Published in final edited form as:

J Neurooncol. 2009 January ; 91(2): 141–149. doi:10.1007/s11060-008-9692-4.

Motexafin gadolinium enhances the efficacy of aminolevulinic acid mediated-photodynamic therapy in human glioma spheroids

Steen J. Madsen,

Department of Health Physics, University of Nevada, 4505, Maryland Pkwy, PO Box 453037, Las Vegas, NV 89154-3037, USA, steen.madsen@unlv.edu

Marlon S. Mathews,

Beckman Laser Institute and Medical Clinic, University of California, Irvine 92612, USA;
Department of Neurological Surgery, University of California, Irvine Medical Center, Orange, CA 92868, USA

Even Angell-Petersen,

Department of Surgical Oncology, The Norwegian Radium Hospital, Oslo 0310, Norway

Chung-Ho Sun,

Beckman Laser Institute and Medical Clinic, University of California, Irvine 92612, USA

Van Vo,

Department of Health Physics, University of Nevada, 4505, Maryland Pkwy, PO Box 453037, Las Vegas, NV 89154-3037, USA

Rogelio Sanchez, and

Beckman Laser Institute and Medical Clinic, University of California, Irvine 92612, USA

Henry Hirschberg

Department of Health Physics, University of Nevada, 4505, Maryland Pkwy, PO Box 453037, Las Vegas, NV 89154-3037, USA; Beckman Laser Institute and Medical Clinic, University of California, Irvine 92612, USA

Abstract

Photodynamic therapy (PDT) has been investigated as a postoperative treatment in patients with high grade gliomas. The purpose of this in vitro investigation was to determine whether motexafin gadolinium (MGd), a known radiation sensitizer, could potentiate the effects of 5-aminolevulinic acid (ALA)-PDT. Human glioma (ACBT) spheroids (250 μm diameter) were incubated in 5-aminolevulinic acid (ALA) with and without MGd and irradiated with 635 nm light for a total light fluence of 6, 12, or 18 J cm^{-2} delivered at a fluence rate of 5 mW cm^{-2} . Spheroid growth was monitored for a period of 4 weeks following each treatment. In another set of experiments, 400–500 μm diameter ACBT spheroids were implanted into a gel collagen matrix and subjected to ALA-PDT (fluence: 3 or 6 J cm^{-2}), MGd, or a combination of ALA-PDT and MGd. The

migration distance of surviving glioma cells in each treatment group was recorded over a 5-day period. The results showed that MGd interacted with PDT in a synergistic manner resulting in greater cytotoxicity than that achievable with either treatment modality alone. The degree of synergism was shown to increase with increasing light fluence. At the highest light fluence investigated (18 J cm^{-2}), the percentage of spheroids demonstrating growth 4 weeks following exposure to MGd, ALA-PDT, or MGd + ALA-PDT was 100%, 75%, and 15%, respectively. The results of cell migration studies revealed that the combination of PDT and MGd produced a significant inhibitory effect on glioma cell migration: the addition of MGd resulted in an approximately three times reduction in migration distance compared with PDT alone. Overall, the results suggest that MGd can potentiate both the cytotoxic and migration inhibitory effects of ALA-PDT and hence, this combined therapeutic approach has the potential to extend treatment volumes in patients with malignant gliomas.

Keywords

Glioma; Photodynamic therapy; Motexafin gadolinium; 5-aminolevulinic acid; Spheroid; Invasive growth

Introduction

The prognosis for patients with high grade brain tumors such as glioblastoma multiforme (GBM) remains poor. Employing current therapeutic regimens consisting of maximal resection followed by postoperative fractionated radiotherapy combined with temozolomide chemotherapy, median survival is around 15 months [1]. Since approximately 80% of patients develop tumor recurrence within 2 cm of the original tumor site, the aim of postoperative therapy is to eliminate the nests of tumor cells remaining in the margins of the resection cavity [2].

Photodynamic therapy (PDT) has been investigated as a postoperative treatment option following surgical tumor resection [3–5]. PDT is a local form of treatment involving the administration of a tumor-localizing photosensitizing drug that is spatially activated by light of a specific wavelength [6]. The resultant photochemical and photobiological events cause irreversible photo-damage to tumor tissues. PDT with endogenous protoporphyrin IX (PpIX), a potent photosensitizer induced by 5-aminolevulinic acid (ALA), or its derivatives, has been established for routine clinical treatments of several pre-malignancies and malignancies. The combination of increased tumor-to-normal brain tissue localization [7–13], short period of skin photosensitization (24–48 h) and oral administration, makes ALA an appealing compound for PDT diagnostics and therapeutics. For example, the high ALA tumor selectivity has been exploited for fluorescence-guided resection of glial tumors [12, 14]. One of the limitations of PDT is the rapid attenuation of light in the brain [15]. As a result, the eradication of infiltrating tumor cells found deep in the resection cavity is difficult to achieve since the delivery of toxic light fluences require long treatment times. One approach to extending treatment volumes is to use redox active drugs that enhance PDT efficacy thus lowering the fluence level required to obtain a cytotoxic effect.

Motexafin gadolinium (MGd) is an expanded form of porphyrins with a 20% larger core size than that of the porphyrins. This is due mainly to the substitution of a five core nitrogen ring for the four core nitrogen ring in porphyrins, allowing gadolinium to be incorporated at the center of the compound [16]. MGd is an agent previously reported to enhance the cytotoxic potential of radiation therapy, as well as several chemotherapeutic agents by causing redox stress to cancer cells [17, 18]. The depletion of intracellular reducing metabolites and production of reactive oxygen species caused by MGd may render cells more susceptible to the oxidative damage caused by PDT and this hypothesis provided the rationale for the present study.

