td>
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<td>EtOH</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
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<tr>
<td>hh</td>
<td>Gene encoding hedgehog protein</td>
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<td>Hh</td>
<td>Hedgehog protein</td>
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<tr>
<td>Ihh</td>
<td>Gene encoding Indian hedgehog protein</td>
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<tr>
<td>KAAD</td>
<td>3-Keto-N-(aminoethyl-aminocaproyl-dihydrocinnamoyl)</td>
</tr>
<tr>
<td>MYCN</td>
<td>V-myelocytomatosis viral related oncogene</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
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<td>nM</td>
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NM………………Neurosphere media
NMGF………….Complete neurosphere media (with growth factors)
PBS……………Phosphate buffered saline
PFA……………Paraformaldehyde
Ptch…………..Patched protein
qPCR………….Quantitative reverse transcription polymerase chain reaction
Shh……………Gene encoding sonic hedgehog protein
Smo…………..Smoothened protein
Sufu…………..Gene encoding suppressor of fused protein
SuFu…………..Suppressor of fused
TMZ………….Temozolomide
TRAIL………….TNF-related apoptosis-inducing ligand
UT……………Untreated
Vit. D…………..1α,25-dihydroxyvitamin D3
Vitamin D3…….1α,25-dihydroxyvitamin D3
VDR………….Vitamin D3 receptor
INTRODUCTION

The World Health Organization classifies Glioblastoma multiforme (GBM) as a grade IV astrocytoma, indicating this deadly cancer’s ability to grow and spread quickly. GBM is not only the most malignant, but also the second most common primary brain tumor in adults (CBTRUS, 2011). Additionally, brain tumors including GBM represent a leading cause of cancer-related death in children (Dirks, 2010). Despite aggressive multimodal treatments, including radiation and chemotherapy, patients diagnosed with GBM have a median survival of only fifteen months (ACS, 2010). Moreover, the 1-year survival rate for GBM patients in the United States from 1995-2007 was only 34.60%, lower than any other type of malignant brain tumor (CBTRUS, 2011). Tremendous research efforts have been made within the last few decades to improve GBM treatment, but these efforts have encountered very little success, and finding new treatment targets remains difficult. Because of its location and aggression, GBM treatment is one of the most significant challenges faced by cancer researchers today.

Brain tumor stem cells (BTSCs) have recently emerged as a potential target for GBM treatment. BTSCs are transformed cells that retain stem cell-like properties, including the ability to differentiate into many cell types and the capacity for self-renewal (Vescovi, Galli, & Reynolds, 2006). Although BTSCs represent only a fraction of the cells in a tumor, they are thought to be largely responsible for tumor formation and recurrence (Clark, Treisman, Ebben, & Kuo, 2007; Galli et al., 2004; Kondo, 2004). While traditional cancer treatment methods like radiation and chemotherapy often target the most rapidly dividing cells in a tumor, BTSCs avoid destruction because of their
limited replication and enhanced ability to repair DNA damage (Bao et al., 2006). BTSCs that remain in the body after traditional cancer treatments may drive the formation of secondary tumors, which often cause GBM death by spreading to vital areas of the brain (Holland, 2000). Clinically, increased BTSC representation in GBM tumors negatively correlates with overall and progression free-survival (Pallini et al., 2008). While further research is necessary to fully understand and characterize BTSCs, developing new therapies that effectively target these cells will have drastic implications for GBM treatment.

To this end, a number of developmental pathways thought to be important in both normal neural stem cell function and BTSC function are being investigated (Clark, et al., 2007). Among them, the hedgehog signaling pathway is active in a subset of primary GBM tumors and implicated in BTSC-related tumorigenesis (Bar et al., 2007). Hedgehog signaling acts in normal stem and progenitor cells to regulate embryonic development. The hedgehog ligand (Hh) binds to a cell surface receptor called Patched (Ptch), which then relieves its inhibition of another protein Smoothened (Smo). Once Smo is free, it initiates a cascade of molecular signals, resulting in the activation of several members within the Gli family of proteins. Gli proteins act within the nucleus to regulate the expression of multiple target genes involved in cell growth and proliferation, including v-myc myelocytomatosis viral related oncogene (MYCN), cyclin D2, and IGF2 (Marcotullio et al., 2004). In addition, hedgehog signaling upregulates transcription of response genes Gli1, Gli2, Ptc1, and Hip (the gene that encodes the hedgehog interacting protein), which have been used in the literature to assess hedgehog pathway
activation (Aparicio et al., 2007; Clement, Sanchez, de Tribolet, Radovanovic, & Ruiz i Altaba, 2007).

1α,25-dihydroxyvitamin D3 (vitamin D3), also known as calcitriol, has been shown to block hedgehog signaling by binding to Smo and has independently been associated with positive treatment outcomes for GBM patients (Bijlsma et al., 2006; Trouillas, Honnorat, Bret, Jouvet, & Gerard, 2001). This study sought to explore the effects of vitamin D3 on GBM cell proliferation in vitro, with the hypothesis that vitamin D3 would block hedgehog signaling and thus inhibit GBM cell proliferation. Because hedgehog signaling is more active within the BTSC population than in differentiated cells, vitamin D3 treatment may be an effective way to target BTSCs specifically. A secondary goal of the study was to compare vitamin D3’s effectiveness with that of cyclopamine, a toxic Smo inhibitor not suitable for therapeutic use. Should vitamin D3 effectively disable GBM growth in vitro, it may make a safe and inexpensive supplement to standard GBM treatments.
CHAPTER 1: LITERATURE REVIEW

Introduction

Glioblastoma Multiforme is an aggressive, incurable type of brain malignancy. Recent evidence suggests that the hedgehog signaling pathway may be implicated in GBM tumorigenesis and important for BTSC function. Vitamin D3, a safe, natural hedgehog pathway inhibitor, has been previously correlated with reduced tumor burden, although its mechanism of action is incompletely understood. This chapter highlights advances in the seemingly disparate research areas of hedgehog signaling and vitamin D3 research in the context of GBM treatment, emphasizing the overlap between these different areas and identifying gaps in our current knowledge.

A Brief Overview of Hedgehog Signaling

Originally discovered in *Drosophila melanogaster*, the hedgehog signaling pathway is present in many taxa, both vertebrate and invertebrate (Hooper & Scott, 2005). While the cellular machinery to execute hedgehog signaling has been largely conserved between insects and mammals, the pathway’s target genes vary based on tissue type and phyla (Hooper & Scott, 2005). In general, the main roles of hedgehog signaling include controlling developmental patterning, regulating proliferation, and regulating differentiation of stem and precursor cells (Yang, Xie, Fan, & Xie, 2010). The pathway is activated when a secreted signaling protein encoded by the hedgehog gene (\textit{hh}) binds to a receptor on the cell’s surface to initiate a series of downstream effects.
In humans and other mammals, three hedgehog genes have been characterized: sonic hedgehog, indian hedgehog, and desert hedgehog (Shh, Ihh, and Dhh, respectively) (Hooper & Scott, 2005). For the purposes of this review, the signaling ligands encoded by these three genes will be simply referred to as Hh, despite potential differences in biologic activity. Hedgehog signaling is initiated when the Hh ligand binds to Patched (Ptch), a 12-transmembrane receptor protein found on the cell surface, causing it to be internalized via endocytosis and undergo lysosomal degradation (Hooper & Scott, 2005). Hh binding functions to relieve Ptch’s inhibition of a seven-transmembrane protein Smoothened (Smo), which in turn initiates a cascade of downstream molecular signals. Although the mechanisms by which Ptch inhibits Smo is not well understood, Ptch contains evolutionarily conserved sterol-sensing domains that have been deemed necessary for Smo repression (Hooper & Scott, 2005). It has been proposed that Ptch acts as a pump, moving 3β-hydroxysteroid molecules into the extracellular space that then bind to Smo and inhibit its function, potentially acting both intercellularly and intracellularly (Bijlsma, et al., 2006).

