Photochemical delivery of bleomycin in malignant glioma cells

Joseph William Blickenstaff III

University of Nevada, Las Vegas

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PHOTOCHEMICAL DELIVERY OF BLEOMYCIN
IN MALIGNANT GLIOMA CELLS

by

Joseph William Blickenstaff III
Bachelor of Science in Physics
Carnegie Mellon University
Pittsburgh, PA
2003

A thesis submitted in partial fulfillment of the requirements for the

Master of Science Degree in Health Physics
Department of Health Physics
Division of Health Sciences

Graduate College
University of Nevada, Las Vegas
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The Thesis prepared by

JOSEPH BLICKENSTAFF

Entitled

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Examination Committee Chair

Dean of the Graduate College

Examination Committee Member

Examination Committee Member

Graduate College Faculty Representative
ABSTRACT

Photochemical Delivery of Bleomycin
in Malignant Glioma Cells

by

Joseph William Blickenstaff III

Dr. Steen Madsen, Examination Committee Chair
Professor of Health Physics
University of Nevada, Las Vegas

Despite continued efforts, glioblastoma multiforme (GBM) remains an incurable form of primary brain cancer. Surgical resection followed by radiation treatment and chemotherapy have been the most effective modalities in prolonging median survival time to 14 months. This research aims to study the response of two different cell culture models to a novel drug delivery method termed photochemical internalization (PCI). PCI is a technique to improve the utilization of macromolecules (e.g. the chemotherapeutic drug bleomycin) in site-specific cancer therapy. This concept is based on the use of specially designed photosensitizers (e.g. aluminum phthalocyanine disulfonate; AlPcS2a) which preferentially localize in the membranes of endocytic vesicles.

The utility of PCI for treating malignant gliomas was investigated in vitro using: (1) F98 rat glioma monolayer cells, and (2) biopsy-derived human glioma spheroids. For both in vitro systems, PCI was found to interact in a synergistic manner resulting in significant toxicity. For example, the combination of 1.5 J/cm² photodynamic therapy (PDT) and 0.25 μg/mL bleomycin resulted in approximately 25% survival in F98 rat glioma cells while only 35% of human glioma spheroids were observed to be growing
two weeks post treatment. Overall, the degree of synergism was found to be less pronounced in the spheroid model. Collectively, the results show that AlPcS2a-mediated PCI can be used to enhance the efficacy of chemotherapeutic agents such as bleomycin in malignant gliomas.
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1.1 Malignant glioma

While primary malignant brain tumors account for only 2% of all cancer cases in the United States, their prognoses are among the worst as they also account for 2% of all cancer related deaths each year (Chandana et al. 2008; Newton 2008). Brain tumors are classified, according to the World Health Organization (WHO), by cellular origin and histologic appearance. For all primary brain tumors, the Central Brain Tumor Registry of the United States (CBTRUS) compiles incidence statistics by histology (Fig. 1).

Figure 1. Primary brain and CNS tumor distribution by histology; collected 2000-2004 (n=73,583). (Adapted from CBTRUS 2008)
All primary brain tumors present with similar symptoms, regardless of histology. Some of these symptoms include (in decreasing occurrence rate): headache, memory loss, seizures, personality change, visual problems, nausea or vomiting (Chananda et al. 2008). The locations of headaches and severity of other symptoms can aid in diagnosis. A cranial magnetic resonance imaging (MRI) scan with gadolinium contrast is typically all that is necessary to diagnose a primary brain tumor. However, computed tomography (CT) can also be used to better localize and measure the tumor’s dimensions. CBTRUS reports an age-adjusted incidence for primary brain tumors to be 6.45 in 100,000 people per year (CBTRUS 2008). This figure has increased in recent years, yet numerous studies have failed to connect most environmental factors to an increased risk of brain tumor development. Factors such as electromagnetic fields, pesticides, cellular phones, head trauma, and N-nitroso compounds have all been studied with inconclusive results. Increased exposure to ionizing radiation is the only proven factor to increase risk. Even low doses have been shown to increase incidence of meningiomas by a factor of 10 and glial tumors by a factor of 3-7. The latency period for such occurrences is 10-20 years after being exposed (DeAngelis 2001). The median survival depends significantly on the histology and malignancy of the tumor, but there are factors that show a favorable prognosis for patients. These factors include: patients younger than 60 years, patients presenting with seizures, tumor located in the frontal lobe, absence of tumor necrosis, and total or near-total bulk resection (Chandana et al. 2008). Primary brain tumors rarely metastasize outside the central nervous system (CNS) leading to no real standard of staging.
Gliomas comprise the vast majority of malignant brain tumors, and still remain incurable. Gliomas account for 36% all primary brain tumors and 81% of malignant tumors (CBTRUS 2008). Symptoms are similar to those of other primary brain tumors with headaches occurring in 50% of patients. In malignant gliomas, seizures are less prevalent occurring in only 15-25%, while mental-state abnormalities in general occur in 40-60% of all patients. Gliomas can be pathologically graded according to the presence or absence of nuclear atypia, mitosis, microvascular proliferation and necrosis (DeAngelis 2001). Grades I and II tumors are both low-grade (i.e. and grades III and IV are high-grade. Of all malignant gliomas, glioblastoma multiforme (GBM) is the most malignant and common comprising (along with other astrocytomas) 75% of all gliomas (Fig. 2, CBTRUS 2008).

Figure 2. Primary brain and CNS glioma distribution by histology subtypes; collected 2000-2004 (n=26,630). (Adapted from CBTRUS 2008)
GBM is the most common brain tumor in adults and accounts for approximately 40% of primary tumors and 80% of high-grade primary CNS neoplasms and is classified as a grade IV astrocytoma (Newton 2008). GBM comprises 18.5% of all reported brain tumors and affects 3 in every 100,000 people per year with a median age at diagnosis of 64 years (CBRTUS 2008). GBM growth is characterized as having a high cellular motility and a resistance to apoptosis. As with other gliomas, GBMs are heterogeneous both histologically and genetically. These factors result in extreme difficulties when treating GBMs. Gross GBM tumor pathology is characterized by a central core of necrosis surrounded by pseudopalisading cells along with angiogenesis. Typically these cells will infiltrate several centimeters or more away from the bulk tumor into the surrounding brain (DeAngelis 2001). This infiltration, along with the production of glutamate which acts to kill surrounding brain cells, facilitates rapid tumor expansion. Furthermore, glioma cells have demonstrated the ability to quickly change shape and volume as they move further into surrounding parenchyma (Lefranc 2009). All of this leads to GBM being an extremely aggressive and difficult cancer to treat. The median survival rate remains only 14.6 months when utilizing the current treatment standard of maximum tumor bulk resection followed by radiotherapy and chemotherapy with temozolomide (TMZ) (Newton 2008; Lefranc 2009).

While there have been recent advancements in the treatment of GBM, relative survival rates have increased very little. The one, three, five, and ten-year relative survival rates for GBM are 29.6, 5.2, 3.4, and 2.4 respectively (CBTRUS 2008). These values have improved only slightly compared with data collected over a decade prior. The current standard treatment once a patient is diagnosed with GBM begins with a
surgical resection of the tumor. This resection, or de-bulking, is performed by aggressively removing as much tissue as possible without adversely affecting the patient's quality of life. Resection not only removes tumor tissue, but will reduce intracranial pressure that leads to many of the common symptoms.

Following surgery, the patient is typically administered radiotherapy and chemotherapy in combination. Radiotherapy is generally given in the form of external beam, administered in 2 Gy fractions daily for 30 fractions. A total dose of 60 Gy is often followed by a subsequent boost dose. Several chemotherapy options exist, with the most recent standard being TMZ. This is typically given in 75 mg/m$^2$ per day for the 6 weeks during radiotherapy treatment. This is followed by at least 6 cycles of 150-200 mg/m$^2$ per day for 5 days given every 28 days (Newton 2008). This protocol was shown to improve the median survival rate of patients from 12.1 to 14.6 months when compared to radiotherapy alone (Chandana et al. 2008; Newton 2008). An increase in two-year survival rate from 10.4% to 26.5% was also seen when compared to traditional radiotherapy alone. Even more impressive increases in the three and four-year survival rates were also observed. The three-year survival rate increased from 4.3% to 16.7% and the four-year went from 3.8% to 12.9% (Newton 2008). The survival rates given here by Newton (2008) are slightly different than those found in the CBTRUS 2008 Report as their pool of data was collected over different time scales.

Beyond the standard treatment protocol, several alternative medicines are being studied. Some of these alternative medicine treatments include: methyl guanine and methyl transferase (MGMT) promoter methylation, epidermal growth factor receptor (EGFR) inhibitors, antiangiogenics, as well as other growth factor and signal transduction
targeted drugs. MGMT has shown some promise, but it, along with the other drugs, is still in clinical trials (Chandana 2008; Fadul et al. 2008; Newton 2008).

The most likely reason for chemotherapy’s poor results lies in poor drug transmission across the blood-brain barrier. The difficult delivery of some drugs to the CNS as well as the genetic differences from one tumor to another has led some to propose genotyping each tumor and custom fitting treatments, in order to fit each individual patient’s needs. This type of individual treatment will unfortunately take many years to develop. It can be agreed that while recent advancements have made incremental steps forward, there is a dire need to develop new and more effective therapies for GBM (DeAngelis 2001; Fadul et al. 2008).

1.2 F98 monolayer

F98 is an undifferentiated rat glioma cell line that shares many characteristics with human GBMs, and is used in this study to examine all therapeutic effects on monolayer configurations. The F98 cell line is morphologically different from normal glial cells, which typically form a monolayered configuration similar to that of epithelial cells. Glial cells have a polygonal cell body with profuse cytoplasm and a flat nucleus (Fig. 3). The nucleus often contains multiple nucleoli, but the amount of cytoplasm present in the cell grants it a low nuclear-cytoplasmic ratio. Glial cells typically have long processes that are thin and interdigitated with other glial cells at confluence. Normal glial cells have a low mitotic index. Ko et al. (1980) measured average glial cell body diameters to be $39.8 \pm 5.0 \, \mu m$. Also measured by Ko et al. were the average number of processes ($2.6 \pm 0.2$) and process length ($114.1 \pm 11.2 \, \mu m$). Glioma cells are distinctive in the criss-crossed
alignment of their processes at confluence, high nuclear-cytoplasmic ratio, and higher mitotic index (Fig. 4). F98 cells originate from undifferentiated gliomas and possess a high degree of morphological homogeneity. The F98 cell line was characterized by Ko et al. to have a cell body diameter of \(29.5 \pm 2.0\) \(\mu m\), as well as an average number of \(4.6 \pm 0.3\) processes with a length of \(28.5 \pm 3.1\) \(\mu m\). The F98 cell line demonstrated a high morphological stability and retained its distinctive characteristics throughout multiple \textit{in vitro} propagations. It was concluded that this stability, growth, and morphology would make F98 an excellent model system for studying therapeutic effects (Ko et al. 1980).