In the experiments reported here, the response of human glioma spheroids to ALA-PDT with and without the addition of MGd was investigated. Three-dimensional multicell spheroids were used in the present work since they mimic micro-tumors and metastases. In comparison to monolayer cultures, or cells in suspension, a significant advantage of spheroid models is that their micro-environment more closely mimics the *in vivo* situation and therefore, gene expression and the biological behavior of the cells are likely similar to that encountered in tumor cells *in situ*. The effects of PDT on the dynamics of tumor spheroid growth and the infiltrating behavior of tumor cells migrating out of the spheroids were investigated. In order to mimic the light conditions typically found at 1–2 cm in the resection cavity wall, light fluences were intentionally kept low.

Materials and methods

Cell cultures

The grade IV GBM cell line (ACBT) used in this study was a generous gift of G. Granger (University of California, Irvine, USA) and was established directly from a patient's biopsy. The response of ACBT spheroids to a range of light fluence and fluence rates commonly used in ALA-PDT was determined in a previous study [19]. The cells were cultured in DMEM (Gibco, Grand Island, NY) with high glucose and supplemented with 2 mM l-glutamine, penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹), and 10% heat inactivated fetal bovine serum (Gibco, Grand Island, NY). Cells were maintained at 37°C in a 7.5% CO₂ incubator. At a density of 70% confluence, cells were removed from the incubator and left at room temperature for approximately 20 min. The resultant cell clusters (consisting of approximately 10 cells) were transferred to a petri dish and grown to tumor spheroids of varying sizes. Spheroids were grown according to standard techniques [20]. Spheroids of approximately 250 µm diameter were selected by passage through a screen mesh (Sigma, St. Louis, MO). It took approximately 14 days for spheroids to reach a size of 250 µm. The spheroid culture medium was changed twice weekly.

PDT treatments

ALA (Sigma, St. Louis, MO) was prepared in growth medium (DMEM with 10% fetal bovine serum) and pH adjusted to 7.4. Spheroids were incubated in 100 µg ml⁻¹ ALA for approximately 4 h. In all cases, spheroids were irradiated with 635 nm light from a diode laser (Intense-HPD, North Brunswick, NJ). Light was coupled into a 200 µm-diameter optical fiber containing a micro-lens at the output end. Spheroids were irradiated in petri

dishes, each containing approximately 40–50 spheroids. Four different control cultures were considered: (1) true controls— no ALA, no light, (2) ALA-only, (3) MGd-only, and (4) ALA and MGd (0.02 mg ml⁻¹), no light. The treatment groups were irradiated using light fluences of 6, 12, and 18 J cm⁻² delivered at a fluence rate of 5 mW cm⁻². Each fluence group was divided into two subgroups: with and without MGd. The MGd group was first incubated for 24 h in the presence of 0.02 mg ml⁻¹ MGd. Thereafter, prior to light irradiation, the cells were washed and incubated in ALA as previously described. Following irradiation, spheroids were washed, resuspended in medium and placed in separate wells of a 48-well culture plate. Determination of spheroid size was performed by measuring two perpendicular diameters of each spheroid using a microscope with a calibrated eyepiece micrometer. The size of each spheroid was measured on five separate occasions: day 0 (immediately following irradiation), and thereafter weekly for a total of 4 weeks. The minimum criterion used for scoring a spheroid as growing was a 10% change in any one of the two diameters during the 1-week observation points. The growing fraction is simply the ratio of growing spheroids to the total number of spheroids measured at each of the 4-weekly observation points.

The degree of interaction between ALA-PDT and MGd was evaluated by a technique proposed by Drewinko et al. [21]. In this scheme, the degree of interaction is given by:

$$\alpha = \frac{SF^M \times SF^P}{SF^{MP}}, \quad (1)$$

where SF^M and SF^P represent growing fractions with MGd and PDT respectively, and SF^{MP} is the growing fraction following combined treatments. In this analysis, $\alpha = 1$ indicates an additive effect (or absence of any effect), $\alpha > 1$ indicates a synergistic effect, and $\alpha < 1$ indicates an antagonistic effect.

Migration assay

The migration assay used in these experiments has been described previously [22]. Briefly, the matrix used consisted of a collagen gel composed of 70% type I rat tail collagen at a concentration of 4 mg ml⁻¹, 20% 5 × DMEM, and 10% reconstituted buffer made of 260 mM sodium bicarbonate, and 200 mM HEPES. The pH of the collagen gel mix was adjusted to 7.4 with sterile 1 N NaOH.

The spheroids were grown as bulk cultures in petri dishes, each containing approximately 36 spheroids. Some of the spheroids were incubated for 24 h in the presence of 0.02 mg ml⁻¹ MGd, washed and re-suspended in medium. Single ACBT tumor spheroids of 400–500 μm were placed in 35 mm diameter imaging dishes coated with a thin layer of dried rat tail type I collagen. After an hour to allow for spheroid adhesion, 200 μl of the collagen gel matrix was deposited over the spheroid and allowed to gel. The cultures were then overlaid with 400 μl of culture medium containing 10% FBS and incubated at 37°C in a 5% CO₂ atmosphere. Some of the individual cultures were incubated with ALA (100 μg ml⁻¹) for approximately 4 h and were thereafter irradiated at either of two fluence levels (3 or 6 J cm⁻²) at a fluence rate of 5 mW cm⁻² using 635 nm light from a diode laser. A 9-cm

diameter laser beam size with a flat beam profile in the actual irradiation area (35 mm) of each culture was used in all experiments. The large total beam size allowed the treatment of four parallel cultures simultaneously in each group. Control cultures were either incubated in ALA but received no light (dark controls) or incubated in MGd and received light treatment but no ALA (MGd-only control).

Invasion distance was calculated daily as the distance in μm from the spheroid edge to the population of invasive cells most distant from the spheroid edge. Figure 1 shows a phase contrast light micrograph of a typical dark control culture 4 days following spheroid implantation.