When the hedgehog pathway is inactive, members of the Gli family of transcription factors are retained in a cytoplasmic complex which includes proteins such as Fused, Suppressor of Fused (SuFu), and structural protein KIF7 (di Magliano & Hebrok, 2003; Hooper & Scott, 2005). When the Hh ligand binds, Ptch inhibition of Smo is relieved, allowing Smo to regulate the activity of downstream molecules (Figure 1). Although Smo’s mechanism of action remain unclear, it is thought that Smo acts as a type of G-Protein coupled receptor (Ayers & Thérond, 2010), that causes the release of Gli proteins from the cytoplasmic complex, allowing them to move into the nucleus and
regulate transcription of target genes (Hooper & Scott, 2005). The Gli family of proteins includes Gli1, Gli2, and Gli3, which are expressed in different tissue types at varying stages of development and have unique roles within the cell. Gli1 and Gli2 act as transcriptional activators, while Gli3 primarily represses transcription (Hooper & Scott, 2005). Depending on the ratio of activation and repression by Gli1 transcription factors, target genes can be specifically regulated. A sample of the many target genes regulated by Gli family proteins act to increase cellular proliferation, including v-myc myelocytomatosis viral related oncogene ($MYCN$), cyclin $D2$, and $IGF2$ (Marcotullio, et al., 2004). In addition, hedgehog signaling upregulates the transcription of its own pathway members $Gli1$, $Gli2$, $Ptch1$, and $Hip$ in a positive feedback loop. Gli1 and Ptch1 in particular have been used in the literature to assess hedgehog pathway activation (Aparicio, et al., 2007; Clement, et al., 2007).

**Figure 1: General hedgehog signaling mechanism.** When the hedgehog ligand (Hh) is unbound, patched (Ptch) inhibits smoothened (Smo) and the fused/Sufu complex retains Gli proteins in the cytoplasm, rendering the pathway inactive (left). Upon Hh ligand binding, Ptch inhibition of Smo is relieved, allowing Gli proteins to move into the nucleus and regulate target gene expression (right).
Hedgehog Signaling in GBM and Other Cancers

The hedgehog signaling pathway has recently been implicated in human disease, including cancer (Hooper & Scott, 2005). Aberrant hedgehog signaling has been noted in malignancies such as leukemia, breast cancer, prostate cancer, pancreatic cancer, basal cell carcinoma, gastrointestinal cancers, medulloblastoma, glioma, and some forms of lung cancer (Aparicio, et al., 2007; Berman et al., 2002; Chi et al., 2006; Clement, et al., 2007; Watkins et al., 2003; Yang, et al., 2010). The pathway can be altered at several points to become oncogenic. Loss or inactivation of the Sufu gene, which encodes a member of the cytoplasmic complex that retains Gli in the cytoplasmic compartment, and overexpression of the Hh gene have been described in subsets of lung cancers, indicating that movement of free Gli to the nucleus is associated with a malignant phenotype (Chi, et al., 2006). Likewise, Ptch and Smo mutations have been implicated in medulloblastoma and prostate cancer (Aparicio, et al., 2007; Ellison et al., 2011). The natural suppression of the hedgehog signaling pathway can be disrupted at a number of different levels to cause disease, and until the pathway is fully characterized, we will lack a complete understanding of its aberrant effects.

Only recently has the hedgehog signaling pathway been implicated in GBM progression. In 2007, Eli Bar and colleagues described Smo, Ptch, and Gli1 expression in several GBM tumor cell lines as well as GBM-derived neurospheres—concentrated populations of brain tumor stem cells isolated from tumor samples (2007). Treating GBM cells with cyclopamine, a hedgehog pathway inhibitor that binds to the Smo protein, decreased Gli1 expression and reduced the growth of GBM cells in culture. Cyclopamine treatment depleted the stem cell population and prevented the formation of new
neurospheres, indicating that hedgehog signaling is indeed an important part of BTSC establishment and function (Bar, et al., 2007).

Furthermore, Bar’s group demonstrated that hedgehog inhibition by cyclopamine blocked the formation of new tumors. When untreated control GBM cells were implanted into mouse brains, aggressive tumors formed, rapidly killing the mice. Pre-treating the GBM cells with cyclopamine, however, completely abolished tumor engraftment and growth, leading to complete survival of the cyclopamine-treated cohort of mice (Bar, et al., 2007). These data suggest that blocking the hedgehog signaling pathway could potentially prevent recurrence or metastasis of GBM. These and other findings warrant a closer look into hedgehog signaling pathway mechanisms and investigation of potential hedgehog pathway inhibitors for GBM and other malignancies.

Inhibition of Hedgehog Signaling as a Means of Cancer Treatment

While cyclopamine is often used to experimentally inhibit the hedgehog signaling pathway, it has many potential clinical drawbacks (Bijlsma, Peppelenbosch, & Spek, 2007). In addition to being a known teratogen, cyclopamine is known to cause widespread toxicity, and is not safe for human use. Cyclopamine’s side effects include severe nausea, vomiting, bradycardia, syncope, paresthesia, weakness, diaphoresis, salivation, and hypotension ("Cyclopamine," 2002). In addition, cyclopamine is difficult to produce in mass quantities, making it an expensive, unsafe, and unrealistic treatment option (di Magliano & Hebrok, 2003).

Many groups are working to develop novel compounds that inhibit hedgehog signaling with less toxicity. One such synthetic analogue is 3-Keto-N-(aminoethyl-
aminocaproyl-dihydrocinnamoyl)cyclopamine (KAAD-cyclopamine), which is 10-20
times more potent than cyclopamine and helps modulate TNF-related apoptosis-inducing
ligand (TRAIL) in glioma cells (Siegelin, Siegelin, Habel, Rami, & Gaiser, 2009; Taipale
et al., 2000). The safety of KAAD-cyclopamine currently remains largely untested, but it
is suspected that its structural similarity to cyclopamine may cause similar unwanted
effects (Figure 2). Other classes of Smo antagonists (SANT1-SANT4) (Chen, Taipale,
Young, Maiti, & Beachy, 2002) and Sonic Hedgehog-neutralizing antibodies (Yang, et
al., 2010) have been identified, but again have not yet been tested for clinical use.
Forskolin, a Gli1 inhibitor, has been shown to reduce tumor growth in
Rhabdomyosarcoma muscle tumors with limited adverse effects in mice (Yamanaka,
Oue, Uehara, & Fukuzawa, 2011). However, Forskolin has also been shown to increase
the complexity of the blood-brain barrier, a tight network of capillaries surrounding the
brain, which suggests that it may not be well-suited for GBM treatment due to delivery
complications (Wolburg et al., 1994).

**Figure 2: Chemical structures of cyclopamine and KAAD-Cyclopamine** ("Chembase," 2011).
Developing these types of therapeutics aimed to inhibit the hedgehog signaling pathway is time-consuming and costly, and a safe, effective hedgehog inhibitor has yet to be identified.

**Vitamin D3 as an Exogenous Hedgehog Pathway Inhibitor**

1α,25-dihydroxyvitamin D3 (vitamin D3) is a safe, naturally occurring secosteroid that directly inhibits the hedgehog signaling pathway (Bijlsma, et al., 2006). With a mode of action very similar to that of cyclopamine, vitamin D3 binds to Smo, preventing it from inducing the signal cascade and thus preventing the transcription of hedgehog target genes. It has been proposed that the 3β-hydroxysteroid molecule involved in normal Ptch-dependent repression of Smo is indeed a form of vitamin D3 (Bijlsma, et al., 2006), although this has been debated and requires further investigation (Ayers & Thérond, 2010; Wilson, Chen, & Chuang, 2009). It has also been suggested that treatment with exogenous vitamin D3 to enhance natural hedgehog pathway repression might be an effective way to disable BTSC function (Bijlsma, et al., 2007; Bijlsma, et al., 2006).

