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The effect of 5-aminolevulinic acid based photodynamic therapy and photochemical internalization of bleomycin on the F98 rat glioma cell line

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THE EFFECT OF 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC
THERAPY AND PHOTOCHEMICAL INTERNALIZATION OF BLEOMYCIN ON
THE F98 RAT GLIOMA CELL LINE

by

Khishigzaya Kharkhuu

Bachelor of Science
Moscow Power Engineering Institute
2003

A thesis submitted in partial fulfillment
of the requirement for the

Master of Science Degree in Health Physics
Department of Health Physics
School of Allied Health Sciences
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Graduate College
University of Nevada, Las Vegas
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The Effect of 5-Aminolevulinic Acid Based Photodynamic Therapy and
Photochemical Internalization of Bleomycin on the F98 Rat Glioma Cell
Line

is approved in partial fulfillment of the requirements for the degree of

Master of Science in Health Physics

Examination Committee Chair

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Graduate College Faculty Representative

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ABSTRACT

The Effect of 5-Aminolevulinic Acid Based Photodynamic Therapy and Photochemical Internalization of Bleomycin on the F98 rat glioma cell line

by

Khishigzaya Kharkhuu

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The F98 rat glioma cell line, a model for human malignant glioma, was used to investigate the efficacy of 5-aminolevulinic acid (ALA)-mediated photodynamic therapy (PDT) under varying light irradiation conditions. Cells in monolayer were pre-incubated in ALA for 4 hours prior to 635 nm light exposure. Using clonogenic survival assays, it was found that F98 glioma cells were sensitive to ALA-PDT: survival was inversely proportional to light fluence. As evidenced from the LD₅₀ (4 J cm⁻² at a fluence rate of 35 mW cm⁻²), ALA-PDT was found to be more effective in F98 cells compared to that found in another commonly used rat glioma cell line (BT₄C). In contrast to findings in BT₄C cells, F98 survival was independent of fluence rate.

ALA-PDT was tested for its ability to enhance the cytotoxicity of Bleomycin (BLM), a procedure termed photochemical internalization (PCI). F98 cells were incubated in ALA and BLM prior to illumination. In all cases, no difference in survival was observed

between F98 cells subjected to ALA-PCI delivery of BLM and cells treated with ALA-PDT. These findings suggest that ALA is not an ideal compound for PCI applications.

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CHAPTER 1

INTRODUCTION

1.1 Glioblastoma Multiforme

Glioblastoma multiforme (GBM) is a high grade glioma and is one of the most highly malignant, aggressive and difficult to treat tumors. It is the most frequent primary brain tumor in adults accounting for approximately 1 % of all cancer deaths (Madsen and Hirschberg 2006). The prognosis of patients with these tumors is poor, and traditional therapies, such as surgical tumor resection followed by external beam radiation and/or chemotherapy, have done little to alter the fatal outcome of this disease. No significant advancements in the treatment of GBM have occurred in the past three decades. Although current therapies remain palliative, they have been shown to increase progression-free survival. From the time of diagnosis, the mean survival time is approximately 17 weeks without treatment (Rao 2003). Mean survival is inversely correlated with age. Patients treated with optimal therapy, including surgical resection, radiation therapy, and chemotherapy, have a median survival of approximately 9 to 12 months, with 2-year survival rates in the range of only 8 to 12% (Stupp et al. 2002). The 5-year survival rate of the disease has remained unchanged over the past 30 years, and stands at less than 5 % (McLendon and Halperin 2003).

GBMs present with an irregular, contrast enhancement pattern on Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) scans. They tend to have a hemorrhagic component and are characterized histologically by the presence of vascular endothelial proliferation and wide areas of necrosis. Consistent with these areas of necrosis from astrocyte cell death, is significant disruption of the blood-brain barrier (Rao 2003).

Treatment failure is due to the presence of subpopulations of highly invasive cells that disseminate into the surrounding brain parenchyma and result in tumor recurrence at the edges of the resection cavity and/or at distant sites in the brain (Loustau et al. 2004).

Surgery and radiotherapy are the mainstay of GBM treatments. Surgery is an important first step in the treatment of GBM. However, it is extremely difficult to remove all tumor tissue without causing severe damage to normal brain. Surgical studies have concluded that complete resection of the tumor is observed in less than 20 % of patients, and is primarily due to difficulties involving indistinct tumor boundaries (Stummer et al. 2006). Conventional radiotherapy can prolong survival, but it cannot cure GBM - the tolerance of healthy brain being lower than the dose required for tumor control. Chemotherapy is also not very effective, probably because most cytotoxic agents cannot penetrate the blood-brain barrier in sufficient amounts. All-in-all, there is an urgent need to find new therapies for this extremely aggressive disease. In particular, much effort has been directed towards the development of localized therapies with the goal of eradicating infiltrating glioma cells in the resection margin.

1.2 Photodynamic Therapy

Photodynamic therapy (PDT) is an emerging modality for the treatment of neoplastic and non-neoplastic diseases. It is a local form of treatment that involves the administration of a tumor-localizing photosensitizing drug followed by photoactivation. This therapy results in a series of photochemical and photobiological events that cause irreversible photo-damage to tumor tissues. It is based on the concept that certain photosensitizers preferentially accumulate in tumor tissue followed by activation of appropriate wavelengths of light to generate active molecular species, such as free radicals (especially singlet molecular oxygen) that are toxic to cells. Because of the tumor-specific photosensitizer accumulation and the carefully directed light application, PDT represents a more selective method of tumor treatment than chemo- or radiation therapies.

The basic principles of PDT are illustrated in Fig. 1. Upon absorption of light, a photosensitizer molecule is excited to a short-lived singlet state. Following excitation, the sensitizer loses its energy by a variety of competing pathways. From the singlet state the sensitizer can readily decay back to its ground state and emit energy in the form of fluorescence. Alternatively, it can convert, by intersystem crossing, to the more stable triplet excited state. In this metastable triplet state, the sensitizer can decay to its ground state by emission of longer lived phosphorescence or, because of its relatively long lifetime (100 μ s), it can interact with the local environment, i.e. tumor tissue, via a type I and or type II reaction mechanism that initiates biochemical reactions (Ochsner 1997).