Live/dead cell viability assay

To determine the viability of the spheroids and infiltrating cells after PDT treatment, the cultures were co-stained with Hoechst 33342 (Invitrogen, Carlsbad, CA) and Ethidium Homodimer 1 (E1169, Invitrogen, Carlsbad, CA) at 5 and 1 $\mu\text{g ml}^{-1}$ of culture medium, respectively. The viability assays were performed 5 days following treatment. The collagen-embedded spheroid cultures were incubated with the dye mixture for 1 h at 37°C. After incubation, the cultures were washed three times with Hank's Buffered Saline Solution (HBSS). The cultures were examined using an inverted laser scanning microscope (LSM 510 META, Zeiss, Oberkochen, Germany) with a 20 $\times$ /0.5 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 60 μm into the spheroids with simultaneous excitation of the two dyes, and the images were pseudo-colored blue and red.

Results

The data presented in Fig. 2 show that PDT and PDT + MGd produced significant growth suppression in human glioma spheroids. In both cases, the extent of growth suppression was most pronounced on day 7 post-treatment (Fig. 2a). Thereafter, the percentage of spheroids demonstrating growth increased until a plateau was reached approximately 4-weeks post-treatment (Fig. 2c). As expected, treatment efficacy was dependent on light fluence: higher fluences produced greater growth suppression. Spheroids in all four control groups (true controls, ALA-only, MGd-only, ALA + MGd) demonstrated similar growth throughout the 4-week observation period. Representative data from two control groups are included in Fig. 2a–c. Differences in growth between spheroids subjected to PDT and those exposed to PDT + MGd became evident at the end of the 2nd week (Fig. 2b). In order to determine the extent of potentiation, the data in Fig. 2c was used to compute the interaction coefficient defined in Eq. 1. As illustrated in Fig. 3, MGd potentiated the effects of ALA-PDT in a synergistic manner—the degree of synergism increased with increasing light fluence.

Spheroid growth kinetics at a low light fluence (6 J cm⁻²) are illustrated in Fig. 4. The data clearly demonstrate the growth suppressing effect of MGd. For example, after 2 weeks in culture, spheroids subjected to the combination treatment showed minimal growth. In

contrast, the growth kinetics of PDT-treated spheroids was similar to the true controls: at the 2-week observation point, PDT-exposed spheroids had quadrupled in diameter.

The cellular migration results illustrated in Fig. 5 clearly show the enhanced inhibitory effects of the combined treatment. For example, the data show that on day 5 following low light fluence (3 J cm^{-2}) exposure, maximum migration distances for glioma cells surviving PDT and PDT + MGd were approximately 500 and 150 μm , respectively. This shows that, in this case, the addition of MGd resulted in an approximately three times reduction in migration distance compared with PDT alone. The complete inhibition of cellular migration observed in the case of the high fluence (6 J cm^{-2}) PDT + MGd was attributed to the high toxicity of this combined treatment and the resultant lack of surviving cells as illustrated in Fig. 6.

The two-photon fluorescence images presented in Fig. 6 illustrate the enhanced toxicity achieved by combining PDT and MGd. At a light fluence of 3 J cm^{-2} very little necrosis (red fluorescence) was observed (Fig. 6b), however, as indicated by the increase in the number of red fluorescent cells, a significant diminution in cell viability was observed when MGd was combined with PDT at this fluence (Fig. 6c). As shown in Fig. 6e, the lack of blue fluorescent cells suggests that no viable cells were present at this spheroid depth (60 μm) following exposure to MGd and PDT at a relatively low light fluence of 6 J cm^{-2} .

Discussion

The primary finding of this study is that the efficacy of PDT at sub-threshold light fluences can be augmented with MGd. Simple calculations indicate that MGd interacts in a synergistic manner with ALA-PDT and therefore, in principle, it should be possible to extend treatment volumes using low fluences. It is quite possible that MGd-PDT efficacy can be extended to even lower fluence rates than those investigated in this study, particularly in combination with repetitive or chronic PDT regimens. For example, *in vitro* studies suggest that fluence rates as low as 0.07 mW cm^{-2} produce significant tumor spheroid toxicity [23]. Although a small sub-population of spheroids appear to survive even at the highest fluence investigated (18 J cm^{-2}), a significant reduction in survival was seen compared to a single treatment without MGd. Previous studies have shown that glioma spheroids that survive acute doses of PDT continue to remain sensitive to subsequent PDT treatment [24]. Taken together, the data suggest that, in addition to its radiation sensitizer properties, MGd can enhance the efficacy of single acute doses of PDT even at the low light fluences typically found in the brain adjacent to tumor (BAT) region.

A secondary finding of this study is that MGd potentiates the inhibitory effects of ALA-PDT on glioma cell migration. This is an important observation since GBMs are characterized by rapid cell migration and invasive growth. Infiltration of glioma cells into functionally intact brain is a major cause of tumor recurrence and, as such, eradication of these cells is a requirement for long-term survival. Unfortunately, this problem is unlikely to be solved with conventional treatments consisting of radiation and chemotherapy. Invading tumor cells may be chemoresistant by virtue of their location in areas of the brain where the blood-brain

barrier is intact, and their tendency to reside in the non-proliferative G₀ phase of the cell cycle [25].