Importantly, vitamin D3 naturally circulates throughout the body and crosses the blood-brain-barrier, making it an attractive therapeutic agent for GBM (Eyles, Burne, & McGrath, 2011). Because vitamin D3 is involved in calcium regulation, one potential side effect of high-dose vitamin D3 treatment might be hypercalcemia, or the overabundancy of calcium in the blood. This side effect is relatively common in cancer patients, affecting 10-20%, but is only rarely observed in GBM patients under standard
treatments (Seif, Azar, Barake, & Sawaya, 2006) Once vitamin D3’s therapeutic efficacy has been determined, possible approaches to reduce the risk of hypercalcemia can be explored, including individual dose determination, dietary restriction, and treatment screening based on preexisting risk factors (Heaney, 2008; Vieth, 2007). Additionally, one study showed that 1-α-dihydroxyvitamin D3 does not cause adverse side effects in mice, while its inactive counterparts 25-hydroxyvitamin D3 and vitamin D3 induced hypercalcemia and weight loss, suggesting that vitamin D3’s side effects may be dependent on concentration of its various forms (DeLuca, Prahl, & Plum, 2011).

Currently, temozolomide (TMZ) is the clinically accepted standard of care for glioblastoma patients. Temozolomide acts by methylating guanine residues at the N-7 or O-6 position, which induces DNA damage and triggers apoptosis. Temozolomide and vitamin D represent an attractive treatment combination, since temozolomide affects the rapidly dividing cells in a tumor, while inhibitors of hedgehog signaling affect BTSCs, which have limited replication and increased chemotherapeutic resistance. Clement and colleagues recently described a synergistic effect of TMZ plus an intermediate dose (5mM) of cyclopamine, demonstrating reduced proliferation and increased apoptosis in both glioma stem cell cultures and U87 glioblastoma cells (Clement, et al., 2007). Interestingly, however, interactions between vitamin D and temozolomide have not been previously described.

Vitamin D3 has long been associated with positive cancer outcomes. A recent review points out that numerous studies correlate vitamin D3 deficiency with increased risk for general malignancy, although the risk for specific, individual cancers is difficult to test, and remains unclear (Anaizi, 2010). In the United States and Canada, one group
has gone so far to suggest that raising year-round serum vitamin D levels to 40 to 60 ng/mL would prevent 58,000 new cases of breast cancer and 48,000 new cases of colorectal cancer every year in addition to preventing three-fourths of deaths from these diseases (Garland, Gorham, Mohr, & Garland, 2009). It is clear that vitamin D3 is a potentially useful cancer therapeutic, despite our incomplete understanding of its cellular mechanisms (Beer & Myrthue, 2004). Further investigation will be necessary to determine whether vitamin D3’s inhibition of the hedgehog signaling pathway contributes to its anti-cancer effects. Ultimately, vitamin D3 may be a useful addition to a therapeutic regimen for GBM and other malignancies.

**Conclusion**

The hedgehog signaling pathway is implicated in the tumorigenesis of GBM and other cancers, making it an attractive therapeutic target. Although synthetic antagonists of the pathway are currently being developed, vitamin D3 and its analogues may provide a safe, efficient, and low-cost alternative to synthetic novel compounds for hedgehog pathway inhibition. While the potential for vitamin D3 as a GBM treatment option is evident within the literature, its efficacy and mechanisms of action are complex, and remain elusive. Studies designed to further elucidate vitamin D3’s effects on GBM cells both *in vitro* and *in vivo* will shed light on its ability to act as a hedgehog pathway inhibitor and help determine its overall potential as a therapeutic agent.
CHAPTER 2: CULTURE AND MAINTENANCE OF GBM CELLS

Introduction

Cell culture has long been used as a model system for GBM studies. Recently, advances in technology and approach have allowed for culture of primary neurospheres, or free-floating populations of stem and progenitor cells, from isolated BTSCs. The experiments in subsequent chapters of this thesis involve both established GBM cell lines purchased from American Type Culture Collection (ATCC) and primary GBM BTSC-derived neurospheres isolated from patients at the Hermelin Brain Tumor Center at Henry Ford Hospital and Marquette General Hospital. This chapter outlines the procedures used to isolate, culture, and maintain the GBM cell lines that were used as model systems for experiments in later chapters.

Adherent GBM Cell Culture

Differentiated GBM cells grow as adherent cultures that have distinct morphological features including rapid growth and a lack of contact inhibition (Figure 3). GBM cell lines LN 229, U-87 MG, U-138 MG, and T 98 (ATCC) were used for the described experiments, cultured under standard conditions (37 °C, 5% CO₂). LN229 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS). U 87 MG, U 138 MG, and T 98 lines were grown in Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% FBS.

Passage of cell lines: Cells were passed as needed, when their confluency approached 100%. First, the cells were detached from the flask bottoms using
Trypsin/EDTA. Cells were then resuspended in the appropriate media and centrifuged at 500 times gravity for approximately five minutes. After removal of the supernatant, the cell pellet was resuspended in the appropriate media and cells and a cell count was performed using the trypan blue exclusion method. Cells were then replated at the desired confluency in new flasks.

*Cryopreservation of cell lines:* Cell stocks were kept in liquid nitrogen in the appropriate media with 10% Dimethyl Sulfoxide (DMSO).

**Figure 3: Morphological differences between differentiated GBM U 87mg cells and GBM-derived 2503 neurospheres.** Differentiated lines (left) grow as an adherent monolayer. Tumor-derived neurospheres (right) grow as free-floating spheres. Images were obtained using an Olympus CKX41 Inverted Fluorescent Microscope at 10x magnification.

**Neurosphere Isolation and Culture**

While differentiated GBM cells adhere to culture flasks, BTSCs grow as free-floating neurospheres, allowing for their selective culture in specified neurosphere media. BTSCs were isolated from both fresh and frozen GBM tissue samples obtained from Marquette General Hospital (Marquette, MI) and Henry Ford Hospital (Detroit, MI) with institutional review board approval. Tumor samples were cut into one millimeter cubed pieces using a sterile scalpel and then washed twice in Dulbecco’s Modified Eagle
Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco). Cells were dissociated overnight on a rotating table in collagenase (two milliliters for each half gram of tissue). The following day, tissues were mechanically homogenized, passed through a 40 µM cell strainer, washed, and then purified over a mouse Lympholyte column (Cedarlane Laboratories Limited, Hornby Ontario, Canada). Briefly, the cell suspension was slowly layered over a five milliliter lympholyte column which was then centrifuged for 20 minutes at 1300 xg at room temperature. The nucleated cells formed a layer between the aqueous media and the lympholyte, which was transferred to a separate tube and pelleted via centrifugation (100 xg, five-ten minutes at room temperature). The pellet was washed twice with 10 mL of DMEM/F12 and resuspended in Neurosphere Media (NM),

NM was comprised of Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco) supplemented with 500 µL N2 supplement (Gibco), 25 mg bovine serum albumin (Sigma), 125 µL Gentimicin (Gibco), and 250 µL Antibiotic/Antimycotic (Gibco) per 50 mL aliquot. Semi-complete media was filter-sterilized before use and stored at four degrees Celsius for no more than 10 days. Recombinant Human Fibroblast Growth Factor-basic (PeproTech) and Recombinant Human Epidermal Growth Factor (PeproTech) were added to sterilized media at 100 µg/mL to make complete media (NMGF), which was freshly prepared before each use. Neurospheres were cultured under standard conditions (37 °C, 5% CO2) in flasks designed specifically for non-adherent cell cultures (NUNC).