**Figure 3.** Monolayer of normal rat glial cells. Arrows indicate mitotic figure in early telophase (Ko et al. 1980).
F98 glioma cells are specifically applicable for this study, because they share many characteristics of *in vivo* growth with human GBM cells (Madsen et al. 2007). When used to induce tumors in Fisher rats, F98 tumors exhibit rapid growth, infiltrative behavior, extensive neovascularization, and absence of encapsulation (Barth RF 1998). F98 cell cultures will be used to study several therapeutic effects in a monolayer cell model.

1.3 Multicellular tumor spheroids

While monolayer cell cultures such as F98 have proven to be well established for use in biomedical experiments, there are limitations in utilizing these two-dimensional *in vitro* models. Monolayer cell cultures allow for a well-controlled cellular environment that is easy to analyze in comparison to *in vivo* studies. Experiments performed *in vivo* better replicate the cellular environment, but are much more complex, costly, and unpredictable. Thus arose the need for a middle ground between control and complexity.
Multicellular tumor spheroids (MTS) are not new to the field of biomedical research. They have been in use since the 1940s and 1950s when Holtfreder and Moscona separately worked on using these spheroidal aggregates of cells to study embryonic and malignant cells (Mueller-Klieser 2000). It was, however, Robert M. Sutherland and others that pioneered the use of MTS in cancer research beginning in the 1970s. The bulk of Sutherland's research revolved around the response of MTS to therapy. Most of the initial work focused on ionizing radiation therapies. Since then, MTS have been widely used to investigate various biological mechanisms and specific cellular responses to a broad range of therapeutic techniques. Their well organized morphology incorporates cell-cell interactions including gene and growth factor expressions, as well as oxygen and nutrient gradients (Kim 2005). Not all cell lines are able to form MTS, but several techniques have made it possible for many cell types to bridge that experimental gap between two-dimensional cell cultures in vitro and tumors in vivo.

MTS are meant to simulate the characteristics of avascular tumor nodules and microregions in vivo. They take advantage of homotypic aggregation, a common characteristic of malignant cells. Their natural tendency towards cell-cell adhesion allows for this self-propelled growth in vitro. As the spheroids grow in diameter, they begin to develop a complex cellular environment, directly applicable to what is found in human tumors. Three classes of cells are commonly found in human tumors. The first class being that of proliferating cells found on the tumor's perimeter and always near ready sources of nutrients and oxygen. These proliferating cells are characterized by rapid growth and a high mitotic index. The second class of cells is in a quiescent state and these cells are located further away from capillaries and other sources of nutrients
that limit growth and division. These cells are still provided sufficient nutrients to survive, but have stopped cycling. Further separated from any nutrient and or oxygen sources is the third class, the necrotic cells (Venkatasubramanian et al. 2006).

MTS are ideally suited to mimic this type of cellular organization within tumors. MTS are characterized by a viable ring (100-220 μm; Sutherland 1988) of proliferating cells followed by quiescent cells. The proliferating cells compose the outermost 3-5 cell layers (75 μm; Sutherland 1988). Once an MTS is of sufficient size (100-600 μm in diameter; Kunz-Schughart 1999) a necrotic core will begin to develop. The size at which an MTS will develop this necrotic core varies greatly between cell types. These variations are the result of differences in substrate consumption rates, cell packing densities, and concentrations of substrate within the medium (Sutherland 1988).

MTS growth is very similar to that of solid tumors. Tumors begin at the microscopic level and undergo avascular growth followed by angiogenesis (Guiot et al. 2003). This ensures the tumor’s continued expansion and infiltration into the local tissue. The tumor can progress to metastasize depending on the type of cancer. In much the same fashion, MTS growth can be described to occur in three phases. First, the spheroid will undergo an initial exponential growth period (typically up to 200 μm in diameter). In this phase, several cells have loosely aggregated and all are highly proliferative. Slowly, intercellular links, such as gap junctions, form and a more compact spheroid begins to appear (Fig. 5). At this point the second phase of MTS growth begins. This phase is marked by an increasing accumulation of a second cell layer in which cells are no longer proliferating. These quiescent cells act to retard the growth process. The MTS is now
increasing linearly and a necrotic core has begun to emerge. This linear growth will continue until the MTS asymptotically approaches a maximum size.

Figure 5. 1400x magnification of a human colon adenocarcinoma spheroid depicting the compact shape while still showing individual cells (Santini et al. 1999).

Proliferative cells still remain on the surface, but only act to replace cells lost through necrosis and shedding (Folkman and Hochberg 1973; Mueller-Klieser 1987; Dubessy et al. 2000). No matter how preferable the environmental conditions are, the MTS will act to self-regulate its size without the addition of angiogenesis that allows human tumors to continue to grow (Folkman and Hochberg 1973). The growth curve is illustrated in Figure 6. Mathematical analysis by Chignola et al. (2000) was able to forecast the growth of both rat and human glioblastoma MTS using a modified Gompertz growth equation. Their work showed that MTS growth exhibits both variability and saturation.
Variability refers to MTSs of identical cell lines whose kinetics at any time during growth will demonstrate different volumes. The same spheroidal volumes will eventually both saturate in growth and equal each other (Chignola et al. 2000). This demonstrates that given different initial conditions, MTS of identical cell lines should reach similar maximum volumes.

One could conclude that while many experiments, both in vitro and in vivo, end before maximum volume is attained, this finding would allow the prediction of when that maximum volume would have been achieved. This would facilitate the direct comparison of growth delays caused by therapeutic effects. MTS maximum growth is directly regulated by cell death at the spheroid center. Cell death is believed to be

---

**Figure 6.** Illustration of solid tumor and MTS growth curve (Mueller-Klieser 1987).
primarily caused by necrosis inside the MTS. The development of this necrotic core is poorly understood and most likely depends on a complex set of factors that include hypoxia (Mueller-Klieser 2000). It has been shown that maximum volume is positively correlated with the thickness of the MTSs viable rim, while not being correlated to: species, cell type, monolayer or spheroid growth rates, clonogenic capacity, cell packing density (Freyer 1988). Those MTS studied exhibited 50-70% of their maximum volume to be central necrosis. The conclusion was that certain products generated during the necrotic process acted as cell proliferation inhibitors. It is interesting to note that the doubling time of some spheroids has been shown to correlate with the growth rates of those tumors from which the MTS are generated and this doubling time is longer than that of a monolayer culture from that same tumor (Dubessy et al. 2000). This suggests that MTS more closely mimic the heterogeneous environment in human tumors when compared to monolayer experiments.

MTS have several advantages and limitations for use in studying the efficacy of therapeutic techniques when compared to monolayer cell cultures. These differences manifest primarily through the cell-cell and cell-matrix interactions that an MTS model provides. In fact MTS and monolayer should be regarded as two distinct models. The different phenotypes of cells within an MTS are arranged in an orderly fashion making the correlation between structure and function possible. Cellular organization is possible through multiple intercellular membrane contacts such as microprojections, tight junctions, and desmosomes. Intercellular communication through gap junctions and the production of extracellular matrix are made possible by these contacts (Dubessy et al. 2000). The very structure of the MTS changes the cells’ shapes and cellular environment
which affects gene expression and therefore biological behavior (Mueller-Klieser 2000). This leads to the primary difference between a two-dimensional and three-dimensional cell culture. In MTS, an extensive extracellular matrix (ECM) comprised of proteins and polysaccharides is formed. In addition to many proteins and growth factors, the ECM contains fibronectin, collagen, laminin, hyaluronate, heparan sulfate, and elastin, all in a proteoglycan gel (Carlsson and Nederman 1989; Santini et al. 2000). The ECM and proteins responsible for cell adhesion, such as integrin, play a vital role in cell survival, growth, and death (Santini et al. 2000). Cell adhesion actually acts to inhibit apoptosis. Apoptosis is a complex phenomenon whose role in tumor growth is still being studied. Recent findings have shown that contrary to common belief, tumor growth is not solely the result of the tumor cell's higher proliferative capacity. It is not simply that tumor cells grow and divide quicker than normal cells. In fact, tumor growth is the result of an imbalance between the production and loss of cells throughout their cell cycle (Santini et al. 2000). Through the loss of apoptotic regulation, tumor cells will proliferate in an uncontrolled manner.

All of these complex interactions are what make MTS ideal for studying tumor response to a multitude of treatments. MTS have been show to exhibit a multicellular-mediated resistance to various chemotherapeutics that was lost when the same cells were disaggregated and treated in a monolayer (Mueller-Klieser 2000). Their three-dimensional structure creates oxygen, nutrient, and pH gradients that result in quiescent cells which are more resistant to chemotherapy, ionizing radiation, and photodynamic therapy. Quiescent cells possess the ability to repair both potentially lethal damage and sublethal damage (Dubessy et al. 2000). For all these reasons, MTS have been suggested
as mandatory test models in cancer research (Mueller-Klieser 2000). There are, however, several limitations to the use of MTS in modeling treatment efficacies. One of the very fundamental issues is that many of the unique properties that allow MTS to replicate characteristics of solid tumors are poorly understood. These properties include the formation of a necrotic core, apoptotic suppression, in addition to the effects of cell adhesion, ECM, and epidermal growth factors affect cell viability. The ECM in MTS is actually different from that of tumors in vivo since it is of tumor cell origin as opposed to being produced from the surrounding tissue stroma. This will change the gene expressions within the ECM (Kim 2005).

All of these are important distinctions from monolayer cell cultures; which are easier, more inexpensive, and faster to work with. Monolayer cells are also grown in conditions of optimal growth and re-oxygenation which can be useful in describing a cell line's specific characteristics. Monolayer cell cultures do lack gradients and are not very representative of actual tumor response to treatment. Normal epithelial cells grown as monolayer have demonstrated highly plastic characteristics and actually mimicked certain traits of tumor cells in vivo (Kim 2005). The final limitation of MTS is their variability with respect to growth which results from a sensitivity to environmental conditions. MTS of identical genotype within the same culture have demonstrated a coefficient of variance of up to 10% despite efforts to normalize all spheroids to the same size (Mueller-Klieser 2000). Freyer and Sutherland also found that fluctuations in oxygen or glucose concentrations within their culture medium caused large changes in the MTS growth rates and extent of central necrosis (Freyer and Sutherland 1986). Human glioma spheroids have shown a large variance in hypoxic markers bound next to
the necrotic core suggesting that oxygen levels are not necessarily static and predictable once necrosis has formed (Mueller-Klieser 2000). It can become very difficult to monitor all these parameters and thus growth rate variations should be expected.