The present work extends the findings of a previous study which showed that ALA-PDT was an effective inhibitor of glioma cell migration in a 3-D collagen matrix [22]. In that study, measurements of cell survival at sublethal fluence levels suggested a direct migratory inhibition effect on the invading cells rather than cytotoxicity as the most likely mechanism for this observation. Results from the present work clearly show that the addition of MGd enhances the inhibitory effects of ALA-PDT on glioma cell migration. In some cases, the combination of MGd and ALA-PDT resulted in almost complete migratory inhibition. Live/dead assays shown in Fig. 6 indicate that at a light fluence of 6 J cm⁻², spheroids incubated with MGd consisted of only dead cells and, hence, the migration inhibition was attributed to direct cytotoxicity. At the lower fluence level (3 J cm⁻²), there were clearly viable cells in the PDT + MGd treated spheroids (Fig. 6c) and the data presented in Fig. 5 suggest that cell migration was clearly inhibited by the addition of MGd to PDT. A comparison of Fig. 6d and e illustrate an important point, namely that the addition of MGd can extend the treatment volume of ALA-PDT as evidenced by the uniform distribution of necrotic cells throughout the spheroid section (Fig. 6e). In contrast, spheroids subjected only to PDT (Fig. 6d) contain a significant number of viable cells residing at the center of the 60 µm deep section. Presumably, these cells are located in hypoxic regions where there is insufficient oxygen for the PDT effect.

Mechanistic studies of the effects of MGd and/or ALA-PDT on cell motility have not been investigated. The results presented in Fig. 5 show that PDT inhibited cell migration while MGd did not appear to affect cell motility. The explanation for these observations is not entirely clear and bears further investigation. Migration and invasion of glioma cells into normal brain are complex processes involving a wide variety of molecules including proteases, adhesion molecules, small G-proteins, cytoskeletal proteins, cytoskeleton associating proteins, extracellular matrix molecules, growth factors, and guidance molecules [26]. In general, glioma cell invasion can be considered to consist of three interrelated processes. The first of these is cell adhesion to matrix proteins followed by dis-adhesion. Adhesion molecules are involved in cell–cell and cell–substrate contact. On contact to their ligands in the extracellular matrix, a signaling cascade causes events such as cytoskeleton reorganization and transcription. It is hypothesized that this allows the cells to separate from the spheroid rim and migrate out into the collagen matrix. In a previous study [22], ALA-PDT treatment of ACBT spheroids prior to implantation in culture, significantly decreased the ability of the cells in the spheroid rim to adhere to the collagen substrate. In addition, PDT appeared to completely block the migration of cells into the matrix.

The second step in glioma invasion involves enzymatic degradation of the components of the matrix (collagen in this case) by tumor secreted proteases. This degradation facilitates glioma cell infiltration. Glioma cells have been shown to overexpress certain proteases including matrix metalloproteinases (MMP) which have been shown to play a crucial role in glioma cell invasion [27–29]. In addition, down-regulation of gelatinase MMP-2 has been shown to inhibit U251MG and U87MG cell invasion. [30] These studies suggest inhibition of protease function might be a potential target for the effects of ALA-PDT.

Cell locomotion is the third requirement for cell invasion. This parameter is dependent on changes to the cell cytoskeleton and associated proteins such as actin which has been shown to be essential for cell migration. Actin responds to adhesion signals mainly from integrins and adhesion complexes undergoing cytoskeletal reorganization [31]. Cytochalasin B, which inhibits assembly of actin fibers, has been shown to reduce glioma cell motility [32]. These observations would suggest that actin fibers constitute a potential target for ALA-PDT and therefore bears further investigation.

PDT efficacy depends on a number of factors including, light fluence, fluence rate, tissue oxygenation status, photosensitizer concentration, and intrinsic tissue sensitivity to the PDT effect. The high degree of fluorescence observed in glial tumors following oral administration of ALA suggests that PpIX levels in tumor tissue and in the BAT are sufficient for effective PDT [12–14]. A limiting factor for successful PDT is the inability to attain threshold light fluences throughout the BAT. This is due to the rapid attenuation of light in brain tissues. For example, in vivo measurements in mammalian brains show that light fluence rates (630–635 nm) decrease exponentially with distance from the resection margin with a penetration depth of 1–3 mm [15, 33–37]. The limited penetration depth is particularly problematic in novel PDT regimens consisting of fractionated or continuous low-fluence rate light delivery [38, 39]. Hence, the motivation for the present work was to evaluate the ability of a known radiation and chemotherapy sensitizer (MGd) to enhance the cytotoxic effect of low-fluence rate ALA-PDT with the aim of extending treatment volumes beyond the immediate vicinity of the BAT. This is a requirement for improving survival in glioma patients since malignant cells typically migrate cm distances beyond the original resection margin.

Studies have elucidated a number of properties that makes MGd particularly suited for the treatment of cancer: (1) it selectively targets tumor cells which have increased rates of metabolism, (2) it remains inside cells for days [40], (3) it disrupts energy production and inhibits cellular repair by stimulating the production of reactive oxygen species (ROS) such as free radicals [41], (4) it is detectable by magnetic resonance imaging [42], and (5) it does not accumulate in normal brain protected by an intact blood–brain barrier [43]. The mechanisms by which MGd enhances the effects of ionizing radiation, chemotherapeutic agents (e.g., doxorubicin and bleomycin) and PDT have not been elucidated. Several possible mechanisms of action have been hypothesized [18] with the process known as futile redox cycling considered the most likely. Cells employ antioxidants (reducing metabolites) for the repair of oxidative damage caused by PDT. MGd might interfere with this repair function by transferring electrons from reducing metabolites to oxygen to produce hydrogen peroxides. This process is hypothesized to inhibit the repair of potentially lethal and sub-lethal damage, via decreased ATP production and increased generation of reactive oxygen species, inducing apoptosis. MGd has also been shown to increase the oxygen tension in tumors. In a recent study, the oxygenation of EMT6 mammary tumor in mice was measured with and without the injection of MGd [44]. Increased median pO_2 levels in tumors were significantly increased by MGd compared to controls. This is of importance since PDT is an oxygen-dependent treatment and the availability of oxygen is often the determining factor for the cytotoxic effect.

Since MGd has been shown to persist in tumor tissues for extended periods after discontinuation of drug injection [42, 45], it would appear to be ideally suited for enhancing the efficacy of fractionated and continuous low-fluence rate PDT regimens which have been shown to be more effective than single acute doses in a number of in vitro studies [46].