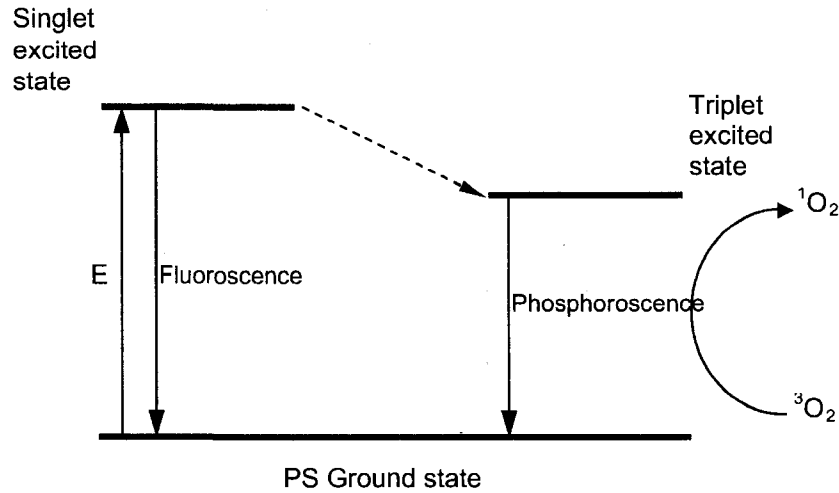


Fig. 1. Photochemical reactions of the excited photosensitizer (PS)

Type I photochemistry involves direct electron transfer between the triplet sensitizer and a nearby substrate to create radicals that further react with oxygen. This reaction will result in peroxy radicals which will undergo typical radical chain reactions (Calzavara-Pinton 2007). Type II photochemistry involves energy transfer from the excited triplet state of the sensitizer to ground state molecular oxygen resulting in the production of singlet oxygen – a highly reactive form of oxygen. Singlet oxygen is a very powerful oxidizing agent that can oxidize many different biomolecules resulting in damage to a variety of cellular structures. Due to its extremely short lifetime (less than $0.04 \mu\text{s}$ in biological tissue) and, accordingly, a short range of action (10–20 nm) inside the cell, the activity of singlet oxygen is restricted to its point of production making the photodamage highly localized (Dolmans et al. 2003).

The photophysics of PDT dictate that it is a light and oxygen catalyzed process with no toxicity in the absence of oxygen, and photosensitizing drugs are inactive until

illuminated. In summary, PDT efficacy depends on the availability of photosensitizer, light and oxygen.

The importance of tissue oxygenation in PDT has been demonstrated by several researchers (See et al. 1984; Henderson et al. 1987). The level of tissue oxygenation must be adequate to sustain singlet oxygen formation. Hypoxic cells, those with less than 5 % oxygenation, have been found to be resistant to PDT (Macdonald and Dougherty 2001). Henderson et al. (1987) have reported that oxygen consumption depends on the fluence rate of light, and that tissue destruction might be enhanced by using lower fluence rates. Lower fluence rates do not deplete the tissue's oxygen supply as rapidly and, as a result, the tissue is exposed to greater concentrations of singlet oxygen for a longer time during treatment.

While an ample oxygen supply is necessary during PDT treatment, eventual destruction of the tumor microvasculature is also crucial to starve tumor cells of oxygen and nutrients since gliomas are known to progress by inducing angiogenesis. Sitnik et al. (1998) have suggested that fluence rates can be judiciously chosen to maximize exposure to singlet oxygen during treatment, while the total fluence can be adjusted to ensure that the tumor microvasculature eventually collapses, starving the tumor of oxygen and nutrients.

The optimal light fluence used to activate a photosensitizer in human cancers is unknown. Light fluence is expressed in J cm^{-2} , but the PDT dose depends on the spectrum of the light source, irradiation geometry, depth of penetration, light scattering in the tissue, concentration of the photosensitizer in the tissue, hemorrhage within the tumor,

ambient oxygen concentration and other factors. This makes the PDT dose difficult to calculate (Webber et al. 2000).

The most commonly used photosensitizers for brain tumor therapy are Photofrin® and hematoporphyrin derivative. However, these sensitizers have the disadvantage of causing prolonged skin sensitization. Perhaps more importantly, damage to normal brain tissue has been reported in preclinical studies (Ito et al. 2004). There are a number of second-generation photosensitizers and prodrugs under preclinical development. For example, 5-aminolevulinic acid (ALA) has been successfully used to diagnose and treat neoplastic tissue (Tsai et al. 2004). ALA itself is not a photosensitizer and serves as the naturally occurring biological precursor for protoporphyrin IX (PpIX), a photosensitizer, in heme biosynthesis (Fig. 2). Heme synthesis is regulated by negative feedback control on ALA production. When ALA is administered exogenously, this inhibitory control can be bypassed and enzyme ferrochelatase (an enzyme that incorporates iron into PpIX in heme synthesis) activity becomes rate-limiting enabling the accumulation of PpIX in cells which renders them photosensitive (Solban et al. 2006).

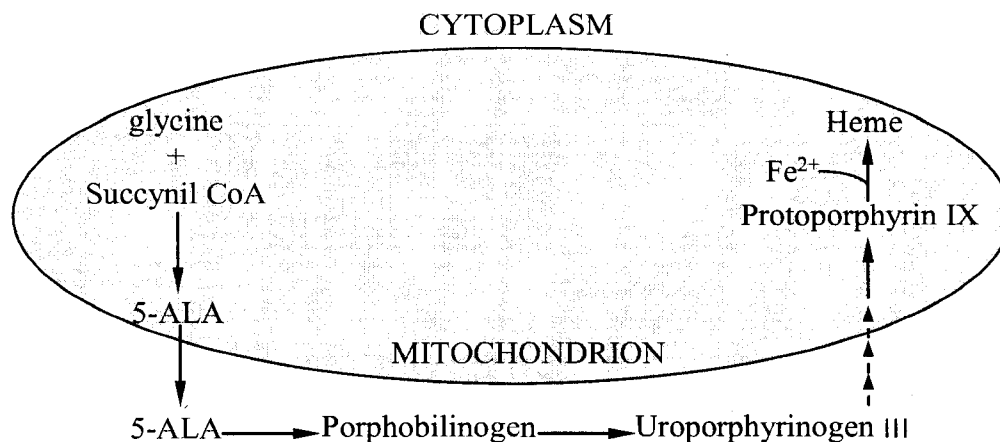


Fig. 2. Heme biosynthetic pathway

ALA-induced PpIX has been shown to selectively accumulate in a number of neoplastic tissues, which is probably due to altered activity levels of the enzymes of the heme biosynthetic pathway within transformed cells (Leunig et al. 2000). For example, uroporphyrinogen has been reported to be higher in some tumors, whereas ferrochelatase is often lower (Peng et al. 1997). The preferential accumulation of ALA in tumors has obvious clinical relevance since it results in selective destruction of neoplastic tissues.

Natural PpIX from ALA is formed within the mitochondria of target cells. Mitochondria occupy 15–50% of the total cytoplasmic volume of most animal cells, and participate in more metabolic functions than any other organelle (Chakrabarti et al. 1998): their proper function is essential to maintain normal cellular metabolism. Mitochondria have been implicated as an important target for PDT and their damage has been shown to directly correlate with cell death, in particular, apoptosis (Dougherty et al. 1998).

The fluorescence-guided resection of glioblastoma multiforme by ALA -induced PpIX has been investigated in a number of clinical studies and the results suggest a role for PDT as a therapeutic modality (Stummer et al. 2000). ALA-induced PpIX has several

light absorption maxima, from the largest peak in the Soret band at about 405 nm, to smaller peaks in the Q-bands at approximately 510, 545, 580 and 630 nm. From a clinical viewpoint, photosensitizers with absorption spectra in the 650 – 800 nm regions are preferred since they allow deeper light penetration in tissue (Calzavara-Pinton et al. 2006).

A number of studies examining the utility of ALA in various brain tumor models have been performed in the past several years (Friesen et al. 2002). The combination of excellent tumor-to-normal brain tissue localization (Angell-Petersen et al. 2006; Lilge et al. 1998) short period of skin photosensitization (24–48 h) (Kennedy and Pottier 1992) and the possibility of oral administration makes ALA a promising photosensitizer for use in fractionated or repeated PDT treatments of GBM patients.