While the MTS model does have its limitations, the ability to better mimic the tumor microenvironment far outweighs these disadvantages. MTS have been shown to be a crucial link between monolayer in vitro culturing and tumor work in vivo. MTS are particularly useful when studying resistance to ionizing radiation, photodynamic, and drug therapies.

1.4 Bleomycin

The drug under investigation for this research is a chemotherapeutic called bleomycin (BLM) (Fig. 7). Bleomycin is a glycopeptic antibiotic whose molecular weight is 1.5 kDa. It was discovered by Umezawa et al. in 1966 and is cytotoxic to eukaryotic cells due to its ability to bind to and degrade DNA (Stubbe and Kozarich 1987).

![Figure 7. The structure of bleomycin (Stubbe and Kozarich 1987).]
The bleomycin molecule has a large hydrophilic structure comprised of four parts: a terminal amine, bithiazole, pseudopeptidic, and glycannic (Mir et al. 1996). The terminal amine group interacts with nucleic acids. The bithiazole part binds to DNA and interacts with the DNA minor groove. Binding to transition metals and DNA sequence recognition are performed by the pseudopeptidic part. The final, glycannic, part's function is still unknown. Bleomycin forms metal oxide complexes by both binding to oxygen as well as redox-active transition metal ions \( \text{Fe}^{2+}, \text{Co}^{2+}, \text{Zn}^{2+}, \text{Ni}^{2+}, \text{and Cu}^{2+} \) (Stubbe and Kozarich 1987). Bleomycin requires the presence of oxygen in order to initiate DNA breaks. Bleomycin has limited ability to penetrate the cell membrane and is found to accumulate in endocytic vesicles. Therefore, bleomycin typically enters the cell through receptor-mediated endocytosis by binding to a 250 kDa receptor on the membrane (Berg et al. 2005). Once inside the cytosol, bleomycin is a very potent drug, however, it must diffuse to the nucleus in order to interact with DNA.

Bleomycin is typically used as a chemotherapeutic for the treatment of squamous cell carcinomas of the head and neck, esophagus, bronchus, and skin, as well as testicular cancer and Hodgkin’s and non-Hodgkin's lymphoma (Mir et al. 1996; Berg et al. 2005). Side effects include pneumonitis (occurs in 46% of patients) and lung fibrosis (occurs in 3%) which can present after a total cumulative dose of 300 mg BLM/m\(^2\) (Mir et al. 1996). Patients are typically treated two to three times per week or given a continuous low dose for several days. The drug has a biological half-life in blood of 2-4 hours after intravenous injection, with 70% excreted after the first 24 hours (Mir et al. 1996). Renal excretion is the primary mode of clearance from the body. Wolff et al. (1999) performed a meta-analysis on over a thousand articles in order to summarize LC\(_{50}\) (drug
concentration required to kill 50% of the cells treated) values for several chemotherapeutic drugs. All the data were taken for the treatment of glioma cells and bleomycin was found to have an LC$_{50}$ value of 18.6 mg/L.

Once bleomycin has entered the cell through an endosome, it faces several barriers to its final target, the nucleus. The first barrier is degradation by hydrolytic enzymes, of late endosomes and lysosomes. The protein, bleomycin hydrolase, is believed to inactivate bleomycin by catalytically deaminating the molecule. The exact location and role of bleomycin hydrolase is not well understood, but its function has been demonstrated (Mir et al. 1996). Furthermore, bleomycin resistance proteins (BRP) have been shown to inactivate bleomycin by forming a stable complex with it and preventing the molecule from reaching DNA. BRP are a group of binding proteins with a high specificity for bleomycin. These proteins are found in the microorganisms that produce bleomycin and bleomycin derivatives. It is no surprise that with all these barriers only 0.1% of the bleomycin added to a medium will make it inside the cells (Roy and Horwitz 1984). If bleomycin were able to therapeutically bypass these barriers, then its true potency could be realized.

Bleomycin cytotoxicity lies in its ability to create single- and double-strand breaks in DNA and other nucleic acids. In this way, bleomycin has a very similar effect to that of ionizing radiation damage. Bleomycin's cytotoxic effect does vary between different tumor genotypes and is affected by interactions with the cellular membrane, the cell's ability for DNA repair, bleomycin hydrolase activity, the cellular uptake mechanisms, and the rate of bleomycin efflux (Berg et al. 2005). DNA cleavage occurs at the GC base pairs. Bleomycin is secured onto the DNA by the bithiazole and terminal amine parts and
often produces two breaks, one each on opposite strands of the DNA. Bleomycin could be considered a miniendonuclease because one molecule can produce 8-10 DNA breaks. There's also one double-strand break for every 6-8 single-strand breaks on average (Povirk et al. 1977). Bleomycin is known to cleave the DNA linker between two consecutive nucleosomes, telomere synthetic sequences, and RNAs. This extensive chromosome fragmentation does lead to some genotoxic effects. Chromosome aberrations such as deletions, dicentrics, multicentrics, rings, exchanges, breaks, and gaps are all induced (Mir et al. 1996). Bleomycin does not however induce sister-chromatid exchanges. There is no limit to the number of targets that bleomycin can attack once inside the cell. These attacks occur less than 30 seconds after the cell’s membrane has been breached (Tounekti et al. 1993). Selective electropermeabilization has demonstrated that as little as a few hundred internalized bleomycin molecules are sufficient to induce cell death.

There are a number of different types of cell death including necrosis and apoptosis. Necrosis is characterized by cell swelling, membrane ruptures, and the dissolution of organized structures, while apoptosis is marked by the shrinkage of cells and chromatin condensation (Tounekti et al. 1993). Necrosis is a far slower process in which the DNA is finally degraded by lysosomal enzymes and apoptosis, also referred to as programmed cell death, is a far swifter process where internal endonuclease is activated which then digests the DNA. A third type of cell death is observed following exposure to ionizing radiation. This is termed mitotic death in which the cell will complete one mitotic cycle before simply disintegrating. This is generally caused by exposure to low-dose ionizing radiation in which the cell remains viable, just no longer able to reproduce. Tounekti et
al. (1993) demonstrated that bleomycin acted in an identical fashion to ionizing radiation in how it attacked and killed cells. They were able to relate the number of bleomycin molecules internalized with the type of cell death produced. At low bleomycin concentrations, cells arrested in the G2/M phase and died a certain amount of time later. This period was equal to that of three cell doubling times and correlated well with mitotic cell death. For high concentrations of bleomycin, cell death occurred in an identical fashion to apoptosis. Tounekti et al. (1993) were also able to rule out several other possible contributors, such as free radical species and endonuclease in order to determine that cell death was a direct result of the bleomycin. This report concluded that approximately 3000 bleomycin molecules resulted in mitotic death, just as seen with low dose ionizing radiation and that 3 million internalized bleomycin molecules caused cell apoptosis, which is seen with high doses of ionizing radiation (Tounekti et al. 1993). There is no doubt as to the powerful cytotoxicity of bleomycin. Relatively few molecules can create severe damage once they have passed the cell membrane. These characteristics of bleomycin makes it ideally suited for use in photochemical internalization, where drugs are selectively released into the cell cytosol. The next requirement is the use of an appropriate membrane localizing photosensitizer.

1.5 Aluminum phthalocyanine disulfonate (AlPcS2a)

The photosensitizer chosen for this study is a metal-based phthalocyanine which belongs to a group of second-generation photosensitizers characterized by their high-efficiency in the red visible wavelength.
The chemical structure of AlPcS₂₈ gives this photosensitizer unique properties useful for PCI applications. Phthalocyanines are traditionally water insoluble, but the sulfonation of these molecules allows them to become biologically active (Berg et al. 1989). Thus, AlPcS₂₈, which is sulfonated on two of its adjacent isoindole units, is water soluble and has an asymmetrical distribution of its polar groups (Edrei et al. 1998; Bonneau et al. 2004) (Fig. 8). The amphiphilic nature of AlPcS₂₈ allows it to favorably interact with cell membrane structures. The photosensitizer alone possesses no local or systemic toxicity and is resistant to dimerization and aggregation once incorporated into the cell (Edrei et al. 1998; Maman et al. 1999). The primary advantage of AlPcS₂₈ and other metallated phthalocyanines is their absorbance spectrum.

Figure 8. The structure of AlPcS₂₈.

In addition to its chemical properties, AlPcS₂₈’s light absorbance and fluorescence characteristics are well suited for administration in biological tissue. AlPcS₂₈’s peak absorbance in aqueous solution is 675 nm (Kunz et al. 2007). The optimum range of wavelengths for light penetration into tissue is between 650 – 680 nm (Edrei et al. 1998). Earlier, first-generation photosensitizers such as Photofrin, had peak absorbance around
630 nm, which resulted in about half the penetration depth (Chan et al. 1997). In addition to light absorbance in a favorable wavelength range, AlPcS$_2$ has the benefit of low absorption in other parts of the visible spectrum (Chan et al. 1997). This reduces the risk to cutaneous photosensitization post-treatment. Compared to Photofrin, patients do not have to wait as long for the drug to clear their system. These absorption benefits only occur in the photosensitizer's monomer form, further supporting the need for low aggregation. The optimum absorption wavelengths for AlPcS$_2$ in PBS are 672 nm and 640 nm in the monomer and aggregate bands respectively (Edrei et al. 1998). Aggregation is a result of both chemical structure and localization of the photosensitizer within the cell. Changes in fluorescence localization are easily measured and can be used to determine changes in photosensitizer distribution.

AlPcS$_2$ primarily localizes in lipid membranes which favors cellular uptake by endocytosis (Edrei et al. 1998; Maman et al. 1999; Bonneau et al. 2004; Kunz et al. 2007). The photosensitizer's amphiphilic chemical structure allows lipid bilayer penetration. The charged sulfonate group is oriented in the outer aqueous phase, where it is hydrated, and the neutral hydrophobic functional group becomes buried in the lipid structure (Maman et al. 1999). Transport across the lipid bilayer has been studied and found to be an extremely slow process (on the order of hours). Due to this slow nature of cellular uptake, passive diffusion of this molecule is highly unlikely (Maman et al. 1999; Bonneau et al. 2004). The primary mechanism for cellular uptake is the incorporation of AlPcS$_2$ into endosomes and lysosomes. These endocytic vesicles are disrupted by photosensitizer-mediated production of singlet molecular oxygen which leads to the diffusion of AlPcS$_2$ into the cytosol. The exact mechanism of this disruption will be
detailed in the following section. Uptake concentrations are highly dependent on environmental factors such as cell line, incubation conditions and time, medium pH, and the photosensitizer’s lipophilicity (Edrei et al. 1998). Optimum incorporation occurs at a pH of 7.3, which correlates with typical physiological pH’s (Maman et al. 1999). Berg et al. found that phthalocyanine photosensitizers’ lipophilicity increased with decreasing pH which correlates with increased uptake (Berg et al. 1989). This would suggest a mechanism for selective uptake into tumor tissue which is often characterized by relatively lower pH levels compared to surrounding tissue due to hypoxia and waste product accumulation. Macrophages have also been shown to exhibit the highest uptake of AlPcS$_{2a}$ in several *in vitro* and *in vivo* studies (Kunz et al. 2007). Since macrophages constitute significant portions of the tumor matrix, this could also be another factor in AlPcS$_{2a}$’s preferential localization in tumor tissue. The tumor to peritumoral tissue ratio was measured by Chan et al. (1990) to be approximately 17, although such measurements are highly dependent on variables such as delivery method and time after administration. A mixture of various sulfonated phthalocyanines known as Photosens® has been used in Russian diagnostic and clinical phototherapy studies of various cancers since 1994 (Edrei et al. 1998). AlPcS$_{2a}$ is the most cytotoxic and thus most promising of the sulfonated phthalocyanines for use in phototherapy due to its high lipophilicity, low aggregation, optimal wavelength absorbance, and high selectivity for tumor tissue.