MGd has been given to a large number of patients to evaluate its utility as a radiation sensitizer [47–52]. Serious side effects were uncommon and the compound was found to have high tumor-specificity and intracellular localization [53, 54]. The data presented here suggest that treatment volumes can be extended to include the BAT where the blood–brain barrier (BBB) is disrupted. Further extension of treatment volumes into normal brain is complicated by the intact BBB which excludes MGd. The delivery of MGd, as well as photosensitizing drugs, to these regions will require methods of selective BBB disruption. The ability of localized PDT treatment to open the BBB in a limited region of the brain is currently being evaluated in animal models [55, 56]. If these studies prove successful, it should be possible to translate them into clinical protocols combining PDT with MGd.

Although only one glioma cell line was investigated in this study, we have previously examined the effects of ALA-PDT in two rat glioma cell lines (BT₄C and F98) [57, 58]. No significant variations in response to ALA-PDT were found in the three glioma lines (including ACBT): in all cases, glioma cells were found to be very sensitive to low light fluence rate ALA-PDT. A complete genetic characterization of the ACBT cell line has not been attempted and therefore the status of a number of proteins that might mediate PDT and/or MGd effects is unknown. A number of studies have shown that low-fluence rate ALA-PDT induces apoptosis in ACBT cells [59, 60]. This is attributed to the localization of ALA-induced PpIX in the mitochondrial membrane following short incubation times resulting in mitochondrial damage after light exposure [61, 62]. It is conceivable that variations in pro- and/or anti-apoptotic proteins between glioma cell lines might result in differing levels of apoptotic cell death and hence differences in overall survival following ALA-PDT, however, the potential for such variations can be minimized by incubating cell lines in ALA for longer times since this is associated with a translocation of PpIX from mitochondrial to plasma membranes resulting in necrosis following light exposure [61].

In summary, the efficacy of ALA-PDT in human glioma spheroids can be enhanced with the addition of MGd. The results suggest that MGd and PDT interact in a synergistic manner and, as such, it should be possible to extend treatment volumes with the low light fluences found in the BAT during PDT. A secondary finding of this study is that the combination of PDT and MGd results in a significant inhibitory effect on glioma cell migration. The mechanisms by which MGd interacts with ALA-PDT remain to be elucidated and are the subject of ongoing studies in this laboratory.

Acknowledgments

Steen Madsen is grateful for the support of the UNLV Office of Research. Portions of this work were made possible, in part, through access to the Laser Microbeam and Medical Program (LAMMP) and the Chao Cancer Center Optical Biology Shared Resource at the University of California, Irvine. These facilities are supported by the National Institutes of Health under grants RR-01192 and CA-62203, respectively. In addition, Beckman Laser Institute programmatic support was provided by the Department of Energy (DOE #DE-FG03-91ER61227), and the Office of Naval Research (ONR #N00014-91-C-0134).

References

1. Stupp R, van den Bent MJ, Hegi ME. Optimal role of temozolomide in the treatment of malignant gliomas. *Curr Neurol Neurosci Rep.* 2005; 5:198–206.10.1007/s11910-005-0047-7 [PubMed: 15865885]
2. Wallner KE, Galicich JH, Krol G, Arbit E, Malkin MG. Patterns of failure following treatments for glioblastoma multiforme and anaplastic astrocytoma. *Int J Radiat Oncol Biol Phys.* 1989; 16:1405–1409. [PubMed: 2542195]
3. Muller, PJ.; Wilson, BC.; Lilge, LD.; Yang, V.; Hetzel, FW.; Chen, Q., et al. Photofrin photodynamic therapy for malignant brain tumors. In: Dougherty, TJ., editor. Optical methods for tumor treatment and detection: mechanisms and techniques in photodynamic therapy. Proceedings SPIE. Vol. 4248. SPIE Publishers; Bellingham, WA: 2001 Apr. 2001 p. 34-41.
4. Eljamel MS, Goodman C, Moseley H. ALA and Photofrin® fluorescence-guided resection and repetitive PDT in glioblastoma multiforme: a single centre phase III randomised controlled trial. *Lasers Med Sci.* 2007;10.1007/s10103-007-0494-2
5. Beck TJ, Kreth FW, Beyer W, Mehrkens JH, Obermeier A, Stepp H, et al. Interstitial photodynamic therapy of nonresectable malignant glioma recurrences using 5-aminolevulinic acid induced protoporphyrin IX. *Lasers Surg Med.* 2007; 39(5):386–393.10.1002/lsm.20507 [PubMed: 17565715]
6. Dougherty TJ. Photodynamic therapy. *Photochem Photobiol.* 1993; 58:895–900.10.1111/j.1751-1097.1993.tb04990.x [PubMed: 8310013]
7. Lilge L, Wilson BC. Photodynamic therapy of intracranial tissues: a preclinical comparative study of four different photosensitizers. *J Clin Laser Med Surg.* 1998; 16:81–92. [PubMed: 9663099]
8. Hebeda KM, Saarnak AE, Olivo M, Sterenborg HJ, Wolbers JG. 5-Aminolevulinic acid induced endogenous porphyrin fluorescence in 9L and C6 brain tumours and in the normal rat brain. *Acta Neurochir (Wien).* 1998; 140:503–513.10.1007/s007010050132 [PubMed: 9728253]
9. Lilge L, Portnoy M, Wilson BC. Apoptosis induced in vivo by photodynamic therapy in normal brain and intracranial tumor tissue. *Br J Cancer.* 2000; 83:1110–1117.10.1054/bjoc.2000.1426 [PubMed: 10993661]
10. Obwegeser A, Jakober R, Kostron H. Uptake and kinetics of ¹⁴C-labelled meta-tetrahydroxylchlorin and 5-aminolevulinic acid in the C6 rat glioma model. *Br J Cancer.* 1998; 78:733–738. [PubMed: 9743291]
11. Stummer W, Stocker S, Novotny A, Heimann A, Sauer O, Kempfski O, et al. In vitro and in vivo porphyrin accumulation by C6 glioma cells after exposure to 5-aminolevulinic acid. *J Photochem Photobiol B Biol.* 1998; 45:160–169.10.1016/S1011-1344(98)00176-6
12. Stummer W, Stocker S, Wagner S, Stepp H, Fritsch C, Goetz C, et al. Intraoperative detection of malignant gliomas by 5-aminolevulinic acid induced porphyrin fluorescence. *Neurosurgery.* 1998; 42:518–526.10.1097/00006123-199803000-00017 [PubMed: 9526986]
13. Terr L, Weiner LP. An autoradiographic study of 5-aminolevulinic acid uptake by mouse brain. *Exp Neurol.* 1983; 79:564–568.10.1016/0014-4886(83)90234-0 [PubMed: 6822280]
14. Stummer W, Novotny A, Stepp H, Goetz C, Bise K, Reulen HJ. Fluorescence-guided resection of glioblastoma multiforme by using 5-aminolevulinic acid-induced porphyrins: a prospective study in 52 consecutive patients. *J Neurosurg.* 2000; 93:1003–1013. [PubMed: 11117842]
15. Angell-Petersen E, Hirschberg H, Madsen SJ. Determination of fluence rate and temperature distributions in the rat brain; implications for photodynamic therapy. *J Biomed Opt.* 2007; 12(1): 014003-1–014003-9. [PubMed: 17343478]
16. Sessler JL, Burrell AK. Expanded porphyrins. *Top Curr Chem.* 1992; 161:177–273.
17. Magda D, Miller RA. Motexafin gadolinium: a novel redox active drug for cancer therapy. *Semin Cancer Biol.* 2006; 16(6):466–476.10.1016/j.semcancer.2006.09.002 [PubMed: 17112739]
18. Evens AM. Motexafin gadolinium: a redox-active tumor selective agent for the treatment of cancer. *Curr Opin Oncol.* 2004; 16(6):576–580.10.1097/01.cco.0000142073.29850.98 [PubMed: 15627019]