Of note, neurosphere cultures from numerous different tumor samples were attempted, but only a few readily generated robust spheres containing BTSCs that were able to regenerate new spheres upon dissociation. Samples that formed neurospheres of
100 µm or larger and survived through seven passages were used for further studies, including samples 190430 and 259274. Additionally, subsequent experiments were performed on neurosphere cell line 2503, which was a generous gift from the Hermelin Brain Tumor Center at Henry Ford Hospital.

Media changes were performed by tilting the NUNC flasks onto their short edges and allowing the neurospheres to settle for five minutes or longer. The top 25-33% of media was removed and replaced with fresh NM. Growth factors were added to each flask individually (usually two microliters per flask, depending on total volume).

**Passage of neurospheres:** To pass neurospheres, cells were transferred to fifteen milliliter conical tubes and allowed to settle for five to ten minutes. Supernatant media was removed, and the spheres were resuspended in 10 mL of sterile, calcium and magnesium free phosphate buffered saline (PBS, Lonza). After fifteen minutes, the solution was pipetted extensively (100 times or more) first with a fine Pasteur pipette and then with a 0.2-10 µL clear pipette tip secured over a 2-200 µL yellow tip secured over a 100-1000 µL blue tip. The series of escalating tip sizes provided a narrow opening to pass the neurospheres through while still allowing for a large volume (one milliliter). Dissociated neurospheres were centrifuged at 800 G for five-ten minutes at room temperature, resuspended in NMGF, and transferred to new flasks.

**Cryopreservation:** Stocks of dissociated neurospheres were kept in liquid nitrogen in DMEM/F12 supplemented with 10% Dimethyl Sulfoxide (DMSO).
Introduction

Hedgehog signaling has been shown to play an important role in glioblastoma stem cell function. Gli1 is a transcription factor that acts as the final messenger in the hedgehog signaling cascade, and is often used as a marker for pathway activity. The immunocytochemistry experiments in this chapter were designed to confirm the presence of Gli1 in LN 229, U 87 MG, U 138 MG, and T 98 cell lines (ATCC) as well as glioblastoma-derived neurospheres (BTSCs). In addition, changes in pathway target gene expression (Gli1 and MYCN) during hedgehog pathway inhibition were observed using the quantitative reverse transcription polymerase chain reaction (qPCR) method. The data indicate that Gli1 is expressed in both differentiated glioblastoma lines and glioblastoma-derived BTSC lines, and that vitamin D3 treatment affects hedgehog target gene expression. This preliminary evidence suggests that Vitamin D3 may be useful as a therapeutic hedgehog pathway inhibitor in GBM patients.

Methods and Materials

Immunocytochemistry

Immunocytochemical staining for Gli1 was performed to assess the activity of the hedgehog signaling pathway in LN 229, U 87 MG, U 138 MG, T 98, and GBM-derived neurosphere cell lines. Adherent cell lines (LN 229, U 87 MG, U 138 MG, T 98) were fixed and stained as previously described (Goldstein & Watkins, 2008). Cells were plated at 100,000 cells per well onto ethanol-sterilized coverslips at the bottom of six-well
plates. After 24 hours (or at approximately 70% confluency), coverslips were fixed using fresh 4% paraformaldehyde (PFA) solution with 1% Triton (Fisher Scientific) made in Phosphate Buffered Saline (PBS). Cells were then stained with primary polyclonal goat-anti Gli1 antibody (Santa Cruz Biotechnology) diluted to five micrograms per milliliter for one hour followed by secondary donkey anti-goat antibody conjugated to fluorescein isothiocyanate (FITC) diluted to 2.5 micrograms per milliliter for one hour. Secondary staining and imaging were performed in the dark. Coverslips were mounted on slides using Vectashield Mounting Medium containing 4’,6-diamidino-2-pheynlyindole (DAPI) for nuclear staining (Vector), and results were visualized using an Olympus BX51 Fluorescent Microscope and an Olympus FV1000 Laser Scanning Confocal Microscope.

A similar procedure was used to image GBM-derived neurospheres, but due to their free-floating nature, they were fixed, cryosectioned, and attached to slides before antibody treatment, as previously described (Jacqueline, Kasia, Laura, Laurie, & Shelley, 2006). Briefly, neurospheres were transferred to a 50 mL tube, allowed to settle for 30 minutes, and resuspended in two milliliters of fresh 4% PFA solution with 1% Triton made in PBS. After settling on ice for 30 minutes, the PFA/Triton solution was removed, and neurospheres were rinsed at least three times with fresh PBS, allowing 30 minutes of settling time between each rinse. The neurospheres were then refrigerated overnight in five milliliters of 30% sucrose dissolved in 0.1 M PBS. The next day, the portion of the liquid containing the settled neurospheres was transferred to a mold between layers of embedding medium for frozen tissue specimens (Sakura Tissue-Tek O.C.T. compound, cat. No. 4583). The mold was then transferred to a cryostat chuck containing a layer of
O.C.T. and cryosectioned into 40 µM slices onto Poly-L-Lysine coated glass microscope slides. Antibody staining then proceeded as described above.

**Polymerase Chain Reaction**

Vitamin D3 and cyclopamine are hedgehog signaling inhibitors which bind and inactivate the cell surface protein smoothened (Smo). To assess hedgehog signaling inhibition in cells treated with vitamin D or cyclopamine, changes in the expression of hedgehog target genes Gli1 and MYCN were measured using qPCR.

Before use, vitamin D3 (Sigma) was diluted to 10 µM and 1 µM aliquots in 100% ethanol (EtOH) and stored in the dark at -20 °C. Cyclopamine was dissolved in dimethyl sulfoxide (DMSO) and aliquoted into 12 mM stocks, also stored in the dark at -20 °C. Neurospheres from line 2503 were dissociated into a single cell suspension, counted using the trypan blue exclusion method, and plated in a 12-well plate at 2,143 cells per milliliter (4,286 cells per well). Wells were then separated into five treatment groups: untreated (UT), ethanol control (EtOH), vitamin D3 (Vit. D), dimethyl sulfoxide control (DMSO), and cyclopamine (CYC). RNA was isolated using the Total RNA Mini Kit (IBI) following the Total RNA Mini Kit (Tissue) Cultured Cell Protocol without the optional steps. RNA yield was quantified using the NanoDrop2000c spectrophotometer (Thermo Scientific). Reverse transcription was accomplished using the Reverse Transcription System (Promega, product A3500). Complimentary DNA (cDNA) was then diluted to 100 µL in nuclease-free water, and qPCR amplification was performed in triplicate using a StepOne Real-Time PCR System (Applied Biosystems) with the following primers: Gli1: 5’-TTCCTACCAGAGTCCCAAGT-3’ and 5’-CCCTATGTGAAGCCCTATTT-3’; MYCN: 5’-AAAAGGCCACTGAGTATGTCC-3’ and 5’-TGTCAGTTTGTGAGAAGCGTC-3’ (Integrated DNA Technologies). Primers
were diluted to a 10 µM concentration before use. Fold change was calculated using the
$2^{-\Delta\Delta CT}$ method and plotted using Microsoft Excel.