1.6 Photodynamic therapy (PDT)

PDT can be defined as the activation of a photosensitizer (PS) by an appropriately chosen light source that, in the presence of oxygen, produces destructive reactive oxygen
species (ROS) causing sufficient cellular damage and leading to cell death (Castano et al. 2005a; Bonneau et al. 2004). PDT is an inherently selective treatment. Photosensitizers are chosen based on their ability to localize in targeted tissues, and light is selectively administered only to those regions of interest (Castano et al. 2004). The very short lifetime and range of ROS further contributes to PDT’s high degree of localization.

PDT has a long history in medicine, but only the most recent developments in cancer therapy will be discussed here. While PDT has been the subject of much research for several decades, clinical applications for a variety of cancers have only occurred over the past 30 years (Stylli and Kaye 2006a). PDT has been approved by the U.S. Food and Drug Administration for the treatment of microinvasive lung cancer, obstructing lung cancer, obstructing esophageal cancer, premalignant actinic keratosis, and age-related macular degeneration (Zhu and Finlay 2008). In addition, several studies have shown effective treatment of head and neck cancer, mesothelioma, Barrett’s esophagus, prostate, and brain tumors (Zhu and Finlay 2008). PDT has been implemented in the treatment of brain tumors since Perria et al. treated human gliomas in 1980 (Madsen et al. 2002; Stylli and Kaye 2006a). PDT is generally used as an adjunct to traditional therapies of maximal tumor resection, ionizing radiation, and chemotherapy. Several groups have utilized PDT in this way, primarily with GBM and anaplastic astrocytoma, to better control infiltrative tumor cells in the vicinity of the resection margin (Dougherty et al. 1998; Madsen et al. 2002). PDT is mostly used for the treatment of localized solid tumors that are easily accessible on the skin surface, in organs that can be accessed endoscopically, or deep-seated lesions within organs treated by interstitial delivery of light (often directly after surgical resection) (Wilson 2002).
Light delivery is typically performed utilizing recently developed diode lasers. These lasers are reliable, compact, user friendly and relatively inexpensive which make them ideally suited for clinical use (Huang 2005). Laser light is focused using optical fibers that can treat both external and internal tumors with typical treatment times of 5-20 minutes (Brown et al 2004). Muller and Wilson were the first to develop a cavitary illumination technique for the treatment of malignant gliomas in the 1990’s that utilizes a balloon-like device filled with diffusing medium to evenly distribute the light (Huang 2005; Stylli and Kaye 2005b). PDT still remains an inherently complex technology which is highly dependent on the tissue being treated as well as the characteristics of the photosensitizer used.

Although it is not agreed upon what constitute the most important qualities of a PS, several chemical and structural characteristics can be identified as ideal for use in PDT. Characteristics of an ideal PS include: high tumor selectivity, strong phototoxicity, low dark toxicity, high singlet oxygen quantum yield, high intersystem crossing (ISC) yield, triplet-state energy greater than the energy required to excite oxygen (i.e. 94 kJ/mol), long wavelength absorption (600-800 nm), low light absorption at shorter wavelengths (400-600 nm), high tumor tissue retention, rapid clearance from surrounding normal tissue, high water solubility, low aggregation in biological environments, short interval between PS injection and illumination, and a commercially available pure chemical that is easily synthesized and highly stable (Almeida et al. 2004; Castano et al. 2004; Nyman and Hynninen 2004; Huang 2005; Stylli and Kaye 2006; Zhu and Finlay 2008). As with many biological systems, it is the balance of several characteristics that makes a PS suitable for PDT. While it is advantageous for a PS to have strong light absorption in
order to minimize its concentration, too strong an absorption leads to a self-shielding
effect that reduces the treatment depth. Similarly, the PS must be stable enough to avoid
rapid degradation (photobleaching), but not so stable that it significantly extends the
patient’s photosensitivity after the treatment is finished. Another important characteristic
is chemical amphiphilicity. Amphiphilic PS have both hydrophobic and hydrophilic
regions and are thus more photodynamically active since they tend to localize in
hydrophobic-hydrophilic membrane interfaces and protein surfaces, as well as exhibit
lower aggregation (MacDonald and Dougherty 2001). These qualities are important
since they determine how and where a PS will localize within tumor cells.

There are a large number of photosensitizers which target a variety of sub-cellular
organelles. These targets include the plasma membrane, lysosomes, mitochondria, Golgi
apparatus, and endoplasmic reticulum (Buytaert et al. 2007). Since PS do not generally
localize in cell nuclei, PDT does not primarily cause DNA damage which lead to
mutations and carcinogenesis as with other treatments like ionizing radiation and
chemotherapy (Buytaert et al. 2007).

As stated earlier, structure dictates localization, but there are a number of other
qualities that govern PS localization. Molecular weight, ionic charge, PS concentration,
incubation time, and target cell phenotype all factor into how a PS will distribute and
localize (Castano et al. 2004). Thus the efficacy of any PDT treatment depends on many
factors including the type of PS, its concentration, and localization.

In addition to the type of PS, light dose (fluence), dose rate (fluence rate), and oxygen
availability are critical determinants of PDT efficacy (Triesscheijn et al. 2006). As is the
case with ionizing radiation, PDT is an oxygen-dependent therapy and therefore has
limited efficacy in hypoxic regions. Optimization requires judicious choice of parameters. In particular, oxygen levels are sensitively dependent on light fluence rates: high fluence rates result in rapid oxygen depletion and diminished PDT efficacy.

PDT is a complex treatment modality requiring the presence of light, PS, and oxygen. Upon photoactivation, the PS is excited from its singlet ground state to a short-lived (nanoseconds) excited singlet state (Fig. 9). The excited PS can decay through three different transitions: (1) fluorescence, (2) internal conversion into heat, or (3) intersystem crossing (ISC) in which the excited electron's spin is inverted and the PS is converted to a triplet state (Nyman and Hynninen 2004; Castano et al. 2004). This first triplet state is longer-lived (microseconds) allowing it to interact through chemical reactions. The triplet state can decay through three different transitions as well: (1) phosphorescence and subsequent return to the ground state, (2) internal conversion into heat, and (3) non-radiative transfer of energy to molecular oxygen (MacDonald and Dougherty 2001). Oxygen has a unique triplet ground state and low excitation states which make this transfer possible. This transfer of energy takes form in two types of reactions. Type I reactions occur directly between the triplet state PS and the cellular substrate. This electron or proton-exchange produces reactive intermediates such as superoxide, hydroperoxyl, hydroxyl radicals, and hydrogen peroxide. Type I reactions typically occur only under anoxic conditions. If the cellular environment is sufficiently oxygenated, Type II reactions occur between the triplet state PS and ground state molecular oxygen. This process involves an electron spin exchange to convert triplet ground state oxygen into excited singlet state oxygen. The excited triplet state PS is converted back to the ground state. This entire process is illustrated in Figures 9 and 10.
**Figure 9.** PDT action mechanism. Once the ground state photosensitizer (PTS) is activated and excited (PTS*), deactivation occurs through fluorescence or intersystem crossing to the excited triplet state (**3PTS**). Energy is then lost via Type I or II reactions producing free radicals and ROS (Buytaert et al. 2007).

**Figure 10.** Jablonski diagram showing excitation and relaxation of a PS: (A) photoexcitation, (B) fluorescence, (C) intersystem crossing, (D) phosphorescence, (E) non-radiative transfer of energy to singlet oxygen, (F) substrate oxidation by singlet oxygen, (G) internal conversion (MacDonald and Dougherty 2001).
The photogenerated singlet state oxygen is the fundamental contributor to PDT's cytotoxicity. Singlet oxygen is a highly reactive oxidizer with a short half-life of less than 40 ns in biological systems which means its effective radius of action is less than 0.02 μm (Castano et al. 2004; Stylli and Kaye 2005; Buytaert et al. 2007). The short lifetime of singlet oxygen limits biological damage to the immediate vicinity of the PS and therefore the organelle(s) affected. The type of cell death, therefore, is selectively dependent on intracellular PS distributions.

As stated earlier, an ideal PS will have maximal light activation at a wavelength between 600-1300 nm – the so-called optical window (Castano et al. 2004) where light has significant penetration in biological tissues due to relatively low absorption and scattering of major tissue constituents (Fig. 11).

![Figure 11. 'Optical window' in tissue. Absorption spectra for water, oxy- and deoxyhemoglobin, and melanin (Castano et al. 2004).](image_url)
The mode of cell death caused by PDT is determined by cell type, PS localization, and light fluence (Castano et al. 2005a). It has generally been found that low-fluence PDT leads to apoptosis while higher fluences will produce proportionately more necrosis. Both modes of cellular death lead to tumor destruction through a direct cellular toxic effect, vascular damage, or an immune reaction (Castano et al. 2005; Stylli and Kaye 2005). All three mechanisms of tumor destruction begin with the production of ROS by activation of a PS. The first mechanism is the result of ROS-induced necrosis or apoptosis of a significant proportion of cells within the tumor. The second mechanism occurs indirectly when PDT-induced damage of the tumor vasculature restricts the supply of nutrients and oxygen, which ultimately leads to tumor infarction. PDT has also been shown to cause primary vascular leakage further restricting the tumor’s ability to sustain itself. PDT has been found to require a functioning immune system to illicit complete tumor response. An immune response to cancer, induced by PDT, is explained by the induction of inflammation which initiates anti-tumor activity within the tissue followed by a long-term immunological response (Castano et al. 2005b).

While many of the tumor responses to PDT are not well understood, the net cytotoxic effect still demonstrates PDTs ability to act as a potent adjunct therapy with many advantages over those currently employed. PDT is not limited by cumulative or maximum dose considerations (except for heat generation which can be controlled through fractionation), allowing repeated applications that can be combined with other therapies. There are few significant side effects and PDT is not known to cause mutations or be carcinogenic. In fact, normal tissue readily heals after treatment. The primary drawback of PDT is the poor penetration depth of light in tissues which limits
the treatment to relatively small tumors easily accessible by intracavitary light delivery methods.