19. Madsen SJ, Sun CH, Tromberg BJ, Wallace VP, Hirschberg H. Photodynamic therapy of human glioma spheroids using 5-aminolevulinic acid. *Photochem Photobiol.* 2000; 72(1):128–134.10.1562/0031-8655(2000)072<0128:PTOHGS>2.0.CO;2 [PubMed: 10911737]
20. Sutherland RM, Carlsson J, Durand RE, Yuhas J. Spheroids in cancer research. *Cancer Res.* 1981; 41:2980–2994.
21. Drewinko B, Loo TL, Brown B, Gottlieb JA, Freireich EJ. Combination therapy in vitro with adriamycin: observation of additive, antagonistic, and synergistic effects when used in two-drug combinations on cultured human lymphoma cells. *Cancer Biochem Biophys.* 1976; 1:187–195. [PubMed: 975020]
22. Hirschberg H, Sun CH, Krasieva T, Madsen SJ. Effects of ALA-mediated photodynamic therapy on the invasiveness of human glioma cells. *Lasers Surg Med.* 2006; 38:939–945.10.1002/lsm.20445 [PubMed: 17163479]
23. Mathews, MS.; Sun, CH.; Madsen, SJ.; Hirschberg, H. Comparing the effects of repetitive and chronic PDT in human glioma spheroids. In: Kollias, N.; Choi, B.; Zeng, H., et al., editors. *Photonic therapeutics and diagnostics III. Proceedings SPIE.* Vol. 6424. SPIE Publishers; Bellingham, WA: 2007. p. D1-D9.
24. Madsen SJ, Sun CH, Tromberg BJ, Hirschberg H. Repetitive 5-aminolevulinic acid-mediated photodynamic therapy on human glioma spheroids. *J Neurooncol.* 2003; 62(3):243–250.10.1023/A:1023362011705 [PubMed: 12777075]
25. Giese A, Loo MA, Tran N, Haskett D, Coons SW, Berens ME. Dichotomy of astrocytoma migration and proliferation. *Int J Cancer.* 1996; 67:275–282.10.1002/(SICI)1097-0215(19960717)67:2<275::AID-IJC20>3.0.CO;2-9 [PubMed: 8760599]
26. Giese A, Loo MA, Rief MD, Tran N, Berens ME. Substrates for astrocytoma invasion. *Neurosurgery.* 1995; 37:294–302.10.1097/00006123-199508000-00015 [PubMed: 7477782]
27. Binder DK, Berger MS. Proteases and the biology of glioma invasion. *J Neurooncol.* 2002; 56:149–158.10.1023/A:1014566604005 [PubMed: 11995816]
28. Rao JS. Molecular mechanisms of glioma invasiveness: the role of proteases. *Nat Rev Cancer.* 2000; 3(7):489–501.10.1038/nrc1121 [PubMed: 12835669]
29. Uhm JH, Dooley NP, Villemure JG, Yong VW. Mechanisms of glioma invasion: role of matrix-metalloproteinases. *Can J Neurol Sci.* 1997; 24:3–15. [PubMed: 9043741]
30. Koul D, Parthasarathy R, Shen R, Davies MA, Jasser SA, Chintala SK, et al. Suppression of matrix metalloproteinase-2 gene expression and invasion in human glioma cells by MMAC/PTEN. *Oncogene.* 2001; 20:6669–6678.10.1038/sj.onc.1204799 [PubMed: 11709701]
31. Maidment SL. The cytoskeleton and brain tumour cell migration. *Anticancer Res.* 1997; 17:4145–4149. [PubMed: 9428348]
32. Chicoine MR, Silbergeld DL. Assessment of brain tumor cell motility in vivo and in vitro. *J Neurosurg.* 1995; 82:615–622. [PubMed: 7897524]
33. Muller PJ, Wilson BC. An update on the penetration depth of 630 nm light in normal and malignant human brain tissue in vivo. *Phys Med Biol.* 1986; 31:1295–1297.10.1088/0031-9155/31/11/012 [PubMed: 3786415]
34. Wilson BC, Jeeves WP, Lowe DM. In vivo and post mortem measurements of the attenuation spectra of light in mammalian tissues. *Photochem Photobiol.* 1985; 42:153–162.10.1111/j.1751-1097.1985.tb01554.x [PubMed: 4048297]
35. Doiron, DR.; Svaasand, LO.; Profio, AE. Light dosimetry in tissue applications to photoradiation therapy. In: Kessel, D.; Dougherty, TJ., editors. *Porphyrin photosensitization.* Plenum Press; New York: 1983. p. 63-75.
36. Powers SK, Brown JT. Light dosimetry in brain tissue: an in vivo model applicable to photodynamic therapy. *Lasers Surg Med.* 1986; 6:318–322.10.1002/lsm.1900060305 [PubMed: 2942742]
37. Chen Q, Chopp M, Madigan L, Dereski MO, Hetzel FW. Damage threshold of normal rat brain in photodynamic therapy. *Photochem Photobiol.* 1996; 64:163–167.10.1111/j.1751-1097.1996.tb02437.x [PubMed: 8787009]