**Results and Discussion**

Immunocytochemistry revealed Gli1 expression in both differentiated GBM cells (LN 229, U 87 MG, U 138 MG, and T 98) and tumor-derived neurosphere populations (Figures 4 and 5). Due to equipment limitations, it was impossible to accurately determine localization or relative expression of Gli1 between samples, although qualitatively, neurospheres generally appeared to have a more robust Gli1 expression and a higher proportion of nuclear Gli1 than differentiated cell lines, as would be predicted due to their stem-like state (Figure 4). Because Gli1 is a downstream member of the pathway whose expression is regulated by pathway activity, the presence of Gli1 suggested that hedgehog signaling was active within the GBM cells. Further research would need to be done to determine whether other pathway components were present and intact, such as Smo or Ptc.

Because higher levels of Gli1 were found in the neurosphere populations, further characterization of hedgehog pathway expression was focused on the 2503 BTSC line. The polymerase chain reaction was used to quantify Gli1 and MYCN mRNA expression in BTSCs to better analyze hedgehog pathway activity and to assess signaling changes in the presence of pathway inhibitors cyclopamine and vitamin D3. Compared to an untreated control (not shown) all treatment groups displayed decreased Gli1 and MYCN expression. Gli1 expression decreased when BTSCs were treated with vitamin D3.
Figure 5: LN 229 (A), T 98 (B), U 87 MG (C), and U 138 MG (D) GBM cells express Gli1, indicating hedgehog pathway expression. Green staining indicates presence of an anti-Gli1 antibody conjugated to FITC. Blue represents DAPI staining for nuclear material. Images taken at 60X magnification using an Olympus FV1000 Laser Scanning Confocal Microscope.

Figure 4: Gli1 expression in LN 229 bulk GBM cells (A) and GBM-derived 2503 neurospheres (B). Green staining indicates presence of an anti-Gli1 antibody conjugated to FITC. Blue represents DAPI staining for nuclear material. Images taken at 40X magnification using an Olympus BX51 Fluorescent Microscope.
Figure 6: Vitamin D3 reduces Gli1 expression in GBM-derived 2503 neurospheres. Quantitative reverse transcriptase polymerase chain reaction was used to determine Gli1 expression in BTSC line 2503 after treatment with vitamin D3 (Vit. D) and cyclopamine (CYC) as compared to their vehicle controls (ethanol (EtOH) and dimethyl sulfoxide (DMSO), respectively). Amplification was performed using a StepOne Real-Time PCR System (Applied Biosystems).

Figure 7: Vitamin D3 and cyclopamine reduce MYCN expression in GBM-derived neurospheres. Quantitative reverse transcriptase polymerase chain reaction was used to determine Gli1 expression in BTSC line 2503 after treatment with vitamin D3 (Vit. D) and cyclopamine (CYC) as compared to their vehicle controls (ethanol (EtOH) and dimethyl sulfoxide (DMSO), respectively). Amplification was performed using a StepOne Real-Time PCR System (Applied Biosystems).
compared to its vehicle control EtOH, which supports vitamin D3’s antagonistic hedgehog effects (Figure 6). Cyclopamine treatment, on the other hand, caused an unexpected increase in Gli1 expression compared to its vehicle DMSO (Figure 6). Subsequent analysis revealed microscopic crystal formation in cyclopamine after storage at -20 °C, which could have contributed to cyclopamine’s inability to inhibit hedgehog signaling. It would be expected, however, that Gli1 expression would be equivalent to that of the DMSO control if the cyclopamine were inactivated due to crystallization. This particular part of the experiment should be repeated using crystal-free cyclopamine to confirm previous findings that cyclopamine decreases Gli1 expression due to hedgehog signaling inhibition. Interestingly, both vitamin D3 and cyclopamine decreased the expression of MYCN, another pathway target (Figure 7). It is unclear from the data whether vitamin D3 regulates MYCN expression through the hedgehog pathway or through a separate receptor-mediated mechanism. Further studies are needed to elucidate exactly how vitamin D3 regulates MYCN expression in GBM cells.

Conclusion

Hedgehog signaling has been implicated in GBM progression. Components of the hedgehog signaling pathway that are overexpressed in GBM cells may be important treatment targets, especially within the BTSC population. These data confirm the presence of the hedgehog signaling protein Gli1 in both differentiated GBM cell lines and GBM-derived neurospheres. These data warrant further investigation into the effects of vitamin D3 on GBM cell proliferation to further assess the therapeutic potential of vitamin D3 as a hedgehog pathway inhibitor.
CHAPTER 4: EFFECTS OF VITAMIN D3 ON GBM CELL PROLIFERATION

**Introduction**

Vitamin D3 is a natural compound that easily crosses the blood brain barrier, inhibits the oncogenic hedgehog signaling pathway, and has long been associated with positive outcomes for brain tumor patients. This study was designed to directly test vitamin D3’s effects on GBM cells *in vitro* through proliferation and dose response assays. Preliminary data investigating vitamin D3-mediated differentiation and apoptosis within the BTSC population are also provided. Vitamin D3 inhibited proliferation of both bulk GBM cells and BTSCs, prevented neurosphere formation from a single cell suspension of BTSCs, and induced activity of caspase 3 in BTSC populations. These results lend support to vitamin D3’s therapeutic potential and warrant further investigation into its mechanism of action within GBM cells.

**Methods and Materials**

The experiments described in this chapter were performed on standard GBM cell lines LN 229, T 98, U 87 MG, and U 138 MG in addition to GBM-derived BTSC lines 2503 and 294275. Vitamin D3’s effects on cellular proliferation, differentiation, and apoptosis were assessed using microscopy and luminescence-based quantification techniques.

*Proliferation/Dose Response*

Vitamin D3 (Sigma) was dissolved in 100% ethanol (EtOH) and diluted to 10 mM, 10 µM, and 1 µM aliquots for storage. Cyclopamine was stored in 12 mM aliquots.
after being dissolved in dimethyl sulfoxide (DMSO). Temozolomide (TMZ) was also dissolved in DMSO, and was stored in 150 mM aliquots. All three compounds were stored in light-tight containers at -20 °C. Prior to treatment, the appropriate media was used to further dilute each compound to the desired concentrations, which ranged from 100 nM to 15 µM for vitamin D3 (Magrassi et al., 1998), 1 µM to 10 µM for cyclopamine (Wang, Pan, Che, Cui, & Li, 2009), and 1 µM to 400 µM for TMZ (Beier et al., 2008). Differentiated cell lines were plated at 5,000 cells per well in 96-well plates and incubated under standard conditions (37 °C, 5% CO₂). Treatments were applied after cells were allowed to incubate for 24 hours. The length of treatment varied depending on experimental protocol. BTSC populations were dissociated into single cell suspensions, resuspended in media containing the desired treatment agents, plated into 96-well plates, and incubated for the appropriate length of time, depending on the experiment.

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was used to assess proliferation. Briefly, 100 µL of activated CellTiter-Glo reagent were added to each well, the plate was incubated at room temperature for 10 minutes, and luminescence was detected using a Modulus Microplate Reader (Turner Biosystems). CellTiter-Glo quantifies adenosine triphosphate (ATP), generating a luminescent signal proportional to the number of metabolically active cells. All treatments were performed at a minimum in triplicate and most frequently in sextuplet. Percent luminescence was determined, indicating average luminescence values from each treatment group compared to the average luminescence of the control. Significant differences between groups were determined at the 0.05, 0.01, or 0.001 level using one-way ANOVA and Tukey’s Post
Hoc analysis in PASW Statistics 18. Figures were generated in Microsoft Excel and
GraphPad Prism.

Differentiation

In order to assess vitamin D3’s effects on differentiation, BTSCs suspended in
treatment-containing media were plated at 12,500 cells per milliliter (25,000 cells per
well) onto a six-well plate with ethanol-sterilized coverslips placed at the bottom of each
well. After four days of incubation, morphological changes, particularly in sphere
formation and adherence, were observed using an Olympus CKX41 inverted fluorescent
microscope.