1.7 Photochemical internalization (PCI)

PCI is a technique which utilizes the photochemical properties of PDT for the enhanced delivery of macromolecules into the cell cytosol. These macromolecules lack the ability to naturally permeate intracellular barriers such as the plasma and endocytic membranes (Berg et al. 2007). Due to this inability, they are expected to be internalized through endocytosis, localized in late endosomes and lysosomes until they are degraded by hydrolytic enzymes. PCI relies on specific PS that preferentially localize in vesicular membranes. Upon light activation, the ROS produced act to permeabilize the endocytic membrane allowing the release of its contents. In this way, the macromolecules are delivered into the cytosol in a biologically active form (Berg et al. 2006). The precise molecular nature of membrane permeabilization is still not fully understood, with some reports identifying membrane protein damage as being more important than the oxidation of membrane lipids (Hogset et al. 2004). However, this technique can be applied to various molecules and is not inhibited by their size or charge. Ideally, molecules chosen for this technique would have no ability to adversely affect non-targeted cells (Hogset et al. 2004). This would restrict the macromolecular activities to those inside photoactivated cells, sparing all others.

Only specialized PS can be used in PCI. Most importantly, the PS must demonstrate preferential localization in endocytic membranes and exhibit low aggregation (Hogset et al. 2004). The PS should localize specifically in the membrane structure itself and not
simply the endocytic lumen. This is important in order for the short-lived ROS to cause membrane damage. In addition, if the PS is also present inside the compartment near the molecules to be delivered, there is an increased chance of the ROS oxidizing the molecules. Obviously, no PCI effect can be achieved if the molecules are destroyed prior to their release into the cytosol. The aggregation state of the PS is also of importance. A highly aggregated PS will undergo endocytosis, not incorporation into the membrane structure. Highly aggregated PS are also inhibited in their transfer of energy to molecular oxygen. For these reasons, amphiphilic PS are ideally suited for PCI due to their hydrophobic and hydrophilic parts resulting in membrane localization with little risk of passage through to the interior of the endosome (Berg et al. 2005b). The basic process is illustrated in Figure 12.

Figure 12. PCI of bleomycin molecules through the activation of the photosensitizer, AlPcS$_{2a}$: (1) the PS is localized in the endocytic membrane, (2) photoactivation creates an excited state PS, (3) the PS activates singlet oxygen and other ROS, (4) which cause permeabilization of the endocytic membrane and subsequent release of bleomycin into the cytosol.
AlPcS$_{2\alpha}$ and bleomycin were both chosen for this study due to their ideal qualities for use in PCI. AlPcS$_{2\alpha}$ is an amphiphilic PS known to localize in endocytic vesicle membranes and absorb in the far-red (670 nm) region (Castano et al. 2004; Hogset et al. 2004; Berg et al. 2006, 2007; Kunz et al. 2007). As for bleomycin, this chemotherapeutic is known to have poor cellular uptake resulting in its accumulation in endocytic vesicles (Hogset et al. 2004; Berg et al. 2007). It has also been determined by several studies that AlPcS$_{2\alpha}$-mediated PCI of bleomycin results in synergistic inhibition of tumor growth in several in vivo and in vitro applications (Hogset et al. 2004; Berg et al. 2005, 2007). These reports are encouraging for this study and its application to the PCI of bleomycin in glioma cells.

1.8 Scope of study

The primary objective of this thesis is to examine the ability of PCI to enhance the efficacy of the chemotherapeutic drug, bleomycin, in both rat glioma monolayers and human glioma spheroids. In both cases, the plasma membrane localizing photosensitizer, aluminum phthalocyanine disulfonate (AlPcS$_{2\alpha}$), will be used. It is hypothesized that: (1) AlPcS$_{2\alpha}$ is an effective photosensitizer for PDT on both F98 monolayers and ACBT spheroids, (2) high concentrations of the chemotherapeutic agent, bleomycin, are capable of producing significant toxicity in both in vitro glioma systems, and (3) the combination of AlPcS$_{2\alpha}$-PDT and bleomycin, i.e. PCI, exhibits a synergistic toxic effect on both glioma cell lines compared to either treatment modality alone.
CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Lines

All experiments were performed \textit{in vitro} utilizing two cell lines, F98 and ACBT. All work was done inside a Labconco Class II Biohazard Cabinet (Labconco Corp., Kansas City, MO) at the University of Nevada, Las Vegas (UNLV) unless otherwise specified. Cells were incubated at 37°C, 5.0% CO$_2$, and 95% humidity inside a VWR incubator (VWR International, LLC. West Chester, PA). Both cell lines were continually kept in Gibco Dulbecco’s Modified Eagle Medium (DMEM) High Glucose 1X (Invitrogen Corp., Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (FBS), 25mM HEPES buffer (pH 7.4), penicillin (100 U/ml), and streptomycin (100 μg/ml). All monolayer cells were passed using the same technique. After the confluency was checked, the flask was carefully emptied of all media and washed with 5mL of Gibco phosphate buffered saline (PBS) with pH of 7.2 (Invitrogen Corp., Carlsbad, CA). Next 1mL of Gibco 0.25% Trypsin-EDTA (Invitrogen Corp., Carlsbad, CA) was added and left in contact for 5 minutes. Trypsin is a proteolytic enzyme, which acts to detach the cells from the flask surface. After this, while holding the flask horizontal, it was tapped briskly against a solid surface in order to dislodge the cells. Next, 4 mL of DMEM was added to the flask. The contents were then pipetted up and down several times and transferred to a 15 mL centrifuge tube. After vortexing for approximately 10 seconds,
200 μL of this solution was added to 4.8 mL of DMEM inside a new flask. Two flasks were seeded in this manner, which resulted in approximately 100,000 cells passed to each new flask. This ensured a consistent supply of cells for all the experiments.

F98 is a rat glioma cell line, maintained as a monolayer and established from gliomas induced in CDF (Fischer) rats. These gliomas were induced through exposure to ethyl-nitrosourea and established by surgically removing the tumor, mincing the tissue, trypsinizing, and cultivating in Eagle medium (Ko et al. 1980). The F98 monolayer cells were maintained in 25 cm$^2$ BD Falcon tissue culture flasks with 0.2 μm vented caps (T25, Beckton, Dickson and Company, Franklin Lakes, NJ). Monolayer cells were passed twice per week, in order to maintain confluences between 40 and 80%. The confluency is a subjective measurement of the percentage of the flask's area covered by the cells. As this is a subjective measurement, the figure is always recorded with a 10% range.

ACBT cells were derived from a human grade IV GBM (G. Granger, University of California, Irvine) and were analyzed both as monolayers and spheroids. ACBT monolayers were kept in T25 flasks and passed once per week. Spheroids were formed by liquid overlay in Fisherbrand 100 x100 x15 mm square dishes (Fisher Scientific, Pittsburgh, PA) that were coated with 10 mL of 2% agarose (BioWhittaker Molecular Applications, Rockland, ME). A solution of cells was extracted as when passing cells. For spheroid formation, as many cells as possible are desired, therefore, the entire solution was added to the Petri dish along with 20 mL of DMEM. Spheroids began forming after one week of incubation. Stocks of four to eight Petri dishes containing spheroids were kept to maintain a sufficient supply. The DMEM in each dish was changed twice per week, removing and replacing 10 to 15 mL.
2.2 Monolayer survival plating

Treatment toxicity for all monolayers was determined through clonogenic assay techniques. Following the treatments, cells were extracted from the T25 flasks just as with passing and pipetted into 15 mL tubes. After vortexing for approximately 10 seconds, 100 μL was pipetted into a Coulter cup containing 9.9 mL of IsoFlow Sheath fluid (Beckman Coulter Inc., Fullerton, CA). Each cup was counted in a Beckman Coulter Particle Count and Size Analyzer Model Z2 (Beckman Coulter Inc., Fullerton, CA). The contents of each cup were gently stirred followed by two consecutive counts, stirred again, and counted two more times. The average of these four counts was calculated to yield the total cells per milliliter in each solution. Once the concentration was found, 500 μL from each tube was taken and added to 4.5 mL of DMEM in order to create a 10% dilution. This was performed in order to attain the desired concentration of cells in medium, so that between 100 and 800 μL of cells were added to 4 mL of DMEM in a 60 x 15 mm Petri dish (Corning Inc., Corning, NY). Each survival group has a specific number of cells to be plated. Some survival groups have two or three different cell count numbers to be plated. To calculate the exact volume of cells needed for plating in each dish, the number of cells desired was divided by the measured cell concentration. After the cells were pipetted into each Petri dish, the contents were gently swished back and forth then side to side in order to maintain even cell coverage within the dish. All the dishes were then incubated for 7 days. After this time, the media was decanted and the dishes stained with crystal violet. Using a light microscope, any colonies that formed in the dishes were counted. A colony was considered any grouping of 50 or more cells. All colonies were scored and survival rate was calculated by normalizing to the control.
Therefore, all control survival rates reported here are exactly 100% and some treatment survival rates may be greater than 100% due to statistical uncertainty.

2.3 Spheroid growth analysis

The effect of treatment on the spheroids was determined by following spheroid growth post-treatment. This was accomplished by pre-selecting spheroids, whose average cross-sectional diameters were between 400 and 500 \( \mu \text{m} \). Spheroid diameter was measured using a microscope with a calibrated eyepiece micrometer. Two perpendicular diameter measurements were made to the nearest 50 \( \mu \text{m} \), and the average was recorded. For each treatment group, 20 to 30 spheroids were placed in a 35 x 10 mm Petri dish (Beckton, Dickson and Company, Franklin Lakes, NJ) with 2 mL of DMEM. After treatment, 20 spheroids from each dish were extracted and placed into separate wells of a Costar 48 well cell culture cluster (Corning Inc., Corning, NY). Each well was coated with 250 \( \mu \text{L} \) of agarose and filled with 750 \( \mu \text{L} \) of DMEM. In this way, each individual spheroid’s growth could be monitored.

Spheroid growth was monitored over a 28 day period, beginning 24 hours after treatment. The initial diameter measurement was taken 24 hours after treatment, and subsequent measurements were taken twice per week for a total of nine measurements. Following each measurement, approximately 500 \( \mu \text{L} \) of DMEM was removed from each well and replaced with fresh DMEM to ensure the spheroids were given adequate nutrients. Spheroids demonstrating a diameter increase in excess of 50 \( \mu \text{m} \) between weekly measurement intervals were scored viable.
2.4 Bleomycin toxicity

Bleomycin (Sigma, St. Louis, MO) toxicity was investigated in F98 monolayers and ACBT spheroids. In both cases, the drug was added to DMEM to yield concentrations from 0.1-10 μg/mL. In all cases, cells were incubated in bleomycin and DMEM for four hours. Following incubation, all monolayer cells were harvested and plated for clonogenic survival as previously described. ACBT spheroids were placed in individual wells filled with DMEM and monitored for growth as previously described.