38. Madsen SJ, Sun CH, Tromberg BJ, Hirschberg H. Development of a novel indwelling balloon applicator for optimizing light delivery in photodynamic therapy. *Lasers Surg Med.* 2001; 29:406–412.10.1002/lsm.10005 [PubMed: 11891728]
39. Bisland SK, Lilge L, Lin A, Rusnow R, Wilson BC. Metronomic photodynamic therapy as a new paradigm for photodynamic therapy: rationale and preclinical evaluation of technical feasibility for treating malignant brain tumors. *Photochem Photobiol.* 2004; 80:22–30.10.1562/2004-03-05-RA-100.1 [PubMed: 15339204]
40. Sessler JL, Miller RA. Texaphyrins: new drugs with diverse clinical applications in radiation and photodynamic therapy. *Biochem Pharmacol.* 2000; 59:733–739.10.1016/S0006-2952(99)00314-7 [PubMed: 10718331]
41. Khuntia D, Mehta M. Motexafin gadolinium: a clinical review of a novel radioenhancer for brain tumors. *Expert Rev Anticancer Ther.* 2004; 4:981–989.10.1586/14737140.4.6.981 [PubMed: 15606327]
42. Carde P, Timmerman R, Mehta MP, Koprowski CD, Ford J, Tishler RB, et al. Multicenter phase IB/II trial of the radiation enhancer motexafin gadolinium in patients with brain metastases. *J Clin Oncol.* 2001; 19:2074–2083. [PubMed: 11283141]
43. Hirschberg H, Wu GN, Madsen SJ. Evaluation of motexafin gadolinium (MGd) as a contrast agent for intraoperative MRI. *Minim Invasive Neurosurg.* 2007; 50:1–6.10.1055/s-2007-993158 [PubMed: 17546535]
44. Donnelly ET, Liu YF, Fatunmbi YO, Lee I, Magda D, Rockwell S. Effects of texaphyrins on the oxygenation of EMT6 mouse mammary tumors. *Int J Radiat Oncol Biol Phys.* 2004; 58(5):1570–1576.10.1016/j.ijrobp.2003.12.017 [PubMed: 15050338]
45. Wu GN, Ford JM, Alger JR. MRI measurement of the uptake and retention of motexafin gadolinium in glioblastoma multiforme and uninvolved normal human brain. *J Neurooncol.* 2006; 77:95–103.10.1007/s11060-005-9101-1 [PubMed: 16547607]
46. Hirschberg H, Sørensen DR, Angell-Petersen E, Peng Q, Tromberg B, Sun CH, et al. Repetitive photodynamic therapy of malignant brain tumors. *J Environ Pathol Toxicol Oncol.* 2006; 25(1–2): 261–280. [PubMed: 16566723]
47. Viala J, Vanel D, Meingan P, Lartigau E, Carde P, Renschler MF. Phases IB and II multidose trial of gadolinium texaphyrin, a radiation sensitizer detectable at MR imaging: preliminary results in brain metastases. *Radiology.* 1999; 212:755–759. [PubMed: 10478243]
48. Mehta MP, Shapiro WR, Glantz MJ, Patchell RA, Weitzner MA, Meyers CA, et al. Lead-in phase to randomized trial of motexafin gadolinium and whole-brain radiation for patients with brain metastases: centralized assessment of magnetic resonance imaging, neurocognitive, and neurologic end points. *J Clin Oncol.* 2002; 20:3445–3453.10.1200/JCO.2002.07.500 [PubMed: 12177105]
49. Mehta MP, Rodrigus P, Terhaard CH, Rao A, Suh J, Roa W, et al. Survival and neurologic outcomes in a randomized trial of motexafin gadolinium and whole-brain radiation therapy in brain metastases. *J Clin Oncol.* 2003; 21(13):2529–2536.10.1200/JCO.2003.12.122 [PubMed: 12829672]
50. Meyers CA, Smith JA, Bezjak A, Mehta MP, Liebmann J, Illidge T, et al. Neurocognitive function and progression in patients with brain metastases treated with whole-brain radiation and motexafin gadolinium: results of a randomized phase III trial. *J Clin Oncol.* 2004; 22(1):157–165.10.1200/JCO.2004.05.128 [PubMed: 14701778]
51. Rosenthal DI, Nurenberg P, Becerra CR, Frenkel EP, Carbone DP, Lum BL, et al. A phase I single dose trial of gadolinium texaphyrin (Gd-Tex), a tumor selective radiation sensitizer detectable by magnetic resonance imaging. *Clin Cancer Res.* 1999; 5:739–745. [PubMed: 10213207]
52. Miller RA, Woodburn K, Fan Q, Renschler MF, Sessler JL, Koutcher JA. In vivo animal studies with gadolinium (III) texaphyrin as a radiation enhancer. *Int J Radiat Oncol Biol Phys.* 1999; 45(4):981–989.10.1016/S0360-3016(99)00274-6 [PubMed: 10571206]
53. De Stasio G, Rajesh D, Ford JM, Daniels MJ, Erhardt RJ, Frazer BH, et al. Motexafin-gadolinium taken up in vitro by at least 90% of glioblastoma cell nuclei. *Clin Cancer Res.* 2006; 12(1):206–213.10.1158/1078-0432.CCR-05-0743 [PubMed: 16397044]

