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Laboratory Investigation

ALA- and ALA-ester-mediated photodynamic therapy of human glioma spheroids

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Key words: glioma, photodynamic therapy, ALA, ALA esters, spheroid

Summary

The effects of photodynamic therapy (PDT) in human glioma spheroids incubated in 5-aminolevulinic acid (ALA), or ALA esters, are investigated. Spheroid survival and growth are monitored following PDT at representative drug concentrations, light doses, and dose rates. The primary finding of this study is that the response of human glioma spheroids to PDT with lipophilic ester derivatives, such as benzyl-ALA and hexyl-ALA, is equivalent to that observed with ALA, however, this equivalency is obtained for ester concentrations 10–20 times lower than the parent compound. The enhanced efficiency of the esters is likely due to their increased membrane penetrance. Potential clinical advantages of using lipophilic esters in PDT of gliomas are discussed.

Introduction

The results of treatments for malignant brain tumors have not improved significantly despite continuing efforts and a multitude of clinical trials. Failure of treatment is usually due to local recurrence at the site of surgical resection – in 80% of all cases, recurrence is within 2 cm of the resected margin [1]. This would indicate that a more aggressive local therapy could be of benefit. We have recently described the clinical results obtained employing a method of high dose balloon brachytherapy directed to the cavity wall following tumor resection [2]. Although this form of treatment is cost effective, and results in a significant time saving, outcomes were comparable to conventional radiation treatment indicating that additional adjuvant therapy is warranted.

Photodynamic therapy (PDT) has been used successfully in a variety of localized malignancies and has characteristics that may prove useful in the treatment of resected brain tumor margins. The tumorcidal mechanism of this form of treatment is based on the activation, by light, of a photosensitizing drug localized to the tumor tissue. Some of the features that make PDT an effective adjuvant therapy in the treatment of brain tumors are: (1) it is a local form of treatment in which the treated volume is limited by high attenuation of light in brain tissues; (2) resistance to PDT has not been encountered during treatments of brain tumors [3]; and (3) repeated applications of PDT is an option due to low long-term morbidity. A significant drawback of PDT is the poor light penetration in brain tissues [4–6]. As a result, long-treatment times and/or fractionated treatments are required to deliver threshold light doses to centimeter depths in the resection margin.

Porphyrins, such as hematoporphyrin derivative and Photofrin® have been used almost exclusively in clinical PDT trials of the brain. Although favorable results have been reported by a number of investigators [7,8], these photosensitizers are not suitable for use in fractionated PDT treatment regimens due to their accumulation in cutaneous tissues. The uncommonly long period of cutaneous photosensitization (lasting up to several weeks) negatively impacts the patient's quality-of-life. Due to the drawbacks of traditional porphyrins, other photosensitizers, such as the prodrug 5-aminolevulinic acid (ALA) are currently being evaluated for use in PDT of gliomas [3,9,10]. ALA is not

a preformed photosensitizer, however, the addition of exogenous ALA, or its derivatives, forces the accumulation of protoporphyrin IX (PpIX) in cells rendering them susceptible to light irradiation.

The combination of excellent tumor-to-normal brain tissue localization [11], short period of skin photosensitization (24–48 h) and the possibility of oral administration, makes ALA a promising photosensitizer for use in fractionated or repeated PDT treatments of glioma patients. In addition, photodynamic detection (PDD) via PpIX fluorescence in tumor tissue can be used to guide the extent of resection during surgery [9,10]. Maximizing ALA-induced PpIX concentration in tumor tissue is therefore critical for effective PDD or PDT.

Although ALA has several advantages over other photosensitizers, its high hydrophilicity gives rise to relatively poor transport across cell membranes. In order to increase the effectiveness of ALA, lipophilic ALA esters have been developed [12]. The esterification of active substances to obtain a more lipophilic prodrug, which upon entering a cell is hydrolysed by esterases, is a well known concept in pharmacology [13]. The use of ALA esters has been shown to induce comparable PpIX production at much lower drug doses compared to ALA [14]. ALA esters also demonstrate increased spatial confinement when applied to skin and exhibit greater tissue penetrance relative to ALA [15]. Finally, the results of both *in vitro* [16] and *in vivo* [17] studies suggest that a more homogeneous PpIX distribution can be obtained with ALA esters compared to ALA.

In this study, the response of human glioma spheroids to ALA- and ALA-ester-mediated PDT was investigated using various light delivery regimens. Spheroid survival was monitored as a function of drug concentration, light dose (fluence) and dose rate (fluence rate). Spheroids were used in this study since they have a complexity intermediate between monolayer cultures and solid tumors *in vivo* and allow the study of tumor cell-specific phenomena in the absence of complex host dependent factors [18,19].