1.3 Photochemical Internalization

Photochemical internalization (PCI) is a novel technology which may improve the therapeutic effect of macromolecules and other compounds that accumulate in endocytic vesicles (Berg et al. 2005). Molecules trapped in endocytic vesicles are inactive. PCI is based on the photodynamic effect which is used to rupture the vesicular membranes of endosomes so that endocytosed intact macromolecules can be released into the cytosol where they may exert their therapeutic effects instead of being degraded by lysosomal hydrolases. The concept (illustrated in Fig. 3) is based on the use of specially designed photosensitizers, which localize preferentially in the membranes of endocytic vesicles. Upon exposure to light of appropriate wavelengths (usually red light), the photosensitizer induces the formation of reactive oxygen species (e.g. singlet oxygen) which cause

photooxidation of the endocytic membranes and subsequent release of encapsulated macromolecules. The PCI-based relocation and activation of the macromolecules has the advantage of minimal side effects since the effect is localized to the area exposed to light. The endosomal escape of macromolecules including genes, oligonucleotides and proteins by means of PCI has been documented both *in vitro* and *in vivo* and has been shown to increase the therapeutic effect in a synergistic manner (Berg et al. 2005).

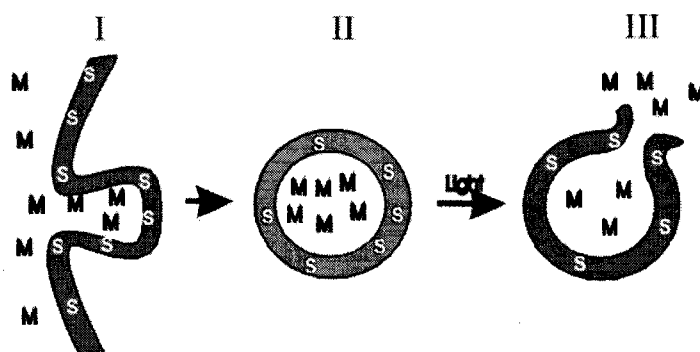


Fig. 3. Transport of molecules to the cytosol by PCI. The photosensitizer (S) and the selected molecule (M) are endocytosed by the cells (I, illustrates the invagination of the plasma membrane) and both compounds end up in the same vesicles (II). When these vesicles are exposed to light, the membranes of these vesicles will be ruptured and the contents released (III) (Berg et al., 2005).

Photosensitizers used in PCI should fulfill certain criteria, perhaps most importantly, they should localize in the endocytic compartments (Høgset et al. 2003). According to Gaullier et al. (1995) PpIX was found to sublocalize to lysosomal compartments in human keratinocytes after long term incubation (42h) with ALA. Thus, ALA-PDT may have the potential to damage endocytic vesicles releasing their contents under certain conditions (Selbo et al. 2001). ALA-based PCI of the immunotoxin MOC31-gelonin has been shown to cause significant cytotoxic effects in WiDr cells (a colon adenocarcinoma

cell line) (Selbo et al. 2001). This is an important observation and provides the rationale for the proposed work in rat glioma cell lines – the PCI effect has not been investigated in brain tumor cell lines. Of particular interest is the ability of ALA-based PCI to enhance the effects of chemotherapeutic agents such as bleomycin (BLM) for the treatment of malignant gliomas. This is an important consideration since chemotherapeutic agents have been relatively ineffective in the treatment of brain tumors.

1.4 Bleomycin

Bleomycin (BLM) is a water soluble glycopeptidic antibiotic used in anticancer chemotherapy and is given as a treatment for several types of cancers including head-and-neck, malignant lymphomas and testicular carcinomas. BLM possesses very high intrinsic cytotoxicity and acts by causing DNA strand breaks. Indeed, as few as 500 BLM molecules introduced into the cytosol are sufficient to kill many cell types (Pron et al. 1999). However, it is known that the BLM effect is limited by its inability to cross the plasma membrane due to its relatively large size (molecular weight of 1.5 kDa) and hydrophilic characteristic. The transport mechanism of BLM into cells has been the focus of several studies. Using radiolabeled BLM, Pron et al. (1993) found an association between receptor proteins on the plasma membrane and the uptake of BLM, suggesting it is taken up by cells through a receptor-mediated endocytotic mechanism. The observation that BLM appears incapable of escaping from endocytic vesicles explains the development of a variety of techniques to permeabilize the barrier membrane, including well known electroporation techniques. Utilizing specifically localized photosensitizers and light to rupture vesicle membranes is a promising approach for exploiting the

therapeutic potential of BLM. In vitro PCI studies with BLM have shown that photochemical treatment can substantially enhance the biological effect of this agent in WiDr colon adenocarcinoma and V79 lung fibroblast cell lines. (Høgset et al. 2003; Berg et al. 2005).

BLM is known as a cell cycle specific chemotherapeutic agent as it mainly affects dividing cells. The fact that healthy neurons and normal glial cells do not divide, or possess extremely low division rates makes BLM advantageous in clinical use of brain cancer therapy since cancer cells are characterized by its high cell division (Leif et al. 1993). The primary motivation for using this chemotherapeutic agent is that it has been used in a number of PCI studies which can serve as benchmarks against which the results of the present work can be compared.

1.5 The F98 Rat Glioma Cell Line

The F98 rat glioma cell line used in this study was originally established by Drs. A. Koestner and W. Wechsler at Ohio State University. It is chemically induced in CDF (Fischer) rats by the i.v. administration of a single dose of N-ethyl-N-nitrosourea (ENU, 50mg kg⁻¹ b.w.) on the 20th day of gestation (Barth 1998). Nitrosourea-induced malignant cell lines reliably produce rapidly growing tumors. The techniques involved are relatively simple, cheap and in vitro, in vivo studies can be carried out in parallel (Whittle et al. 1998). The biological characteristics of this tumor closely resemble those of human glioblastoma. The transplantable tumor displays an infiltrative pattern of growth within the brain and is weakly immunogenic in syngeneic rats (Barth 1998). Knowledge of the immunogenicity of a tumor model is an important factor for

determining its appropriateness in therapeutic studies where survival is the end point. For example, certain immunogenic glioma cell lines, like C6 and 9L are not suitable for studies in immunocompetent rats if the objective is to prolong and register animal survival (Fournier et al. 2003). F98 cells have been found to be resistant to a variety of treatment modalities including chemo-, radio-, and immunotherapy and, to this date, only 25% of tumor-bearing animals have been cured using these standard therapeutic regimens (Barth et al. 2003). The failure of cancer treatments for human gliomas is mainly attributed to its highly invasive nature. F98 cells are highly invasive and, as such, are highly suitable for studying human glioma resistance (Sibenaller et al. 2005).

1.6 Thesis Objectives

The overall objective of this thesis is to determine the efficacy of ALA-mediated PDT and PCI in an F98 rat glioma cell line. The work consists of three specific aims. ALA dark toxicity and PpIX fluorescence yields will be investigated in specific aim #1. It is hypothesized that ALA toxicity and PpIX fluorescence yields in F98 cells are similar to those observed in other rat glioma cell lines.