Apoptosis

The Caspase-Glo 3/7 Assay (Promega) was used to determine whether vitamin
D3 induced BTSC apoptosis, or programmed cell death. BTSC populations were
dissociated into single cell suspensions and resuspended in media containing no
treatment, 10 µM EtOH, 10 µM vitamin D3, 10 µM DMSO, or 10 µM cyclopamine. A
blank control (media only without cells) was also included for each timepoint. Cell
suspensions were plated at 8,500 cells per well into 96-well plates and incubated for 8
and 24 hours before addition of the Caspase-Glo reagent following manufacturer
instructions. Luminescence was detected using a Modulus Microplate Reader (Turner
Biosystems) at 30, 45, 60, and 75 minutes after reagent addition. Significant differences
between groups were determined at the 0.05, 0.01, or 0.001 level using one-way ANOVA
and Tukey’s Post Hoc analysis in PASW Statistics 18. Figures were generated in
GraphPad Prism.
Results and Discussion

Vitamin D3 inhibits proliferation of differentiated GBM cell lines with varying efficacy

Vitamin D3 had varying anti-proliferation effects on the cells, dependent on which cell line was used and the time-point of treatment. An initial proliferation assay using LN 229 cells indicated that Vitamin D3 at 100 nM concentration significantly inhibited cell growth at 24, 48, and 60 hour timepoints after treatment, with p values of 0.008, 0.004, and <0.001, respectively (Figure 8). A second LN 229 proliferation assay also appeared to show a slight vitamin D3 effect, but this effect failed to reach statistical significance (Figure 9). This second proliferation assay also considered TMZ, which significantly reduced the growth of the cells but was statistically indistinguishable from its DMSO vehicle. Because of the large DMSO effect, combined effects of TMZ and vitamin D3 could not be determined.

Multiple dose response assays were subsequently conducted to determine effective levels of vitamin D3 on LN 229 and other cell lines. Growth was analyzed 48 hours after treatment, based on the significant results of the initial proliferation assay. The first dose response assay using LN 229 cells clearly showed that vitamin D3 at 1000 µM (10 times the amount originally used) significantly inhibited GBM cell growth, without significant ethanol interference (p<0.001, Figure 10). However, this result was not duplicated in a subsequent dose response assay using LN 229 and U 87mg cell lines (Figure 11), in which neither cell line showed significant growth inhibition for 1000 µM or 1200 µM vitamin D3 concentrations.
Figure 8: Vitamin D3 slightly reduces LN 229 proliferation over time. Cells were plated at 5,000 cells per well, treated 24 hours after plating, and viable cells were detected using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

Figure 9: Effects of vitamin D3 and Temozolomide (TMZ) on LN 229 proliferation over time. Cells were plated at 5,000 cells per well, treated 24 hours after plating, and viable cells were detected using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega).
Figure 10: 1000 nM Vitamin D3 significantly inhibits LN 229 growth. Cells were plated at 5,000 cells per well, treated 24 hours after plating, and viable cells were detected after 48 hours of incubation using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Reported values indicate the mean of six replicates converted to percent of control (untreated) group. Significant values indicated at p ≤ 0.001 (**).
Further dose response assays were conducted using increasing concentrations of vitamin D3 to determine a consistently effective level. Of note, LN 229 cells showed a significant increase in growth when treated with 5 µM vitamin D3 (p ≤ 0.001, Figure 12A). Only the highest concentration of 15 µM vitamin D3 significantly inhibited LN 229 growth (p ≤ 0.001), but this effect could not be statistically separated from the effects of the ethanol control (p = 0.024, Figure 12). T 98 cells, on the other hand, significantly responded to 10 and 15 µM vitamin D3 concentrations without significant ethanol effects (p = 0.001 and 0.036, respectively, Figure 12). In the U87 cell line, significant ethanol effects were observed at 10 µM concentration, but no significant vitamin D3 effects were observed. The U 138 MG cell line did not appear to respond at all to treatment. (Note: U 87 MG cells and U138 MG cells were not treated at 15 µM due to a shortage of available vitamin D3).

Because T 98 cells responded best to vitamin D3 treatment (perhaps due to increased hedgehog expression, Figure 5), they were chosen to perform the final group of dose responses. This time, cyclopamine and TMZ were also considered. Importantly, new stocks of all three treatment agents were prepared, and cyclopamine and vitamin D3 dilutions were prepared in the dark. All treatments were applied at one, five, and 10 µM concentrations. Results showed that cyclopamine significantly inhibited T 98 growth when applied at one and 10 µM concentrations (p= 0.026 and <0.001, respectively; Figures 13A and 13C). Vitamin D also significantly inhibited cell growth at one and 10 µM concentrations, but these effects could not statistically be distinguished from ethanol effects (Figures 13A and 13C).
Figure 12: Vitamin D3 has varying effects on LN 229, T 98, U 87 MG, and U 138 MG proliferation. Cells were plated at 5,000 cells per well, treated 24 hours after plating, and viable cells were detected after 48 hours of incubation using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Values reported indicate the mean of six replicates converted to percent of control (untreated) group. Significant values indicated at p ≤ 0.05 (*), p ≤ 0.01 (**), and p ≤ 0.001 (***).
Figure 13: T98 cells respond to cyclopamine at 1 and 10 µM, but not 5 µM concentrations. Cells were plated at 5,000 cells per well, treated 24 hours after plating, and viable cells were detected after 48 hours of incubation using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Values reported indicate the mean of six replicates converted to percent of control (untreated) group. Significant values indicated at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***).
Interestingly, no significant results were observed when treatments were applied at five micromolar concentrations in T 98 cells (Figure 13B). Temozolomide at the chosen concentrations had no appreciable effect on proliferation of T 98 cells (Figure 13).

Overall, proliferation assays indicated that GBM cultures were inhibited by vitamin D3 to some extent, but results were inconsistent. Early experiments reached significance at 100 nM concentrations (Figure 8), while later dose response assays required much higher levels of up to 15 µM to affect proliferation within the same cell line (Figures 10 and 12). One explanation for this phenomenon might be that 1-α-dihydroxyvitamin D, the purified, active form of vitamin D3, is extremely sensitive to degradation. Both ultraviolet light and oxygen can affect the compound’s stability, and the effects can occur very rapidly (Lebwohl, Quijije, Gilliard, Rollin, & Watts, 2003).

Because of suspected vitamin D3 degradation, fresh stocks of all chemicals were used for the experiments summarized in Figure 13, which were expected to again yield growth inhibition at low concentrations. Indeed, with the exception of the five micromolar treatment group, vitamin D3 significantly inhibited the cells’ growth. However, a large, unexpected ethanol effect was observed. This effect was surprising, considering that less ethanol was used to dissolve this aliquot of vitamin D3 than the previous batch, and that the final ethanol concentrations were only 0.01%, 0.05%, and 0.10% of the final volume for the one, five, and ten micromolar treatment stocks, respectively.

An unexpected result was that the group of T 98 cells treated with five micromolar vitamin D3 showed no decrease in growth compared to the control (Figure 13B). It is possible that the plate had been contaminated, although this seems unlikely.
since the luminescence values were similar to the other plates in the series. A contaminated plate would be expected to have higher luminescence values due to the presence of a large number of bacterial cells. An alternate explanation might have been that the vitamin D3 was not dissolved completely, which would affect the intended treatment concentrations. The process of vitamin D3 dilution was precarious at best—a 10 µL Hamilton syringe was used to inject only 23 µL of ethanol into the original vitamin D3 packaging, and this volume was not fully recovered during transfer to secondary containers. Furthermore, the dilution process was completed in the dark to prevent vitamin D3 degradation. Even in bright conditions, the vitamin D3 used to develop treatment stocks is sold in 0.01 mg aliquots, an amount too small to be seen. Needless to say, it was difficult to determine whether the substance was completely dissolved. However, it is strange that five micromolar vitamin D3 failed to inhibit growth and even significantly increased growth in earlier assays as well (Figure 12A). Perhaps vitamin D3 may have a dose-dependent effect that stimulates proliferation at some concentrations. This phenomenon should be further investigated in future studies.