Materials and methods

Chemicals

Aminolevulinic acid hydrochloride was purchased from Sigma (St. Louis, MO). ALA methyl, hexyl, and benzyl esters were supplied by PhotoCure (Oslo, Norway). The esters were first dissolved in DMSO (100 mM) before further dilution in culture medium. The octanol/water partition coefficients (P) for ALA and the three ALA esters are shown in Table 1. ALA and methyl-ALA (m-ALA) are soluble in water but less soluble in octanol, whereas hexyl-ALA (h-ALA) and benzyl-ALA (b-ALA) have much lower water solubility coefficients and are significantly more lipophilic. As shown in Table 1, the esterfication of ALA changes the relative lipophilicity by three to four orders of magnitude compared to the parent compound.

Table 1. Molecular weights and octanol/water partition coefficients for ALA and its derivatives

Chemical name	Mol. wt. as HCl salt	Log *P* octanol/water
5-aminolevulinate HCl (ALA)	167	−1.4
Methyl 5-aminolevulinate HCl (m-ALA)	181	−0.8
Hexyl 5-aminolevulinate HCl (h-ALA)	251	+2.2
Benzyl 5-aminolevulinate HCl (b-ALA)	257	+2.4

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). 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% CO_2 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 [19]. Spheroids of 400 μm diameter were selected by passage through a screen mesh (Sigma, St. Louis, MO). It took approximately 20 days for spheroids to reach a size of 400 μm. The spheroid culture medium was changed three times weekly. In general, growth occurred at the periphery of the spheroid, with a quiescent layer of cells at intermediate depths and a necrotic core at the center.

PDT treatments

Spheroids were incubated in: (1) ALA at concentrations ranging from 3.8 to 758 μg ml^{-1} (0.025–5.0 mM), or (2) ALA ester derivatives at concentrations of 6.3–125 μg ml^{-1} (0.025–0.5 mM). In all cases, the incubation time was approximately 4 h. Spheroids were irradiated with 635 nm light from an argon ion-pumped dye laser (Coherent Inc., Santa Clara, CA). Light was coupled into a 200 μm diameter optical fiber containing a microlens at the output end. Spheroids were irradiated in a petri dish containing a 2 cm diameter gasket to confine the spheroids to the central portion of the dish and thus limit the extent of the irradiated field. Following irradiation, individual spheroids were placed into separate wells of a 48-well culture plate and monitored for growth. Determination of spheroid size was carried out by measuring two perpendicular diameters of each spheroid using a microscope with a calibrated eyepiece micrometer. Typically, 16–24 spheroids were followed in each trial. Since each trial was performed 2 or 3 times, a total of 32–48 spheroids were followed for a given set of parameters. Spheroids were followed for up to 28 days. In the case of the fluence/fluence rate studies, spheroids were subjected to a light fluence of either 6, 12, 25 or 50 J cm^{-2} delivered at a fluence rate of 5 or 25 mW cm^{-2}.

Results

The growth kinetics of true controls (no drug, no light) and dark controls (drug only) are illustrated in Figure 1. As can be seen from the figure, spheroids incubated for 4 h in 0.5 mM of h-ALA ester or 5.0 mM of ALA had identical growth rates compared to the true controls. The growth kinetics of m-ALA and b-ALA (at equivalent concentrations to h-ALA) were also found to be identical to the true controls (data not shown). Even at these high drug concentrations, there is no evidence of toxicity. The glioma spheroids reached a limiting size of approximately 1600 μm, 21–28 days post incubation. As shown in Figure 1, an approximate four-fold increase in spheroid diameter was typical in this time period. This represents a 64-fold increase in the number of cells constituting the spheroid.

The results of an initial screening of the effects of ALA- or ALA-ester-induced PDT are shown in Figure 2. The spheroids were incubated in a drug concentration of approximately 0.05 mM, and irradiated with a fluence of 25 J cm^{-2} at a fluence rate of 25 mW cm^{-2}. The spheroids were scored after three weeks in culture. The two water-soluble compounds, ALA and m-ALA, demonstrated a relatively weak PDT effect at these concentrations, with 100% of the spheroids showing growth with only a short growth delay compared to controls. In contradistinction, h-ALA and b-ALA showed significant growth inhibition. Since the methyl-ester seemed to behave very much like the parent ALA compound, it was excluded from further evaluation.