In specific aim #2, the effects of light fluence and fluence rate in the rat glioma cell line will be determined. There are numerous studies suggesting that low fluence rates result in more damage than high fluence rates for the same total fluence. It is very important to elucidate the different fluence rate effects in photodynamic therapy for effective treatment planning. As has been found in other rat glioma cell lines, it is hypothesized that higher light fluences and lower fluence rates are more effective at killing glioma cells.

In specific aim #3, the ability of ALA-PCI to enhance the effects of BLM in the F98 rat glioma cell line will be investigated. It is hypothesized that ALA-PCI with BLM will have a greater cytotoxic effect than that achievable with ALA-PDT or BLM alone.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Dulbecco's modified Eagle medium (DMEM), Phosphate Buffered Saline (PBS), and trypsin-EDTA were purchased from Invitrogen Corp., Carlsbad, CA. 5-Aminolaevulinic acid (5-ALA) and bleomycin in powder form (15 IU per vial) were purchased from Sigma (St.Louis, MO).

2.2 Cell Culture

The F98 cells used in all experiments were provided by Dr. Chung-Ho Sun from the University of California, Irvine. Originally, the F98 cell line was established from gliomas induced in CDF (Fischer) rats by the i.v. administration of a single dose of N-ethyl-N-nitrosourea (ENU, 50 mg kg⁻¹ b.w.) on the 20th day of gestation. Cells were routinely cultured in monolayer in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 50 mM HEPES buffer (pH 7.4), penicillin (100 U ml⁻¹) and streptomycin (100 mg ml⁻¹). They were incubated in a humidified chamber with 5% CO₂ at 37°C. For routine maintenance, cells were subcultured at 80–90% confluence. Proliferating cells received fresh medium every third day. F98 cells were seeded in the medium at a density of about 150×10³ cells per 25 cm²

cell culture flasks, and incubated for 48 hours prior to each experiment. Prior to drug treatment and irradiation, cells were switched to a serum-free medium (0% FBS) since serum can act as an extracellular competitor for PpIX (Hanania et al. 1992).

2.3 PpIX Fluorescence

In one set of experiments, F98 cells were incubated in ALA and a serum-free medium for 4 h. PpIX fluorescence intensity was evaluated for ALA concentrations ranging from 0 to 4 mM using a FACS Calibur cytometer. In another set of experiments, cells were incubated in 1 mM ALA for times ranging from 1 to 24 h and PpIX fluorescence intensity was evaluated using the cytometer.

2.4 ALA Toxicity

F98 cells were incubated in the dark for 4 h with 1, 6, 12, 18 or 24 mM ALA in a serum-free medium. Exposure to light was avoided during incubation with ALA. Following incubation, cells were harvested, counted and prepared for colony growth as described in section 2.5.

2.5 Photodynamic Therapy

F98 cells were seeded in the medium at a density of about 150×10^3 cells per 25 cm^2 cell culture flasks. After 48 h, cells were washed with 5 ml PBS and 5 ml of serum-free DMEM was added. 100 mM ALA stock was prepared immediately before treatment with 100 mM HEPES buffer and pH adjusted to 7.3 - 7.6 using 10 M NaOH. 50 μl of the 100 mM ALA stock was added to the cells in 5 ml serum-free medium to a final

concentration of 1 mM and incubated for 4 h. The serum-free medium (without ALA) was also used for the control group. Cells were assigned into three groups: group 1, control – no ALA; group 2, dark control – ALA only; and group 3, ALA-PDT. After incubation, cells incubated with 1 mM ALA for 4 h (except the dark controls) were subjected to light fluences of 2, 4, 10 or 20 J cm⁻² delivered at fluence rates of 5, 25, or 40 mW cm⁻². All illuminations were performed using a 400 μm core diameter optical fiber (with microlens) coupled to a diode laser (High Power Devices, North Brunswick, NJ) emitting light at a wavelength of 635 nm. Power output at the tip of the fiber was checked with a power meter. The beam was expanded to a spot size of 37.5 mm radius in order to cover the monolayer cell culture homogeneously. Immediately after irradiation, cells were washed in PBS, harvested using trypsin, and resuspended in drug-free culture medium. Cells were counted using a Coulter Counter and plated at various densities in culture dishes and further incubated with the complete medium for colony formation. All experiments, in which cells were exposed to ALA, were carried out in subdued light. Cell survivals were evaluated using a colony formation assay. The clonogenic assay was performed to assess the proliferation ability after ALA-PDT.

2.6 Bleomycin Toxicity

BLM powder was dissolved in PBS and further diluted to achieve the desired concentrations. F98 cells were incubated in varying concentrations (0.1 – 10 μg ml⁻¹) of BLM and serum-free medium for 4 h. Following incubation, cells were washed, trypsinized, counted and prepared for colony growth as described in section 2.5.

2.7 ALA-Based PCI of Bleomycin

F98 cells were subjected to varying incubation times in ALA and BLM prior to illumination. In the initial set of experiments, F98 cells were coincubated with 1 mM ALA and 0.1 $\mu\text{g ml}^{-1}$ BLM in serum-free medium for 4 or 18 h. In later experiments F98 cells were first incubated in ALA for 18 h followed by 4 h incubation in 0.1 $\mu\text{g ml}^{-1}$ BLM. Following incubation, cells were irradiated with 635 nm light (fluence rate = 5 mWcm^{-2} , fluence = 2 J cm^{-2}). Subsequent to irradiation, cells were harvested and prepared for colony growth as described in section 2.5. In addition to the control (no ALA, no light), two dark control groups (ALA only, 4 and 18 h) and a BLM + light group were evaluated. All experiments were carried out in subdued light.

2.8 Colony Formation Assay

All F98 cultures were incubated in the dark for 7 – 9 days after the experiments until visible colonies were observed. Thereafter, cells were stained with crystal violet, and surviving colonies counted manually. Colonies containing more than 50 cells were counted. The number of colonies was expressed as a fraction of the dark control (ALA only).

Survival was determined by:

$$\frac{\text{number of colonies from treated cells/ number of treated cells seeded}}{\text{number of colonies from dark control cells/ number of dark control cells seeded}} \times 100$$

For all measurements, triplicate plates were used for each point and each experiment was carried out more than three times. In all cases, data are presented as mean \pm SD.

CHAPTER 3

RESULTS

3.1 PpIX Fluorescence and ALA Toxicity

The data in Fig. 4 show that PpIX fluorescence in F98 cells increases as a function of ALA incubation time. After 18 h, the fluorescence signal reached a plateau suggesting that the maximum PpIX accumulation had been attained in this cell line. As illustrated in Fig. 5, PpIX fluorescence reached a plateau at an ALA concentration of approximately 2 mM. ALA toxicity was investigated by evaluating the survival of F98 cells after incubation for 4 h with increasing concentrations of ALA (Fig. 6). At concentrations up to 12 mM no significant decrease in cell viability was observed. However, significant dark toxicity was seen in cells incubated at higher concentrations. Surviving fractions markedly diminished to about 24 % and 11 % at concentrations of 18 and 24 mM respectively.