54. Woodburn KW. Intracellular localization of the radiation enhancer motexafin gadolinium using interferometric Fourier fluorescence microscopy. *J Pharmacol Exp Ther.* 2001; 297:888–894. [PubMed: 11356908]
55. Hirschberg, H.; Mathews, MS.; Angell-Petersen, E.; Spetalen, S.; Madsen, SJ. Increased brain edema following 5-aminolevulinic acid administration mediated photodynamic therapy in normal and tumor-bearing rats. In: Kollias, N.; Choi, B.; Zeng, H., et al., editors. *Photonic therapeutics and diagnostics III. Proceedings SPIE.* Vol. 6424. SPIE Publishers; Bellingham, WA: 2007. p. B1-B8.
56. Hirschberg, H.; Zhang, M.; Chighvinadze, D.; Peng, Q.; Madsen, SJ. Targeted opening of the blood brain barrier by ALA mediated PDT. In: Kollias, N.; Choi, B.; Zeng, H., et al., editors. *Photonic therapeutics and diagnostics IV. Proceedings SPIE.* Vol. 6842. SPIE Publishers; Bellingham, WA: 2008. p. O1-O11.
57. Madsen SJ, Angell-Petersen E, Spitalen S, Carper SW, Ziegler S, Hirschberg H. Photodynamic therapy of newly implanted glioma cells in the rat brain. *Lasers Surg Med.* 2006; 38:540–548.10.1002/lsm.20274 [PubMed: 16392143]
58. Madsen SJ, Kharkhuu K, Hirschberg H. Utility of the F98 rat glioma model for photodynamic therapy. *J Environ Pathol Toxicol Oncol.* 2007; 26(2):149–155. [PubMed: 17725541]
59. Madsen SJ, Sun CH, Tromberg BJ, Yeh AT, Sanchez R, Hirschberg H. Effects of combined photodynamic therapy and ionizing radiation on human glioma spheroids. *Photochem Photobiol.* 2002; 76:411–416.10.1562/0031-8655(2002)076<0411:EOCPTA>2.0.CO;2 [PubMed: 12405149]
60. Hirschberg H, Sun CH, Yeh AT, Tromberg BJ, Madsen SJ. Enhanced effects of concurrent 5-aminolevulinic acid-mediated photodynamic therapy by hyperthermia on human glioma spheroids. *J Neurooncol.* 2004; 70:289–299.10.1007/s11060-004-9161-7 [PubMed: 15662970]
61. Iinuma S, Farshi SS, Ortel B, Hasan T. A mechanistic study of cellular photodestruction with 5-aminolevulinic acid-induced porphyrin. *Br J Cancer.* 1994; 70:21–28. [PubMed: 8018536]
62. Verma A, Facchina SL, Hirsch DJ, Song SY, Dillahey LF, Williams JR, et al. Photodynamic tumor therapy: mitochondrial benzodiazepine receptors as a therapeutic target. *Mol Med.* 1998; 4:40–48. [PubMed: 9513188]

Fig. 1.
Phase contrast light micrograph showing migration of glioma cells from a dark control spheroid. The image was acquired 4 days following spheroid implantation

Fig. 2.

Effects of PDT and MGd on spheroid growth evaluated 1 week (a), 2 weeks (b), and 4 weeks (c) following treatment. Three light fluences were considered: 6, 12, and 18 J cm⁻². In all cases, light was delivered at a fluence rate of 5 mW cm⁻². Each data point represents the mean (±standard deviation) of 48–60 spheroids pooled from three distinct trials

Fig. 3.

The degree of interaction between PDT and MGd as a function of light fluence. The horizontal line denotes the boundary between synergism ($\alpha > 1$) and antagonism ($\alpha < 1$). Each data point represents the mean ($\pm$ standard deviation) of 48–60 spheroids pooled from three distinct trials

Fig. 4. Spheroid growth kinetics following treatments at a low light fluence (6 J cm^{-2}) delivered at a fluence rate of 5 mW cm^{-2} . Each data point represents the mean ($\pm$ standard deviation) of 48–60 spheroids pooled from three distinct trials

Fig. 5. Effects of PDT and MGd on cellular migration as a function of time following treatment. Two light fluences were considered: 3 and 6 J cm⁻². In all cases, light was delivered at a fluence rate of 5 mW cm⁻². Error bars denote standard deviations. Each experiment was repeated twice for a total of three trials

Fig. 6. Two-photon fluorescence images of spheroids stained with Hoechst 33342 (blue) and Ethidium Homodimer 1 (red). All images were acquired at a depth of approximately 60 μm , 5 days after treatment: **(a)** control, **(b)** PDT (3 J cm^{-2}), **(c)** PDT (3 J cm^{-2}) + MGd, **(d)** PDT (6 J cm^{-2}), **(e)** PDT (6 J cm^{-2}) + MGd. In all cases, the field of view was $400 \mu\text{m} \times 400 \mu\text{m}$