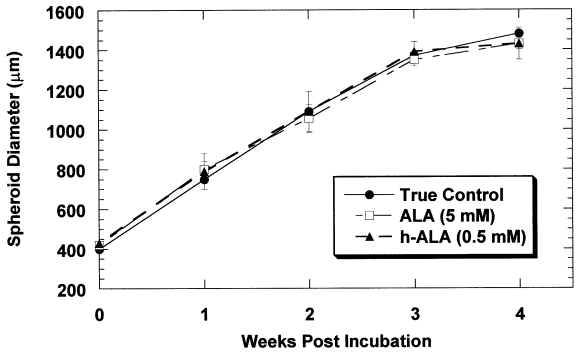

Figure 1. Growth kinetics of non-irradiated spheroids.

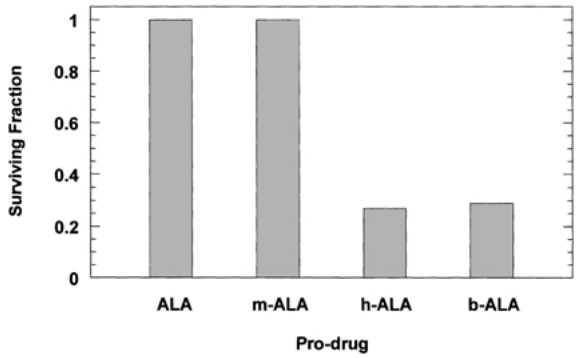

Figure 2. Spheroid survival for ALA and its derivatives following PDT. In all cases, spheroids were incubated in drug concentrations of approximately 0.05 mM and exposed to light fluences of 25 J cm^{-2} delivered at a fluence rate of 25 mW cm^{-2}.

The effects of PDT at drug concentrations ranging from 0.025 to 0.5 mM are shown in Figure 3. Each data point is the mean of three experiments (approximately 64 spheroids) irradiated at a fluence of 25 J cm^{-2} (fluence rate = 25 mW cm^{-2}) and evaluated after four weeks in culture. As illustrated in Figure 3, all spheroids survived treatment at the two lowest ALA concentrations. In contrast, both ALA ester

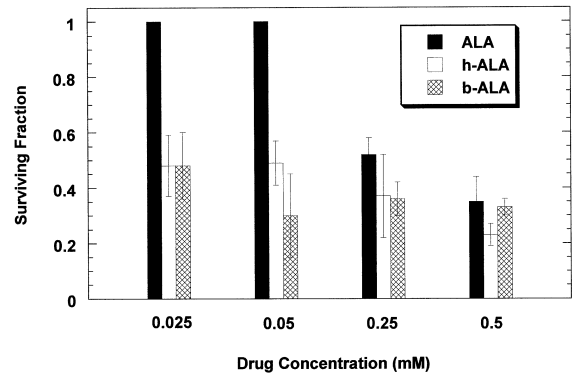

Figure 3. Effects of PDT on spheroid survival at various drug concentrations. Spheroids were exposed to light fluences of 25 J cm^{-2} delivered at a fluence rate of 25 mW cm^{-2}.

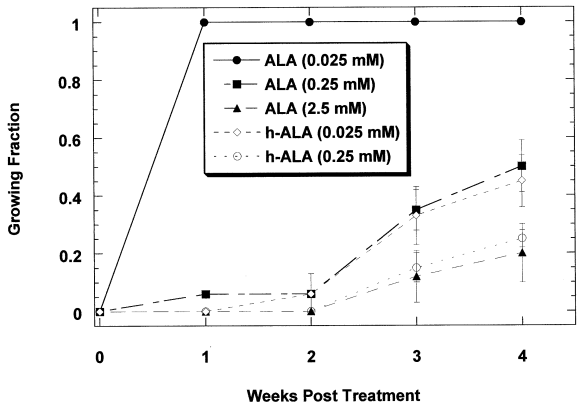

Figure 4. Growth kinetics of spheroids incubated in various concentrations of ALA or h-ALA. PDT treatments were performed using light fluences of 50 J cm^{-2} (fluence rate of 25 mW cm^{-2}).

derivatives demonstrated significant spheroid kill at all concentrations investigated, with a gradual decrease in spheroid survival with increasing concentration. No significant differences between h-ALA and b-ALA were observed in any of the experiments.