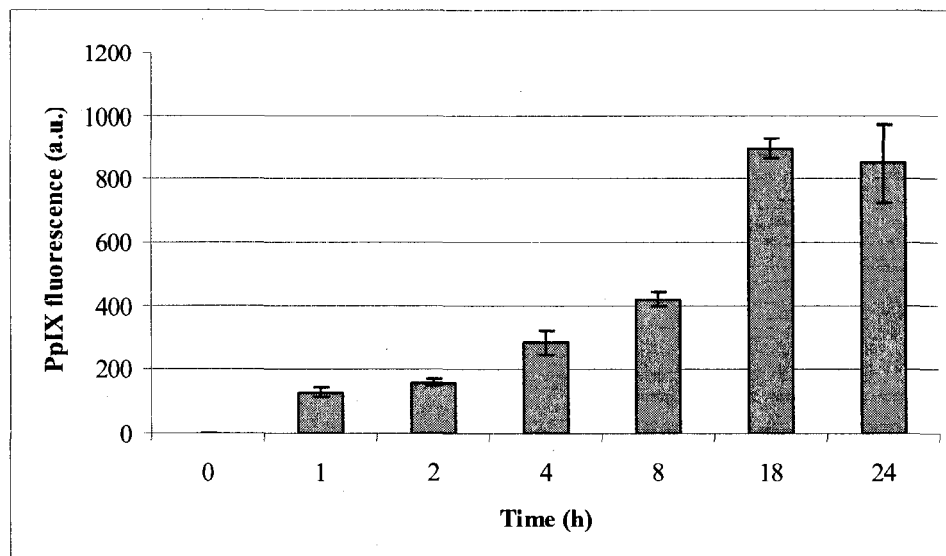


Fig. 4. Time dependent PpIX fluorescence formation in F98 cells. Cells were incubated in 1 mM ALA for different times. Each data point represents the mean (\pm SE) of 2 experimental trials.

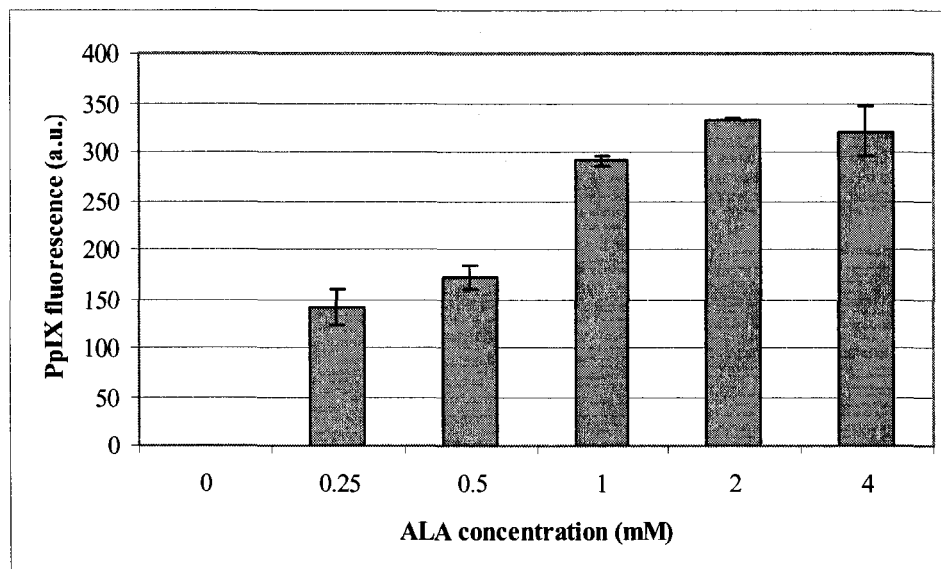


Fig. 5. Dose dependent PpIX fluorescence formation in F98 cells. Cells were incubated in different concentrations of ALA for 4 h. Each data point represents the mean (\pm SE) of 2 experimental trials.

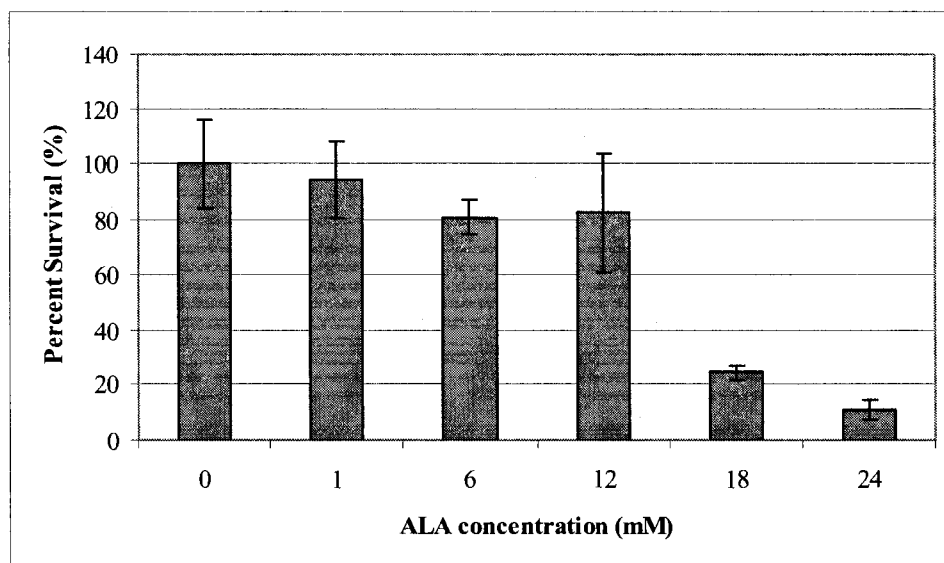


Fig. 6. F98 cell survival as a function of ALA concentration. In all cases, cells were incubated in ALA and serum free medium for 4 h. Each data point represents the mean (\pm SE) of more than 3 experimental trials.

3.2 Photodynamic Effects on F98 Cells

Fig. 7 illustrates the survival of F98 cells after 4 h incubation in 1 mM ALA and exposure to 635 nm light delivered at a range of fluences and fluence rates. In each case, cellular viability was estimated by normalizing the results of the colony formation assay to cells that were exposed to ALA-only (dark control). Control experiments were performed to insure the reduction in cell viability during PDT was caused solely by photosensitized PDT reactions. A fluence-dependent decrease in cell viability was clearly observed at all three light fluence rates investigated. Neither sole incubation with 1 mM ALA, nor the laser light alone, had a significant effect on cell viability. Significant reduction of cell viability, relative to dark control, was observed when irradiating with 4, 10, or 20 J cm⁻² at all fluence rates investigated. At 2 J cm⁻², however, low or no significant phototoxicity was observed when compared with dark control and the mean percent survivals were 95.7 \pm 8.2%, 86.8 \pm 12.1% and 94.4 \pm 13.2% for fluence rates of

5, 25 and 40 mW cm⁻² respectively. At 4 J cm⁻², the cell killing reached approximately half the value of non-irradiated controls (53.2 ± 8.3%, 49.3 ± 15.7%, and 42 ± 6.46% for fluence rates of 5, 25 and 40 mW cm⁻² respectively). After exposure to 10 J cm⁻² cell survival decreased to 23.4 ± 4.9%, 18.8 ± 3.1% and 29.7 ± 9% for fluence rates of 5, 25 and 40 mW cm⁻² respectively. Exposures to 20 J cm⁻² resulted in close to complete cell inactivation at all fluence rates investigated (5.7 ± 1.2%, 1.8 ± 0.3% and 6.4 ± 0.6% for fluence rates of 5, 25 and 40 mW cm⁻² respectively). As illustrated in Fig. 7, there do not appear to be significant differences in survival for the three fluence rates investigated.