2.5 PDT toxicity

The effects of PDT on both the F98 and ACBT cell lines were determined using similar protocols. First, a photosensitizer was administered, and then a light dose was given. Cells (or spheroids) were incubated for 18 hours with 1 μg/mL of AlPcS2a (Frontier Scientific, Inc., Logan, UT) and DMEM. Following incubation, the media was removed and the cells (or spheroids) were placed in DMEM only. Next, the cells (or spheroids) were irradiated using a Class 4 photodiode laser (High Power Devices, Inc., North Brunswick, NJ) coupled to a 200 μm diameter optical fiber containing a micro-lens. The diode laser was calibrated to emit light at a wavelength of 670 nm. All cells (or spheroids) were exposed to an irradiance of 5 mW/cm². The total radiant exposure was determined by exposure time and ranged from 0.75 to 6 J/cm². The effects of PDT on cells and spheroids were determined using clonogenic survival and growth kinetics, respectively (as previously described).
2.6 PCI toxicity

In order to test for a PCI effect, cells (or spheroids) were first incubated in 1 µg/mL of AlPcS\textsubscript{2a} for 18 hours, just as with PDT. After this, cells (or spheroids) were incubated with either 0.1 or 0.25 µg/mL of bleomycin in DMEM for four hours. Next, the cells (or spheroids) were irradiated with 670 nm light (irradiance of 5 mW/cm\textsuperscript{2} and radiant exposure of 1.5J/cm\textsuperscript{2}) as described in section 2.4. The effects of PCI on cells and spheroids were determined using clonogenic survival and growth kinetics, respectively (as previously described).

2.7 Flow cytometry

Flow cytometry was used to determine the fraction of viable, apoptotic, and necrotic cells. The two different fluorescent labels used were Annexin V-FITC (Beckton, Dickson and Company, Franklin Lakes, NJ) to distinguish apoptotic cells, and propidium iodide (PI, Sigma, St. Louis, MO) to label necrosis. Unlabeled cells were assumed to be viable. The procedure to prepare both monolayer and spheroid cells was identical, except for how the cells were brought into solution.

All monolayer cells were first harvested into a DMEM solution, while spheroids were incubated in 15 mL tubes for 20 minutes in 500 µL of a one-to-one mixture of 1% collagenase (Invitrogen Corp., Carlsbad, CA) in Hanks’ Balanced Salt Solution 1x (HBSS with Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, Invitrogen Corp., Carlsbad, CA) to dispase (Beckton, Dickson and Company, Franklin Lakes, NJ). After incubation, the spheroids were vortexed for 5 seconds and 5 mL of PBS was added. The tubes were then centrifuged in a Beckman GS-6 centrifuge (Beckman Coulter Inc., Fullerton, CA) for five minutes at
1000 rpm. The PBS was then decanted, 5 mL of PBS was added and the tube was vortexed for 5 seconds. The tubes were centrifuged again, the PBS decanted, and 500 µL of PBS was added. For a final time the tubes were centrifuged, the PBS decanted, and 1x binding buffer (Beckton, Dickson and Company, Franklin Lakes, NJ) was added to achieve a final concentration of $10^6$ cells/mL. Next, 100 µL of the solution was transferred along with 400 µL of 1x binding buffer to a 5 mL BD Falcon tube. The two labels were then added with 5 µL of Annexin V-FITC and 10 µL of 100 µg/mL concentration PI. The solutions were gently agitated, and then incubated for 15 minutes at room temperature in the dark.

For each experiment, a set of control solutions was also prepared. One control remained unlabeled, the next labeled only with Annexin V-FITC, and the third labeled only with PI. Finally, after 15 minutes of incubating in the dark, each tube was analyzed in a Beckton Dickson FACSCalibur (Beckton, Dickson and Company, Franklin Lakes, NJ) flow cytometer along with CellQuest software.

2.8 Two-photon fluorescence microscopy (TPFM)

All TPFM experiments were performed at the Beckman Laser Institute, University of California, Irvine (UC, Irvine). Two-photon fluorescence images were taken of ACBT spheroids of various diameters and subjected to various treatments. The spheroids were co-stained with Hoechst 33342 (Invitrogen, Carlsbad, CA) and Ethidium Homodimer 1 (E1169, Invitrogen, Carlsbad, CA) 48 hours after treatment. The stains were administered in 5 and 1 µg/mL concentrations in 2 mL of medium for Hoechst and Ethidium Homodimer respectively. Spheroids were incubated for 1 hour and then
washed three times with HBSS before being transferred to new 35 x 10 mm Petri dishes. The spheroids were then fixed by adding 2 mL of 5% formaldehyde in ultrapure water to each dish. After 1 hour, five spheroids from each treatment group were pipetted into separate wells of a Costar 96 well cell culture cluster (Corning Inc., Corning, NY) which were each filled with 250 μL of PBS. The spheroids were imaged using an inverted laser scanning microscope (LSM 510 META, Zeiss, Oberkochen, Germany) with a 20x /0.5 NA lens (EC Plan-Neofluar, Zeiss, Oberkochen, Germany). The blue Hoechst fluorescence from all cell nuclei was imaged using 800 nm two-photon excitation and 390-465 nm detection. The red Ethidium Homodimer fluorescence from necrotic nuclei was imaged confocally with 488 nm excitation and 576-640 nm detection. Scans were made at planes 30-50 μm into each spheroid with simultaneous excitation of the two dyes, and the images were pseudo-colored blue and red.

2.9 Statistical analysis

All data was analyzed and graphed using Microsoft Excel. The arithmetic mean and standard deviation were used throughout to calculate averages and errors. Statistical significances were calculated using the Student’s t-test as well as the Welch’s t-test. Two values were considered distinct when their p-values were below 0.05.

Synergism was calculated when analyzing PCI treatments. Equation 2.1 was used to determine if the PCI effect was synergistic, antagonistic, or additive.

\[
\alpha = \frac{SF^a \times SF^b}{SF^{ab}}
\]

(Eq. 2.1)

In this scheme SF\textsuperscript{i} represents the survival fraction for a specific treatment. If two treatments are to be compared, the survival fractions of each separate treatment are
multiplied together and then divided by the survival fraction when both treatments were applied together. The resulting number (\( \alpha \)) describes the cumulative effect. If \( \alpha > 1 \), the result is synergistic. If \( \alpha < 1 \), the result is antagonistic, and if \( \alpha = 1 \) the result is simply additive.
CHAPTER 3

RESULTS

3.1 AlPcS$_2$ and bleomycin toxicity in F98 monolayers

The first priority was to determine the relative drug toxicities of the photosensitizer, AlPcS$_2$, and bleomycin in the F98 rat glioma cell line. F98 monolayer cells were incubated for 18 hours in DMEM with increasing concentrations of AlPcS$_2$. Photosensitizer concentrations of 0 (control), 0.5, 1, 2, 4, 8, 16, 24, 32, and 64 µg/mL were administered. All experiments were performed in the dark with minimal light, and the data demonstrated no correlation between photosensitizer concentration and percent survival (Fig. 13).

The relative toxicity of bleomycin was also measured after a four hour incubation period. The concentrations of bleomycin tested were: 0.1, 0.25, 0.5, 0.75, 1, 5, and 10 µg/mL. As expected, there was a noticeable decrease in survival fraction directly correlated to increasing concentration of bleomycin. The LC$_{50}$ was shown to occur at approximately 0.5 µg/mL. Concentrations of 0.1 and 0.25 µg/mL resulted in survival rates of approximately 80 and 60%, respectively (Fig. 14).
Figure 13. Percent survival vs. AlPcS$_{2a}$ concentration for F98 monolayers.

Figure 14. Percent survival vs. bleomycin concentration for F98 monolayers.
3.2 PDT toxicity in F98 monolayers

The F98 monolayer cells were incubated for 18 hours in a concentration of 1 μg/mL of AlPcS$_{2a}$. Six different groups were analyzed. The first group was a control containing no photosensitizer. The second group, known as the dark control, was incubated in AlPcS$_{2a}$ and no light was administered. This was used to ensure that the effects seen were those solely of the photosensitizer activated by the laser light. Therefore, the dark control group should exhibit 100% survival. The remaining four groups were incubated in AlPcS$_{2a}$, and subjected to increasing light exposures. The results showed a clear correlation between increasing light exposure and decrease in survival fraction with an LD$_{50}$ of approximately 2.5 J/cm$^2$. An exposure of 1.5 J/cm$^2$ resulted in a survival rate of approximately 80% (Fig. 15). The p-values (Table 3.1) show no distinction between the control and the dark control groups. There is also no statistical distinction between the dark control and the first PDT treatment with 0.75 J/cm$^2$ of light.

Table 3.1: P-values for F98 PDT toxicity

<table>
<thead>
<tr>
<th>Two Groups Being Compared</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control / Dark Control</td>
<td>0.9209</td>
</tr>
<tr>
<td>Dark Control / AlPcS$_{2a}$ + 0.75J</td>
<td>0.6929</td>
</tr>
<tr>
<td>AlPcS$<em>{2a}$ + 0.75J / AlPcS$</em>{2a}$ + 1.5J</td>
<td>0.0106</td>
</tr>
<tr>
<td>AlPcS$<em>{2a}$ + 1.5J / AlPcS$</em>{2a}$ + 3J</td>
<td>0.0000</td>
</tr>
<tr>
<td>AlPcS$<em>{2a}$ + 3J / AlPcS$</em>{2a}$ + 6J</td>
<td>0.0098</td>
</tr>
</tbody>
</table>
3.3 PCI toxicity in F98 monolayers

The final step in evaluating toxicities in the F98 monolayer was to determine the effect of PCI treatment and to compare with previous results of bleomycin alone and PDT. All PCI survivals included nine separate treatment groups. These groups included previously studied parameters such as a control, AlPcS$_2$a dark control, 0.1 μg/mL bleomycin, 0.25 μg/mL bleomycin, and 1.5 J/cm$^2$ PDT with 1 μg/mL AlPcS$_2$a. The inclusion of these five groups allowed for direct comparison to PCI, as well as confirmatory comparisons to previous experiments. The remaining four treatment groups were used to both eliminate possible unwanted drug interactions, as well as illustrate the desired PCI effect. The first unwanted interaction was between AlPcS$_2$a and bleomycin alone. For this group, the photosensitizer and bleomycin were incubated as with the other experiments, however no light was administered. Since the PCI effect should only be

---

**Figure 15.** Percent survival vs. PDT treatments with increasing radiant exposures.
present when the photosensitizer is activated, this group should show identical toxicity to that of bleomycin alone. The other unwanted interaction investigated was that of bleomycin and the laser light. No photosensitizer was added to this group. Bleomycin was not expected to be activated or degraded in the presence of light; therefore, this group should also behave identical to that of bleomycin alone.