Spheroid growth kinetics at various concentrations of ALA or h-ALA are shown in Figure 4. In all cases, spheroids were irradiated to 50 J cm^{-2} at a fluence rate of 25 mW cm^{-2}. As illustrated in Figure 4, increasing the drug concentration resulted in a decrease in the number of spheroids showing growth during the culture period. A significant growth delay was observed for all but the lowest ALA concentration, in which case the growth kinetics were identical to true and dark control cultures. Although similar growth inhibition was observed for both ALA and h-ALA, it should be

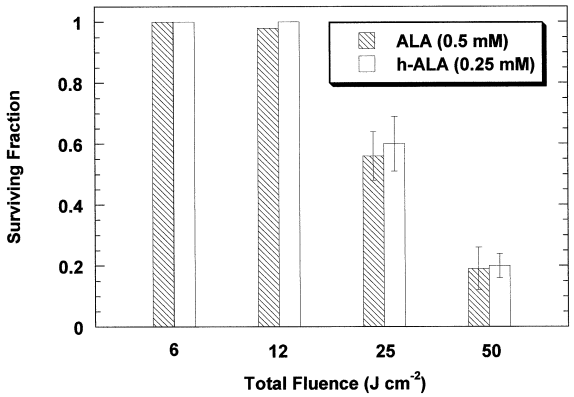

Figure 5. Effects of light fluence on ALA- or h-ALA-incubated spheroids. A fluence rate of 25 mW cm^{-2} was used in all cases.

noted that ALA concentrations were 10 times higher compared to h-ALA.

The effects of total fluence on spheroids incubated in ALA or h-ALA are shown in Figure 5. Spheroids were incubated at drug concentrations previously shown to give significant PDT effects (0.5 mM ALA or 0.25 mM h-ALA) and irradiated at an optimal fluence rate of 25 mW cm^{-2}. At sub-optimal fluence levels of either 6 or 12 J cm^{-2}, almost all spheroids survived treatment. Although higher fluences resulted in greater spheroid kill, no significant difference in survival was observed between the ALA- and h-ALA-incubated spheroids.

Figure 6(a) shows the effects of ALA- and h-ALA-induced PDT using optimal and sub-optimal fluence rates of 25 and 5 mW cm^{-2}, respectively. In all cases, spheroids were irradiated to a total fluence of 25 J cm^{-2}. As illustrated in Figure 6(a), low fluence rates of 5 mW cm^{-2} had no effect on spheroid survival at the lowest ALA and h-ALA concentrations. Increasing the concentration of both prodrugs resulted in significant spheroid growth inhibition comparable to that seen at optimal fluence rates of 25 mW cm^{-2}. At the higher concentrations, no additional growth inhibition was observed with h-ALA compared to ALA although the concentration of ALA required was 10 times greater. Figure 6(b) shows the kinetics of spheroid growth following low fluence rate PDT. Compared to controls, significant growth delay and growth inhibition was observed in both ALA and h-ALA incubated spheroids. In the case of the h-ALA spheroids, these results were obtained at concentrations 20 times lower than those of the ALA spheroids.

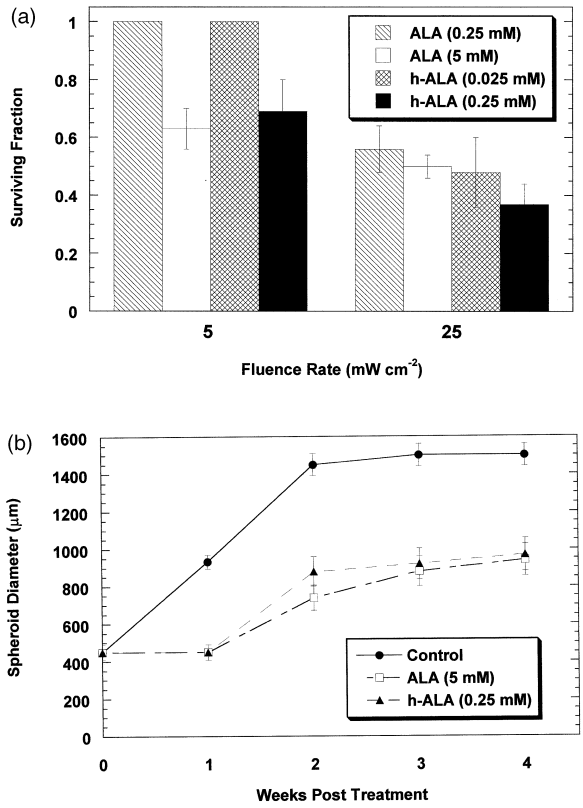

Figure 6. (a) Surviving fraction of spheroids incubated in ALA or h-ALA at two different fluence rates. Spheroids were irradiated to a light fluence of 25 J cm^{-2}. (b) Effects of low fluence rates on spheroid growth kinetics. Spheroids were irradiated to 25 J cm^{-2} at a fluence rate of 5 mW cm^{-2}.