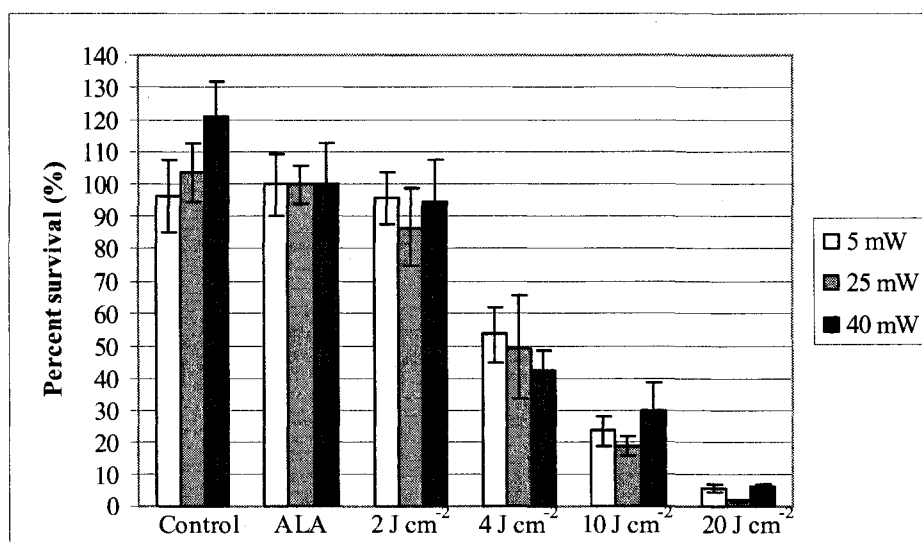


Fig. 7. Fluence and fluence rate effect of ALA-PDT in F98 cells. Cells were incubated with 1 mM ALA for 4 h prior to treatment. Each data point represents the mean (±SE) of more than 3 trials.

3.3 Bleomycin Toxicity

The BLM dose response curve was generated for the F98 cell line following 4 h treatment with increasing concentrations of BLM (Fig. 8). Initial toxicity evaluation was carried out for BLM concentrations ranging from 1 to 60 µg ml⁻¹. For this range, colony

counts rapidly diminished. At concentrations above $10 \mu\text{g ml}^{-1}$ the toxicity was so high that the percent survival became negligible. The results presented in Fig. 8 show that F98 glioma cells are very sensitive to BLM– the LD_{50} is approximately $0.25 \mu\text{g ml}^{-1}$.

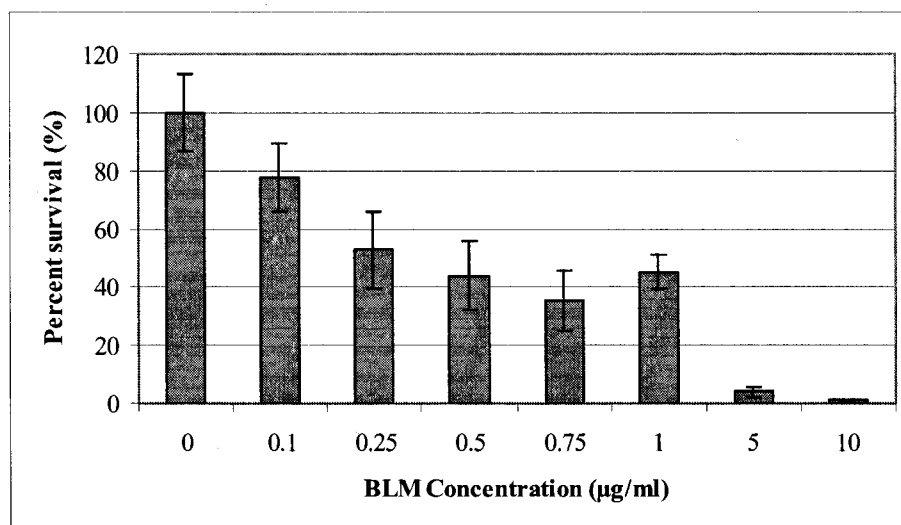


Fig. 8. F98 cell survival as a function of BLM concentration. In all cases, cells were incubated in BLM and serum free media for 4 h. Each data point represents the mean ($\pm\text{SE}$) of more than 3 experimental trials.

3.4 Photochemical Internalization

The effects of ALA-PCI are illustrated in Figs. 9 and 10. In the case of ALA and BLM co-incubation (Fig. 9) the data show that there was no statistically significant difference (95% confidence interval) in survival between cells subjected to PDT and those treated with PCI for both incubation times investigated (4 and 18 h). Not surprisingly, PDT was more effective in the 18 hour ALA-incubated cells: $95 \pm 8 \%$ and $40 \pm 7 \%$ for PDT after 4 and 18 hour ALA incubation respectively. No difference in survival was observed between BLM-only cells and BLM-only cells exposed to light suggesting that bleomycin does not have a photosensitizing effect (data not shown). As

illustrated in Fig. 10, changing the incubation conditions (18 h ALA followed by 4 h BLM) did not result in a statistically significant difference in survival between the PDT and PCI groups.

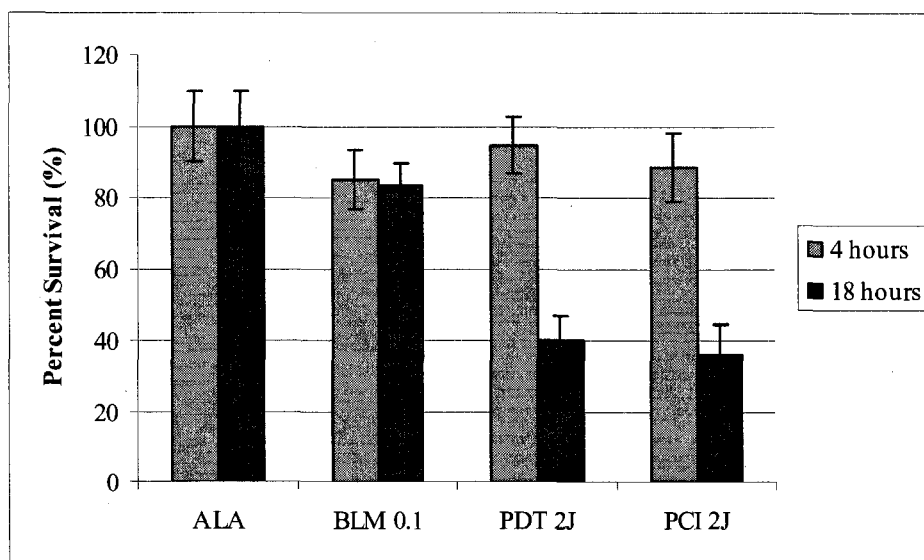


Fig. 9. ALA based PCI of BLM in F98 cells after 4 and 18 h co-incubation with 1 mM ALA and $0.1 \mu\text{g ml}^{-1}$ BLM prior to light exposure (2 J cm^{-2}). Each data point represents the mean ($\pm\text{SE}$) of more than 3 trials.