For the two PCI effect groups, identical concentrations of AlPcS2a, as well as radiant exposures were used. One group was incubated in 0.1 μg/mL and the other in 0.25 μg/mL of bleomycin. Both PCI treatment groups should therefore be comparable to the two concentrations of bleomycin alone and PDT treatment (Fig. 16). The p-values shown in Table 3.2 show statistically significant differences in survival between monolayers subjected to both PCI regimens and either PDT-only or BLM-only treatments.

<table>
<thead>
<tr>
<th>Two Groups Being Compared</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control / AlPcS2a (Dark)</td>
<td>0.9193</td>
</tr>
<tr>
<td>0.1 BLM / 0.25 BLM</td>
<td>0.1325</td>
</tr>
<tr>
<td>0.25 BLM / AlPcS2a + 0.25 BLM</td>
<td>0.7226</td>
</tr>
<tr>
<td>0.25 BLM / 0.25 BLM + 1.5J</td>
<td>0.9586</td>
</tr>
<tr>
<td>0.1 BLM / AlPcS2a + 1.5J (PDT)</td>
<td>0.4651</td>
</tr>
<tr>
<td>0.25 BLM / AlPcS2a + 1.5J (PDT)</td>
<td>0.2469</td>
</tr>
<tr>
<td>AlPcS2a + 1.5J (PDT) / AlPcS2a + 0.1 BLM + 1.5J (PCI)</td>
<td>0.0183</td>
</tr>
<tr>
<td>AlPcS2a + 1.5J (PDT) / AlPcS2a + 0.25 BLM + 1.5J (PCI)</td>
<td>0.0013</td>
</tr>
<tr>
<td>0.1 BLM / AlPcS2a + 0.1 BLM + 1.5J (PCI)</td>
<td>0.0116</td>
</tr>
<tr>
<td>0.25 BLM / AlPcS2a + 0.25 BLM + 1.5J (PCI)</td>
<td>0.0205</td>
</tr>
<tr>
<td>AlPcS2a + 0.1 BLM + 1.5J (PCI) / AlPcS2a + 0.25 BLM + 1.5J (PCI)</td>
<td>0.0257</td>
</tr>
</tbody>
</table>
Since PCI is a technique which relies on the combination of drug and photosensitizer, the resultant toxicities should show more than an additive effect between bleomycin and AlPcS2a-PDT. The degree of synergism was calculated by comparing the survival fractions of bleomycin and PDT alone with that of the PCI treatment (Table 3.3). As evidenced from equation 2.1, the higher the $\alpha$ value, the greater the degree of synergism.

Table 3.3: Degree of potentiation between PDT and bleomycin for F98 monolayers.

<table>
<thead>
<tr>
<th>PCI Survival</th>
<th>$\alpha$</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlPcS2a + 0.1 BLM + 1.5J (PCI)</td>
<td>1.454</td>
<td>0.1433</td>
</tr>
<tr>
<td>AlPcS2a + 0.25 BLM + 1.5J (PCI)</td>
<td>2.432</td>
<td>0.2286</td>
</tr>
</tbody>
</table>
3.4 ACBT Spheroid Growth Assays

The monolayer results clearly demonstrated a PCI effect in the F98 rat glioma cell line. The next step was to determine whether such an effect could be elicited in a more complex model. This was done by performing growth assays on spheroids of the human glioma cell line, ACBT.

A total of seven groups were followed simultaneously in order to better compare the various toxicities. The seven groups included: a control, AlPcS$_{2a}$ dark control, 0.1 μg/mL bleomycin, 0.25 μg/mL bleomycin, 1.5 J/cm$^2$ PDT with 1 μg/mL AlPcS$_{2a}$, and PCI groups with 0.1 and 0.25 μg/mL bleomycin. Each PCI group was incubated in 1 μg/mL AlPcS$_{2a}$ and irradiated with 1.5 J/cm$^2$. Based on the monolayer results (Figures 3.1 and 3.2), sub-optimal bleomycin and light doses were chosen in order to optimize the PCI effect. The exclusion of two groups (AlPcS$_{2a}$ + bleomycin and bleomycin + 1.5 J/cm$^2$) was made since no effect was observed in the F98 experiments.

The p-values (Appendix I) are illustrative in showing how many days post-treatment that the spheroids' diameters have measurable statistical differences in growth. In many cases, the spheroid diameter growth difference was evident after the fifth day. For others, the difference did not appear until the 12th day. For example, the 0.25 μg/mL bleomycin and the PCI with 0.25 μg/mL bleomycin groups demonstrated similar growth patterns until the 12th day. After this point, a clear distinction was discernible, showing a reduction in growth of the latter. A line graph of the average spheroid diameter over time after treatment (Fig. 17) clearly shows a growth delay in the PCI treatment groups. All spheroids in the seven different groups began with an average diameter of approximately
450 μm and grew to a final diameter between 900 and 1300 μm by the final measurement on the 29th day.

The control group showed the most accelerated growth rate over this time, while both PCI treatment groups demonstrated the slowest growth. The growth pattern illustrated in Figure 17 was consistent with Gomperzian growth (initial exponential growth followed by a linear phase and finally reaching an asymptotic limit). It is easier to see the initial differences in diameter, if only the first eight days post-treatment are shown (Fig. 18). Already after five days, a difference in the growth rates was discernible.
Figure 17. Average spheroid diameter vs. days post-treatment.
Figure 18. Average spheroid diameter vs. days post-treatment through the first 8 days.

It is also instructive to look at the viability of the ACBT spheroids from the perspective of percent growing versus time (Fig. 19). This is illustrated by plotting the percentage of spheroids showing growth, thus still viable, between weekly measurement periods. Weeks 1, 2, 3, and 4 correspond to those measurements taken on days 8, 15, 22, and 29 respectively.
Figure 19. Percentage of spheroids viable for weeks 1 through 4 (measured at days 8, 15, 22, 29 respectively). Error bars denote standard deviation of the means.
The degree of potentiation between PDT and bleomycin is shown in Table 3.4. The data suggest an additive effect for the combination of 0.1 μg/mL bleomycin and PDT, while a synergistic effect is observed during the first two weeks post-treatment for the 0.25 μg/mL bleomycin and PDT group. Subsequent proliferation of cells not damaged by the treatments resulted in MTS regrowth, as indicated by the loss of synergism in weeks 3 and 4.

Table 3.4. Degree of potentiation between PDT and bleomycin for ACBT spheroids.

<table>
<thead>
<tr>
<th></th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
<td>+/−</td>
<td>α</td>
<td>+/−</td>
</tr>
<tr>
<td>AlPcS2a + 0.1 BLM + 1.5J (PCI)</td>
<td>1.346</td>
<td>0.3203</td>
<td>1.284</td>
<td>0.3056</td>
</tr>
<tr>
<td>AlPcS2a + 0.25 BLM + 1.5J (PCI)</td>
<td>1.657</td>
<td>0.3970</td>
<td>1.983</td>
<td>0.4748</td>
</tr>
</tbody>
</table>

3.5 Determination of spheroid cell viability by flow cytometry

Flow cytometry was utilized to examine the effects of treatment on cellular viability. This was performed by examining the percentage of viable, necrotic, and apoptotic cells as a function of treatment for each of the seven groups considered in section 3.4 (Fig. 20). All spheroids were between 450 and 550 μm in diameter. The percentage of viable cells was slightly lower in the PCI groups, while a small increase in the apoptotic fraction was noted in these two groups.
3.6 Two-photon fluorescence microscopy

The results of two-photon fluorescence images demonstrated a qualitative enhancement in the toxicity of PCI compared to bleomycin or PDT-only treatments alone. This was inferred from the high proportion of red fluorescing cells (suggestive of necrosis) observed from the PCI-treated spheroids (Fig. 21d). By comparison, a smaller number of red fluorescing cells was observed from PDT-only spheroids (Fig. 21c). As illustrated in Figs. 21a and b, virtually no necrotic cells were observed in control and bleomycin-only spheroids.
Figure 21. Two-photon fluorescence microscopy images of ACBT spheroids stained with Hoechst 33342 (grey) and Ethidium Homodimer (white, necrosis). Images a and b were taken at a depth of 20 μm and images c and d at 30 and 40 μm, respectively. Spheroids were fixed 48 hours after treatment: (a) control, (b) 0.1 μg/mL bleomycin, (c) 1.5 J/cm² PDT, (d) PCI with 0.1 μg/mL bleomycin + 1.5 J/cm². Field of view for all images was 400 x 400 μm.
CHAPTER 4

CONCLUSIONS

The results of this study lead to several conclusions about the responses of two different cell culture models to PCI, a novel drug delivery technique. F98 monolayer colony formation assays revealed the ability of both bleomycin and AlPcS\textsubscript{2a}-PDT to elicit significant cytotoxic effects. They also showed the synergistic nature of PCI treatment over both bleomycin and PDT alone. The results of these experiments also served to eliminate the possibility of extrinsic effects between bleomycin and AlPcS\textsubscript{2a} or bleomycin and light. ACBT spheroid growth kinetics demonstrated that bleomycin and PDT were both inefficient treatments of MTS at low doses, but that together a synergistic effect caused significant growth retardation. The growth curves also supported the modeling of ACBT spheroids according to Gompertzian growth characteristics.

Flow cytometry and two-photon fluorescence microscopy both aided in qualifying the internal composition of spheroids after various treatments. Flow cytometry data showed that PCI treatment caused a decrease in the percentage of viable cells and an increase in the apoptotic fraction. The TPFM images illustrated the increased formation of necrotic cells in both PDT and PCI treated spheroids. The significant increase in PCI-induced necrosis would suggest a synergistic interaction for the PCI compared to PDT and
bleomycin alone due to the smaller number of necrotic cells observed in bleomycin-only and PDT-only treated spheroids.

All these findings can be generalized to describe a major difference between the two cell culture models. For each of the experiments, monolayer cells demonstrated higher sensitivity to the various treatment modalities. This can be attributed to the fundamental differences between the two models. Monolayers lack intercellular communication and are actively proliferating in optimal growth and re-oxygenation conditions. In particular, a readily available supply of oxygen plays a major role in both PDT and PCI cytotoxicity. Monolayer cells have high morphological homogeneity exhibiting identical growth within the model. In contrast, MTS have developed significant amounts of extracellular matrix and present three distinct phases of cell growth (proliferating, quiescent, and necrotic) within each spheroid. MTS also exhibit nutrient, pH, and oxygen gradients.