Discussion

The efficacy of PDT is, in part, dependent on the ability to deliver adequate amounts of photosensitizer to the target tissue. Other parameters that influence the photodynamic cytotoxic effect include the light energy absorbed by the target tissue, the dose rate at which the light is delivered, tissue oxygenation status, and tissue sensitivity to the PDT effect.

Spheroids were used in this study since their three-dimensional geometry results in heterogeneous subpopulations of cells differing in proliferation, nutritional, metabolic and, most importantly, oxygenation status [19]. As a result, cells in different locations throughout the spheroid will experience different environments, thus mimicking the gradients found in solid tumors. In this study, changes in spheroid growth are used to examine the ability of representative ALA derivatives to induce PpIX photosensitizer production compared to the parent ALA compound.

The primary finding of this study is that the response of human glioma spheroids to PDT with lipophilic esters, such as b-ALA and h-ALA, is equivalent to that observed with ALA, albeit at concentrations that are 10–20 times lower. The enhanced efficacy of the esters is, in all likelihood, due to their increased membrane penetrance. The results of this study are consistent with those of Bigelow et al. [16] who found that comparable levels of PpIX fluorescence could be achieved throughout EMT6 spheroids with 100-fold lower h-ALA concentrations compared to ALA.

PpIX production induced by ALA esters is dependent on both the prodrug concentration and on the cleavage of the attached ester by cellular esterases, releasing ALA to the heme pathway [13]. Fluorescence microscopy studies in spheroids suggest that saturation of esterase activity occurs in the outermost cells at h-ALA concentrations of 0.05 mM [16]. In spheroids incubated in lower h-ALA concentrations, it is the ALA availability that is the limiting factor in PpIX production. Although PpIX production may be limited by esterase activity saturation at the high h-ALA concentrations used in this study, the PpIX levels nevertheless appear to be sufficient for the PDT effect. The results presented in this study suggest that it is the availability of the drug that is the limiting factor of the effectiveness of PDT in this system. For example, as shown in Figure 4, the number of spheroids exhibiting growth following treatment decreased with increasing h-ALA concentration.

Previous studies on isolated tissue sections have shown that PpIX fluorescence intensity, induced by h-ALA, is 2–3 times greater than that induced with ALA, even though 100 times more ALA is applied [20]. However, in the test system employed here, no increase in the induced spheroid response is observed for h-ALA compared to ALA at prodrug concentrations high enough to make the availability of PpIX sufficient at the light fluence levels used (Figure 5). This suggests that the previously demonstrated light energy threshold of approximately 50 J cm^{-2} for this spheroid model [21] is independent of the type of prodrug employed. Thus, although effective at much lower concentrations, the ester derivatives do not significantly increase the maximum PDT treatment effect in the human glioma spheroids used in this study.

The observation that lower fluence rates are more effective than higher ones has been demonstrated in a number of spheroid systems [18,21]. Theoretical

models suggest that the spatial distribution of singlet molecular oxygen (the primary cytotoxic species) depends critically on the fluence rate and on the availability of ambient oxygen [22]. At a given spheroid depth, the concentration of singlet molecular oxygen increases as fluence rates decrease. As a result, photodynamic damage will extend further into the spheroid as the fluence rate is lowered. Thus, PDT administered at lower fluence rates yields improved therapeutic response since singlet molecular oxygen is delivered to a larger volume of tumor cells in the spheroid.

In addition to the difficulties associated with delivering adequate amounts of photosensitizer to tumor tissues, the success of ALA-induced PDT in a therapeutic treatment protocol for patients harboring gliomas is likely to be limited by the high attenuation of light in the brain, resulting in very low dose rates at depths greater than 1 cm in the resection margin. We have recently described an indwelling balloon applicator allowing both long-treatment times and the possibility of repeatable PDT treatments [23,24]. Relatively high fluence levels can be achieved by extending treatment times to several hours, but the limitations set by very low fluence rates are not as easily overcome due to the thermal limitations of brain tissue [25]. For example, the high incident laser powers required to obtain sufficient fluence rates at centimeter depths in brain tissue would produce unacceptable thermal damage to tissues near the source. As illustrated in Figure 6(a) and (b) there appears to be no compelling reason to choose h-ALA over ALA, at low fluence rates as both prodrugs produce similar effects at sufficient concentrations.

The high lipopilicity of h-ALA makes it well adapted to topical application. This is advantageous since much higher levels of cellular PpIX can be achieved with this application route compared to IV administration [15]. The effects of direct *in situ* application of h-ALA in the resection cavity following tumor resection would thus offer the advantages of lower systemic side effects and high tissue concentrations compared to other modes of administration. Animal experiments are now in progress to explore this possibility.

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