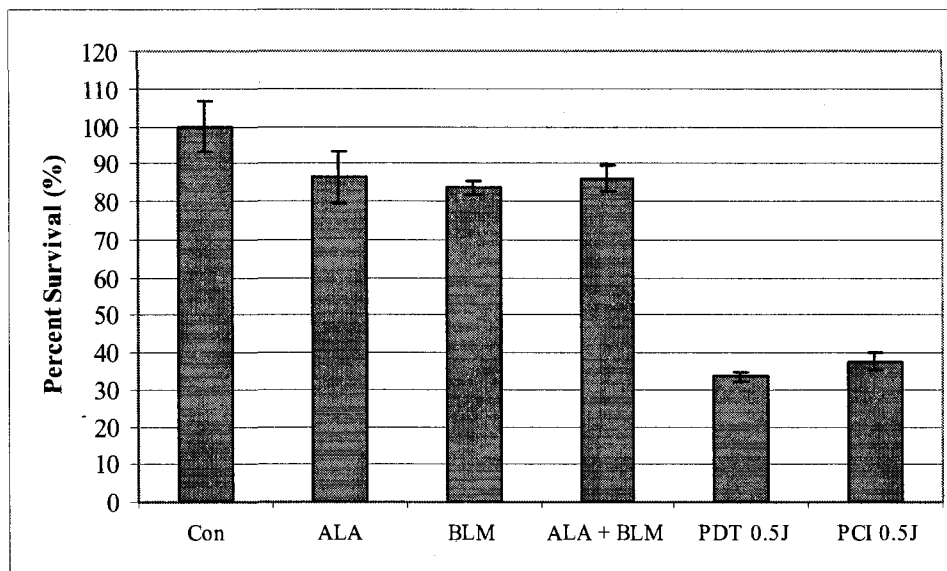


Fig. 10. ALA based PCI of BLM in F98 cells after 18 h – 1 mM ALA followed by 4 h – $0.1 \mu\text{g ml}^{-1}$ BLM incubation prior to light exposure (0.5 J cm^{-2}). Each data point represents the mean ($\pm\text{SE}$) of 2 trials.

CHAPTER 4

DISCUSSION

At present, glioblastoma multiforme is considered an incurable disease. Results have been poor with conventional treatments involving surgery followed by adjuvant radiotherapy and chemotherapy. Because of the failure to control local recurrences with conventional therapies, other methods are currently being developed (Sandeman 1986). PDT is an appealing treatment option for GBM patients due to its selective ability to target brain tumor cells. First generation photosensitizers, such as hematoporphyrin derivatives, are not ideally suited for PDT due to lack of tumor selectivity and complications associated with cutaneous photosensitivity (Dougherty et al. 1990). Thus, new photosensitizers and prodrugs (including ALA) are under active investigation to evaluate their efficacy for PDT of malignant gliomas. ALA is a naturally occurring metabolite in the heme synthetic pathway and a precursor of PpIX, a potent photosensitizer. ALA has been shown to have fewer side effects and a much more transient phototoxicity compared to first generation compounds (Rick et al. 1997).

In the present study, the utility of ALA was assessed in the PDT treatment of weakly immunogenic F98 glioma cells in vitro. Since some cells are not readily photosensitized when exposed to exogenous ALA, it is advisable to perform in vitro studies in tumor cell lines prior to the initiation of animal studies (Betz et al. 2002). Whetsell et al. (1978) and

Percy et al. (1981) have shown that neurons, glial cells and Schwann cells in culture synthesize porphyrins from ALA.

The results illustrated in Figs. 4 and 5 show that F98 glioma cells are readily capable of synthesizing PpIX following ALA exposure. The trend of increasing PpIX fluorescence intensity as a function of ALA incubation time (Fig. 4) has also been observed in other cell lines (Gamarra et al. 2002) including the BT₄C rat glioma line (Ziegler 2007). In F98 cells, a fluorescence plateau was reached after approximately 18 h of ALA incubation indicative of feedback inhibition of porphyrin synthesis (Stummer et al. 1998). The results illustrated in Fig. 5 show that PpIX fluorescence saturates at an ALA concentration of approximately 2 mM which is in good agreement with the findings of Ziegler (2007) in a BT₄C rat glioma cell line. The observation of a plateau is of particular importance, because it predicts that any given increase in ALA dose will not inevitably increase the availability of ALA-induced porphyrins. On the other hand, unwanted side-effects related to ALA may be more liable to occur if ALA doses in patients are increased capriciously (Stummer et al., 1998).

It has been shown that ALA can induce cytotoxicity in the absence of light – the so-called dark toxicity which is due to the generation of reactive oxygen species via ALA oxidation (Xing et al. 2001). In the F98 cell line, a significant reduction in survival was observed at ALA concentrations exceeding 12 mM (Fig. 6). Based on this data, the LD₅₀ would appear to be in the range of 12 to 18 mM. These results are in general agreement with the findings in BT₄C rat glioma cells where the LD₅₀ was estimated at 24 mM (Madsen et al. 2006). The results are also consistent with the dark toxicity observed in a Waf human bladder carcinoma cell line which demonstrated no decrease in survival at

ALA concentrations less than 10 mM (Bachor et al. 1996). Thereafter, survival dropped abruptly to 15 % at a concentration of 15 mM.

Overall, the results presented in Figs. 4-6 provided guidance as to the optimal ALA concentration and incubation time to use for the PDT and PCI experiments. In all cases, a 1 mM ALA concentration and a 4 h incubation time were chosen for the PDT studies and the initial PCI investigations.

The F98 glioma cell line was used as a model system in this study since these cells form rat brain tumors that mimic the fundamental characteristics of human GBM including infiltrative growth and invasiveness. Furthermore, like human GBMs, F98-derived tumors have been shown to be refractory to a wide variety of treatment modalities including standard therapeutic regimens consisting of radiation and chemotherapeutic agents (Barth et al. 2003).

Recently, much effort has been devoted to determining the optimal light dosimetry parameters for PDT. To that end, the response of F98 glioma cells to various light fluences and irradiation conditions were investigated. Not surprisingly, a significant light fluence dependent PDT response was observed as measured by clonogenic survival: higher light doses resulted in reduced survival. As illustrated in Fig. 7, exposure to light doses of 20 J cm^{-2} resulted in less than 10 % survival whereas 2 J cm^{-2} had little impact on cell viability. The results suggest that the F98 cell line is slightly more sensitive to ALA-PDT compared to BT₄C cells. For example, at a fluence rate of 35 mW cm^{-2} , the LD₅₀ for BT₄C and F98 cells occurred at light fluences of approximately 7 and 4 J cm^{-2} , respectively (Madsen et al. 2006). The reasons for this slight discrepancy in ALA-PDT

response among the two glioma cell lines are not known but may be due to differences in: (1) cell metabolism, (2) photosensitizer binding, and/or (3) organelle concentration.

From the results presented in Fig. 9 it is hardly surprising that phototoxicity increased significantly following 18 h incubation. For example, a light fluence of 2 J cm^{-2} resulted in surviving fractions of approximately 95 and 40 % following 4 and 18 h incubation, respectively. The increased PDT efficacy observed at longer incubation times may be attributed to a number of factors including an increase in cellular PpIX concentrations. In addition, several investigators have described the critical role of intracellular sensitizer localization and distribution in cellular phototoxicity and PDT efficacy. Longer exposure to ALA may result in combined targeting of several sites within the cell due to PpIX relocation from mitochondria to other organelles, including the plasma membrane (Lye Yee et al. 2002). Such PpIX relocation is also likely to cause a shift from an apoptotic to a necrotic mode of cell death (Morgan and Oseroff 2001).

As illustrated in Fig. 7, there was no significant difference in F98 survival as a function of fluence rate. There have been numerous *in vitro* and *in vivo* studies investigating the impact of fluence rate on PDT efficacy. In general, the decreased PDT efficacy observed at high fluence rates is attributed to rapid oxygen depletion and there is ample evidence to support this hypothesis in both multicellular 3-D spheroids (Madsen et al. 2000) and animals (Moor et al. 1997). During exposure to high fluence rates, oxygen depletion exceeds replenishment and, as a result, the PDT effect is limited only to cells in close proximity to the oxygen supply, i.e., cells in the outer spheroid layers or tumor cells in close proximity to the vasculature.

Results of fluence rate effects in monolayers have been mixed – some studies have found a fluence rate dependence while others have failed to find such a relationship. The lack of a fluence rate effect is hardly surprising since all cells in monolayers are well-oxygenated. In the present studies, it is hypothesized that oxygenation levels were sufficient to ensure survival of F98 cells under all fluence rates investigated. It is interesting to note that, in a similar study employing BT₄C rat glioma cells, a fluence rate effect was observed (Ziegler 2007): higher fluence rates resulted in increased survival. Differences in growth conditions and cell manipulation notwithstanding, the reasons for the discrepancy between the two cell lines is not understood at present and bears further investigation.

The biological activity of a number of chemotherapeutic agents is limited by their poor ability to enter the cytosol. BLM is a glycopeptidic antibiotic that binds to DNA causing single and double strand breaks. This agent has been used in a number of standard chemotherapeutic regimens for the treatment of head and neck, esophagus and testicular carcinomas (Berg et al. 2005). Unfortunately, the clinical efficacy of BLM is limited due to drug resistance and dose-dependent induction of pulmonary fibrosis (Jani et al. 1992). Improvement in BLM efficacy would allow for the delivery of lower doses resulting in a concomitant decrease in morbidity.

BLM enters the cytosol through the endocytic pathway. Poor escape from endocytic vesicles is a major barrier for delivery of the compound since it is unable to reach the cytosol where it can be transported to the nucleus. Instead, BLM remains trapped in the endocytic vesicles and finally degraded in lysosomes. Thus, the development of endosome-disruptive strategies, such as PCI, is of great importance since they may

enhance the clinical efficacy of BLM. The PCI technique, utilizing amphiphilic photosensitizers to enhance the activity of BLM, has been successfully documented by Berg et al. (2005) on two different carcinoma cell lines. The central question addressed in this study was whether BLM efficacy could be enhanced by ALA-PCI in a rat glioma cell line. The results presented in Figs. 9 and 10 suggest that ALA-PCI was not an effective delivery technique for the selected conditions investigated. In all cases, no significant differences in survival were observed between cells exposed to PDT and those subjected to PCI.

PCI efficacy is critically dependent on incubation time. In Fig. 9 two incubation times of 4 and 18 h were considered based on the work of Selbo et al. (2001) who investigated ALA-PCI efficacy in the WiDr – colon adenocarcinoma cell line. These investigators used fluorescence microscopy which showed significant ALA-induced PpIX translocation as a function of incubation time. Not surprisingly, at short incubation times (a few hours), PpIX fluorescence was confined primarily to the mitochondria. At approximately 4 h, a small increase in PpIX fluorescence intensity was noted in the plasma membrane. At longer incubation times (≥ 24 h) a sharp increase in fluorescence intensity was observed in the cytoplasm suggesting PpIX localization to extra mitochondrial sites and indicative of an endosome/lysosome association. Taken together, the fluorescence studies suggest that a PCI effect should be possible using a 4 h incubation time and that the effect is optimal after 24 h.

In both cases considered in Fig. 9, cells were co-incubated in ALA and BLM. This protocol can be problematic if BLM-forming vesicles detach from the plasma membrane prior to sufficient incorporation of PpIX. To minimize the risk of this occurrence, a series

of experiments were performed in which F98 cells were first incubated in ALA for 18 h, followed by 4 h BLM incubation. Unfortunately, the results illustrated in Fig.10, demonstrate an absence of the PCI effect under these incubation conditions. The lack of an observed PCI effect in the present work is likely due to limitations associated with the choice of pro-drug (ALA). Optimization of the PCI effect requires lipophilic sensitizers that localize in the plasma membrane. In that context, ALA would appear to be a sub-optimal choice since it is a hydrophilic molecule and its associated photosensitizer (PpIX) has strong mitochondrial affinity. It is also possible that the ALA-PpIX conversion kinetics in the F98 glioma cell line are different compared to those in other cell lines such as the WiDr line where ALA-PCI has been shown to have some effect (Selbo et al. 2001).

CHAPTER 5

CONCLUSION

PpIX fluorescence analysis in F98 rat glioma cells yielded results consistent with those observed in the BT₄C rat glioma cell line. Data from the present study showed that maximum PpIX fluorescence was achieved following 18 h ALA incubation. In addition, it was found that the PpIX fluorescence intensity saturated at a relatively low ALA concentration of 2 mM. This has significant clinical implications since it suggests that a therapeutic effect can be achieved at ALA concentrations well tolerated by patients. ALA dark toxicity was found to be similar to that observed in the BT₄C cell line: the LD₅₀ was estimated to be between 12 and 18 mM in F98 glioma cells.

The present study demonstrated that ALA-PDT was very effective against F98 glioma cells. As expected, higher light fluences resulted in reduced survival. The results suggest that the F98 cell line is more sensitive to ALA-PDT compared to BT₄C cells. The LD₅₀ for F98 cells exposed to 35 mW cm⁻² occurred at a light fluence of approximately 4 J cm⁻².

In contrast to the findings in BT₄C cells, a fluence rate effect was not observed in the F98 cell line. The reasons for this discrepancy are not understood and require further investigation in more realistic oxygen-limiting conditions such as those found in multicell 3-D spheroids and animal tumors.

ALA-PCI was not an effective enhancer of bleomycin efficacy in the F98 rat glioma cell line. The lack of an effect is likely due to the ineffectiveness of ALA in PCI applications. Future work will be aimed towards a better understanding of the PCI effect using more appropriate amphiphilic photosensitizers that preferentially localize in plasma membranes.

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Thesis Title: The Effect of 5-Aminolevulinic Acid Based Photodynamic Therapy and Photochemical Internalization of Bleomycin on the F98 rat glioma cell line

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