While many of the specific interactions are not fully understood, these differences clearly confer an increased resistance of MTS to various chemotherapeutic and ionizing radiation treatments. This is often attributed to MTS resistance to apoptosis and the ability for quiescent cells to better repair sublethal and potentially lethal damage which are characteristic of low-dose ionizing radiation. Since bleomycin is known for inducing apoptosis and has cytotoxic similarities to ionizing radiation, these key differences between monolayers and MTS are especially important for this study.

While the synergistic effect was less pronounced in MTS, these findings bode well for possible clinical applications of PCI in the treatment of GBM. The current regimen of surgical resection followed by external beam radiation and chemotherapy could be augmented to include PCI therapy. Post surgical applications could selectively target
glioma cells that were unresectable as well as infiltrative cells two or more centimeters from the resection cavity. As this treatment is not limited by cumulative dose restrictions, it could be easily incorporated into the treatment regime.

It would be instructive to further investigate several aspects of the findings in this study. It was demonstrated that PCI can be effectively used to enhance the delivery of bleomycin into glioma cells. The next step is to optimize the observed effects in the ACBT spheroid model. This would include optimal concentrations of bleomycin, light doses for AlPcS₂₄-PDT, and their combination in PCI. Better characterization of the ACBT spheroid model would also present a clearer picture with regard to spheroid response to treatment. Utilizing theoretical Gompertz growth equations based on experimental data could better illustrate expected values such as diameter at onset of necrosis formation, viable rim thickness, and maximum attainable volume. These values would aid in the analysis of spheroid growth kinetics. The final step would be in vivo studies to test the optimizations and growth predictions.

In conclusion, the results of these experiments clearly demonstrate the synergistic effects of PCI in two different in vitro glioma cell models. Sub-optimal doses of bleomycin and PDT in monolayers demonstrated a decreased efficiency in MTS. Even at these sub-optimal levels, PCI was very effective, resulting in 35% spheroid viability two weeks post-treatment. This result is extremely encouraging considering the low doses of bleomycin and PDT administered. PCI can clearly be used to substantially enhance the delivery of bleomycin in malignant glioma cells resulting in a significant reduction in cell survival. Additional animal studies will be required in order to fully understand the implications of these results.
**APPENDIX I**

**ACBT SPHEROID GROWTH ASSAY P-VALUES**

P-values for spheroid diameter measurements versus days post treatment are given in the table below. For simplicity, the experimental groups are labeled A through G as follows: control (A), AlPcS$_{2a}$ dark control (B), 0.1 µg/mL bleomycin (C), 0.25 µg/mL bleomycin (D), 1.5J PDT with 1 µg/mL AlPcS$_{2a}$ (E), PCI with 0.1 µg/mL bleomycin (F), PCI with 0.25 µg/mL bleomycin (G). Therefore, column AB signifies p-values between the measurements of groups A and B for days 1 - 29.

Table A1. P-values for ACBT spheroid diameter growth assay.

<table>
<thead>
<tr>
<th>Two Groups Being Compared</th>
<th>AB</th>
<th>AC</th>
<th>AD</th>
<th>AE</th>
<th>AF</th>
<th>AG</th>
<th>BC</th>
<th>BD</th>
<th>BE</th>
<th>BF</th>
<th>BG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1</strong></td>
<td>0.254</td>
<td>0.843</td>
<td>0.030</td>
<td>0.175</td>
<td>0.116</td>
<td>0.101</td>
<td>0.172</td>
<td>0.259</td>
<td>0.662</td>
<td>0.608</td>
<td>0.513</td>
</tr>
<tr>
<td><strong>Day 5</strong></td>
<td>0.073</td>
<td>0.001</td>
<td>0.000</td>
<td>0.002</td>
<td>0.000</td>
<td>0.000</td>
<td>0.131</td>
<td>0.000</td>
<td>0.120</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Day 8</strong></td>
<td>0.122</td>
<td>0.000</td>
<td>0.000</td>
<td>0.008</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.207</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Day 12</strong></td>
<td>0.113</td>
<td>0.000</td>
<td>0.000</td>
<td>0.071</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.756</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Day 15</strong></td>
<td>0.090</td>
<td>0.000</td>
<td>0.000</td>
<td>0.081</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.874</td>
<td>0.000</td>
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<tr>
<td><strong>Day 19</strong></td>
<td>0.215</td>
<td>0.000</td>
<td>0.000</td>
<td>0.352</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.756</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Day 22</strong></td>
<td>0.342</td>
<td>0.000</td>
<td>0.000</td>
<td>0.997</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.325</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Day 26</strong></td>
<td>0.195</td>
<td>0.000</td>
<td>0.000</td>
<td>0.906</td>
<td>0.000</td>
<td>0.000</td>
<td>0.012</td>
<td>0.000</td>
<td>0.158</td>
<td>0.000</td>
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<tr>
<td><strong>Day 29</strong></td>
<td>0.051</td>
<td>0.000</td>
<td>0.000</td>
<td>0.903</td>
<td>0.000</td>
<td>0.000</td>
<td>0.681</td>
<td>0.003</td>
<td>0.043</td>
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<table>
<thead>
<tr>
<th>Two Groups Being Compared</th>
<th>CD</th>
<th>CE</th>
<th>CF</th>
<th>CG</th>
<th>DE</th>
<th>DF</th>
<th>DG</th>
<th>EF</th>
<th>EG</th>
<th>FG</th>
</tr>
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<tbody>
<tr>
<td><strong>Day 1</strong></td>
<td>0.017</td>
<td>0.124</td>
<td>0.074</td>
<td>0.065</td>
<td>0.606</td>
<td>0.561</td>
<td>0.703</td>
<td>0.995</td>
<td>0.878</td>
<td>0.868</td>
</tr>
<tr>
<td><strong>Day 5</strong></td>
<td>0.000</td>
<td>0.772</td>
<td>0.006</td>
<td>0.000</td>
<td>0.000</td>
<td>0.004</td>
<td>0.293</td>
<td>0.552</td>
<td>0.039</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Day 8</strong></td>
<td>0.000</td>
<td>0.018</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.299</td>
<td>0.066</td>
<td>0.000</td>
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</tr>
<tr>
<td><strong>Day 12</strong></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.279</td>
<td>0.008</td>
<td>0.000</td>
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</tr>
<tr>
<td><strong>Day 15</strong></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.177</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Day 19</strong></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.225</td>
<td>0.000</td>
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</tr>
<tr>
<td><strong>Day 22</strong></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.497</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
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APPENDIX II

FLOW CYTOMETRY RAW DATA

All data presented here are quadrant and histogram readouts from CellQuest software used to analyze output from the Beckton Dickson FACSCalibur flow cytometer. Sample ID’s represent the size or treatment group. Each data set begins with ‘Unstained’, ‘PI only’, and ‘AnnexinV only’. These represent initial calibration samples that were either given no stain, or stained only with PI or Annexin V respectively. All other samples were stained with both dyes and histograms are given for both separately. All treatment samples were labeled by a letter: control (A), AlPcS2a dark control (B), 0.1 µg/mL bleomycin (C), 0.25 µg/mL bleomycin (D), 1.5 J/cm² PDT with 1 µg/mL AlPcS2a (E), PCI with 1.5 J/cm² PDT and 0.1 µg/mL bleomycin (F), PCI with 1.5 J/cm² PDT and 0.25 µg/mL (G), PCI with 2.0 J/cm² PDT and 0.25 µg/mL bleomycin (H), PDT with 2.0 J/cm² (I).
Sample ID: unstained
Acquisition Date: 25-Mar-09
Quad Events % Total
UL 833 8.33
UR 151 1.51
LL 9007 90.07
LR 9 0.09

Sample ID: PI only
Acquisition Date: 25-Mar-09
Quad Events % Total
UL 314 3.14
UR 61 0.61
LL 9599 95.99
LR 26 0.26

Sample ID: AnnexinV only
Acquisition Date: 25-Mar-09
Quad Events % Total
UL 241 2.41
UR 103 1.03
LL 8020 80.20
LR 1636 16.36
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**Acquisition Date:** 25-Mar-09

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M1 9496 94.96
M2 515 5.15

Sample ID: B
Acquisition Date: 25-Mar-09
Marker Events % Total
All 10000
M1 8877 88.77
M2 1133 11.33

Sample ID: C
Acquisition Date: 25-Mar-09
Marker Events % Total
All 10000
M1 8887 88.87
M2 1123 11.23

Sample ID: D
Acquisition Date: 25-Mar-09
Marker Events % Total
All 10000
M1 8892 88.92
M2 1127 11.27

Sample ID: A
Acquisition Date: 25-Mar-09
Quad Events % Total
UL 867
UR 213
LL 7508
LR 1412
8.67
2.13
75.08
14.12

Sample ID: B
Acquisition Date: 25-Mar-09
Quad Events % Total
UL 8877
UR 1133
LL 7966
LR 679
88.77
11.33
79.66
6.79

Sample ID: C
Acquisition Date: 25-Mar-09
Quad Events % Total
UL 7960
UR 679
LL 1133
LR 8877
79.66
6.79
11.33
88.77

Sample ID: D
Acquisition Date: 25-Mar-09
Quad Events % Total
UL 8892
UR 1127
LL 7986
LR 679
88.92
11.27
79.69
6.79

Sample ID: A
Acquisition Date: 25-Mar-09
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M1 9486
M2 515
94.86
5.15

Sample ID: B
Acquisition Date: 25-Mar-09
All 10000
M1 8877
M2 1133
88.77
11.33

Sample ID: C
Acquisition Date: 25-Mar-09
All 10000
M1 8887
M2 1123
88.87
11.23

Sample ID: D
Acquisition Date: 25-Mar-09
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M2 1127
88.92
11.27
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LL 8999 89.99
LR 711 7.11

Sample ID: E
Acquisition Date: 08-Apr-09
Quad Events % Total
UL 314 3.14
UR 413 4.13
LL 8709 87.09
LR 564 5.64

Sample ID: F
Acquisition Date: 08-Apr-09
Quad Events % Total
UL 316 3.16
UR 560 5.60
LL 7717 77.17
LR 1407 14.07
REFERENCES


VITA

Graduate College
University of Nevada, Las Vegas

Joseph William Blickenstaff III

Local Address:
1800 N. Green Valley Pkwy Apt. 1324
Henderson, NV 89074

Home Address:
2026 Herbert Ave.
Westminster, MD 21157

Degrees:
Bachelor of Science, Physics, 2003
Carnegie Mellon University

Publications:

Thesis Title: Photochemical Delivery of Bleomycin in Malignant Glioma Cells

Thesis Examination Committee:
Chairperson, Dr. Steen J. Madsen, Ph. D.
Committee Member, Dr. Phillip W. Patton, Ph. D., CHP
Committee Member, Dr. Ralf Sudowe, Ph. D.
Graduate Faculty Representative, Dr. Mack D. Rubley, Ph. D., ATC