Vitamin D3 dramatically inhibits proliferation of GBM-derived neurospheres

Although vitamin D3’s effects on differentiated GBM cell lines were inconsistent, vitamin D3 had a much more profound inhibitory effect on BTSC proliferation, which was expected due to BTSCs’ reliance on the hedgehog signaling pathway. Neurospheres that had recently been differentiated by adding fetal bovine serum (FBS) in order to make them adherent (similar to standard cell lines) showed a decrease in growth when treated with vitamin D3 (Figure 14). Neurospheres from the same line that were left as non-adherent cultures displayed an even more robust response to vitamin D3 treatment, with
luminescence decreasing from 100% to approximately 45% after treatment with 10 µM vitamin D3 (Figure 15). An extended repetition of the study showed that five micromolar vitamin D3 decreased luminescence to approximately 38% of the untreated control, while 10 µM vitamin D3 treatment decreased luminescence to approximately 6% of the untreated control (Figure 16). Moreover, vitamin D3 was much more efficient than cyclopamine at inhibiting neurosphere growth. Five and ten micromolar concentrations of cyclopamine only decreased luminescence to approximately 86% and 83% of the untreated control, respectively. Cyclopamine was slightly more effective at inhibiting the growth of neurosphere line 259274, decreasing luminescence to 59% and 41% of vehicle control at five and ten micromolar concentrations, respectively (Figure 17). Vitamin D3, however, was still more effective than cyclopamine in the 259274 line, reducing luminescence to 38% and 2% at five and ten micromolar concentrations, respectively. These results suggest that vitamin D3 inhibits neurosphere growth in a dose-dependent manner, and that it does so more efficiently than cyclopamine.

**Differentiation should be considered as a vitamin D3-related effect**

Because vitamin D3 reduced neurosphere growth more effectively than cyclopamine, alternate vitamin D3 effects in addition to hedgehog signaling inhibition were investigated. One hypothesis was that vitamin D3 may have been causing differentiation of the BTSCs, which would inhibit their ability to grow and survive in the selective neurosphere media. A simple, morphological assay was used to more closely examine vitamin D3s effects on cellular differentiation. Results indicated that,
**Figure 14:** Vitamin D3 drastically decreases newly differentiated BTSC proliferation. BTSCs were exposed to FBS to cause differentiation for approximately two weeks before undergoing treatment. Adherent cells were then plated at 5,000 cells per well and treated after 24 hours. Luminescence was detected after 48 hours of treatment. Significant values indicated at p ≤ 0.05 (*), p ≤ 0.01 (**), and p ≤ 0.001 (***)..

**Figure 15:** Vitamin D3 drastically decreases undifferentiated BTSC proliferation. Neurospheres were dissociated into single cell suspensions and resuspended at 50,000 cells per milliliter in media containing respective treatments. Luminescence was detected after 48 hours of treatment. Significant values indicated at p ≤ 0.05 (***).
**Figure 16:** Vitamin D3 decreases BTSC proliferation more effectively than cyclopamine in the 2503 line. Neurospheres were dissociated into single cell suspensions and resuspended at 50,000 cells per milliliter in media containing respective treatments. Luminescence was detected after 48 hours of treatment. A star above the bar denotes a significant difference compared to the untreated control (UT). A star above a line denotes significance of the two indicated samples in comparison to one another. Significant values indicated at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***)..

**Figure 17:** Vitamin D3 decreases BTSC proliferation more effectively than cyclopamine in the 259274 line. Neurospheres were dissociated into single cell suspensions and resuspended at 50,000 cells per milliliter in media containing respective treatments. Luminescence was detected after 48 hours of treatment. Significant values indicated at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***).
although vitamin D3 did not cause neurosphere cells to adhere to the bottom of the flask like FBS, it did prevent single cell suspensions from generating new spheres (Figure 18).

Too few neurospheres were generated to be able to prepare them for immunofluorescent staining for differentiation markers, but future studies should find a way to address this technical difficulty in order to gain a better understanding of whether vitamin D3 induces BTSC differentiation or affects another cellular function, such as adhesion.

**Figure 18: Vitamin D3 prevents 2503 BTSCs from forming new neurospheres.** Single cell BTSC suspensions were treated, plated, and imaged after a four day incubation period. Vitamin D3 prevented neurosphere formation (lower right), while the other treatments had no observable effect.
Vitamin D3 induces BTSC apoptosis through a caspase-dependent mechanism

Vitamin D3 has been reported in the literature to induce apoptosis at high concentrations (Baudet et al., 1996). The luminescence-based Caspase-Glo assay (Promega) was used to determine whether vitamin D3-related growth inhibition occurs through an apoptotic mechanism. After eight hours of incubation, vitamin D3-treated 2503 BTSCs showed significantly increased enzymatic activity of caspases three and seven compared to the other treatment groups (Figure 19). Cyclopamine, DMSO, untreated cells, and ethanol-treated cells all had the same level of background luminescence (Figure 19). There was no significant difference in caspase activity at 24 hours (data not shown).

**Figure 19:** Vitamin D3 induces caspases three and seven to initiate apoptosis of BTSCs. 2503 BTSCs were plated at 85,000 cells per well and incubated for eight hours before the addition of the Caspase-Glo® reagent (Promega). Following 30 minute incubation, luminescence was detected using a Modulus Microplate Reader (Turner Biosystems). Significance indicated at $p \leq 0.001$ (***)
Conclusion

Clinical trials have indicated vitamin D3’s benefit to GBM patients, although its mechanism of action has not been fully elucidated (Trouillas, et al., 2001). The experiments detailed in this chapter demonstrated that vitamin D3 acts to inhibit GBM cell proliferation—particularly within BTSC populations, prevent neurosphere formation, and induce apoptosis through a caspase-dependent mechanism.

In standard GBM LN 229, T 98, U 87 MG, and U 138 MG cell lines, vitamin D3 significantly inhibited proliferation, although the effective dose varied from experiment to experiment. The inconsistencies in vitamin D3’s effectiveness may have been caused by the rapid degradation of active vitamin D3 within the *in vitro* model system chosen for the study. In addition, the technical difficulty of diluting and storing active vitamin D3 may have contributed to its variability.

Vitamin D3’s inhibitory effects on BTSC (neurosphere) populations were much more dramatic and consistent. Vitamin D3 inhibited neurosphere proliferation in a dose-dependent manner, and addition of 10 µM vitamin D3 nearly halted proliferation in two separate neurosphere lines. Upon morphological examination, vitamin D3 clearly prevented neurosphere formation from a single cell suspension, although the mechanism by which this occurred remains unclear. Future studies should consider whether differentiation or perhaps even changes in cell-cell adhesion are caused by vitamin D3.

Interestingly, vitamin D3 was more effective than the hedgehog antagonist cyclopamine at inhibiting BTSC proliferation (Figures 16 and 17). Previous studies have shown that vitamin D3 more efficiently inhibits hedgehog signaling than cyclopamine (Bijlsma, et al., 2006), which could partially explain its dramatic effects in BTSCs, which
more strongly express the hedgehog signaling pathway than differentiated cells. However, it is likely that vitamin D3’s other actions within the cell, such as induction of apoptosis, contribute to its antiproliferative effects. Indeed, the data indicate that vitamin D3 causes induction of caspase three and seven activity at a 10 µM concentration. Further studies should consider other concentrations and cell types to more fully describe vitamin D3’s effects.

*In vitro*, vitamin D3 inhibits GBM cell proliferation by antagonizing hedgehog signaling, inducing apoptosis, preventing neurosphere formation, and potentially by affecting other cellular mechanisms. These effects may contribute to vitamin D3’s benefits in GBM patients, but more research needs to be done to further understand this complex system. Overall, the findings of this study indicate the need for a closer look into vitamin D3’s actions within GBM cells and identify vitamin D3 as a potential adjuvant therapy for GBM treatment.
Glioblastoma multiforme (GBM) is a devastating type of brain tumor with limited treatment options. Vitamin D3 is currently in clinical trials for GBM treatment adjuvant to standard therapies, though its cellular effects are poorly understood. This thesis lends support to the use of vitamin D3 as a therapeutic agent through \textit{in vitro} studies of GBM cells. Previous studies showing vitamin D3’s ability to inhibit the growth-inducing hedgehog signaling pathway were confirmed through the observation of decreased Gli1 and MYCN expression after vitamin D3 treatment. In addition, Vitamin D3 decreased proliferation of both standard GBM cell lines and tumor-derived neurospheres composed of isolated BTSC populations. Interestingly, vitamin D3 inhibited BTSC proliferation much more effectively than the standard hedgehog antagonist cyclopamine. Further characterization studies provided evidence for vitamin D3’s ability to prevent neurosphere formation from a single-cell suspension and induce apoptosis in BTSCs through the activity of caspases three and seven. Future studies will further elucidate vitamin D3’s mechanisms of action within GBM cells and determine whether these actions are conserved \textit{in vivo}.

Several limitations existed within the described experiments. For one, the software available to analyze the immunocytochemistry results did not allow for quantitative comparison. It appeared as though BTSCs exhibited a higher level of Gli1 compared to the standard cell lines, but further studies with more sophisticated equipment would need to be done to accurately compare fluorescent signal between samples. It would be interesting to use either use quantitative immunocytochemistry or quantitative
polymerase chain reaction (qPCR) to numerically gauge differences in Gli1 expression between bulk GBM cells and BTSCs, as well as between GBM cell lines. Such analyses would be useful in determining whether vitamin D3’s effectiveness correlate with hedgehog pathway expression. Furthermore, Gli1 expression could be investigated as a potential biomarker for tumor aggression or tested for its ability to predict vitamin D3 responsiveness.

The most prominent limitation of the study was vitamin D3’s instability due to its rapid degradation after exposure to oxygen and ultraviolet light (Lebwohl, et al., 2003). Future studies might consider employing an in vivo model, in which a more stable, less expensive, inactive precursor of 1-α-dihydroxyvitamin D could be substituted. 25-hydroxyvitamin D3, for instance, is converted into 1α,25-dihydroxyvitamin D3 in the mammalian liver and may be a plausible alternative to purified 1α,25-dihydroxyvitamin D3. Active vitamin D3 may also be more stable if delivered intravenously. An in vivo system would also allow for the delivery of vitamin D3 in an oil vehicle, which increases its bioavailability compared to ethanol or powder. (Grossmann & Tangpricha, 2010). These methods might circumvent some of the problems with rapid degradation that lead to dosing inconsistencies observed in this study.

An in vivo study would also provide a more complete picture of vitamin D3’s effects on tumor biology and overall well-being. Bar and colleagues point out that GBM tumors are multiforme, meaning that cells in different parts of the tumor may exhibit varying levels of gene expression, including hedgehog expression (2007). In vivo studies using an animal model would better represent human GBM tumors by exhibiting cellular heterogeneity, which is limited in cell culture. Additionally, an in vivo model would
allow for a better understanding of systemic effects of vitamin D3 and help find ways to manage its limited side-effects.

Future studies might also include a broader and more complete range of doses to pinpoint the concentration at which vitamin D3 effectively and consistently inhibits GBM growth. It should be noted that the doses used in this study were much higher than those that would be used in vivo, and therefore should not be considered physiologically relevant. Others have found that certain concentrations of vitamin D3 may actually increase proliferation in certain standard GBM cell lines (Diesel, 2005), which this study corroborates to an extent. Once proper dosing information has been determined to effectively inhibit cell growth, vitamin D3’s interactions with Temozolomide (TMZ), the current standard of care for GBM patients, should also be investigated to determine whether synergistic or inhibitory effects may occur.

Interestingly, in direct contrast to the presented experiments, previous studies have shown that T98 cells were resistant to vitamin D treatment and that other GBM cell lines did not respond to vitamin D3 at 10 µM concentrations (Reichrath et al., 2010; Zou et al., 2000). What might be controlling vitamin D3 responsiveness in GBM cells? Can cells from the same lineage undergo changes in gene expression that affect vitamin D3 responsiveness? One suggestion from the literature is that a 220-kd protein may act as a switch to control responsiveness to vitamin D (Zou, et al., 2000). However, the identity or activity of this protein has not been described. Another suggestion is that the effects of vitamin D3 treatment depend on a tumor’s P53 status (Stambolsky et al., 2010). The question remains as to what other genes may predict vitamin D3 responsiveness and mediate vitamin D3’s actions within the cell.
Despite this study’s promising findings, many questions still remain. A recent study using prostate cancer cells showed that 182 genes were upregulated and 80 were downregulated in response to vitamin D3 treatment (Kovalenko, Zhang, Cui, Clinton, & Fleet, 2010). Some of the effects of these genes led to suppression of Wnt, Notch, NF-κB, and IGF1 in addition to the suppression inflammation and angiogenesis (Kovalenko, et al., 2010). Are these same genes regulated by vitamin D3 in GBM cells, and if so, are they regulated in the same way? An interesting aspect of this question will be to find out whether GBM cells in culture or in patients express the vitamin D receptor (VDR), and whether this has an effect on vitamin D3 responsiveness as suggested by other studies (Davoust et al., 1998). If vitamin D3 is indeed able to act by inhibiting the Smo protein in the hedgehog signaling pathway, will vitamin D3 still be able to decrease GBM proliferation in the absence of the VDR? In the absence of the VDR, would vitamin D3’s effects be the same as cyclopamine’s effects, or are other VDR-independent mechanisms intact within the cells? These are all areas for further exploration.

Additionally, it is yet unclear whether vitamin D3 induces differentiation of GBM BTSCs in the same way that it has been shown to induce differentiation and inhibit the clonal (stem cell) population in breast cancer (Elstner et al., 1995). Eyles reviews that in the normal neural development, vitamin D reduces proliferation and induces differentiation and apoptosis (Eyles, et al., 2011). Do these cellular effects occur in normal neural stem cells within the subventricular zone, and if so, will they affect the ability for patients to regenerate parts of the brain damaged by tumor resection? These and other questions become important when considering vitamin D3 as a GBM treatment option.
Vitamin D3 regulates over 200 different genes, and its deficiency is associated with heart disease as well as 17 different types of cancer (Anaizi, 2010; Current Science, 2010). This and other studies show that much more work needs to be done to fully understand how vitamin D3 interacts with and affects tumor growth through the hedgehog signaling pathway as well as through other mechanisms. Basic science will need to catch up with ongoing clinical trials to elucidate the way in which vitamin D3 interacts with bulk GBM cells and BTSCs to provide patient benefits, and how best to capitalize on this phenomenon to develop better treatments.
REFERENCES


Cyclopamine. (2002). Retrieved February 14, 2012, from Hazardous Substances Data Bank:


