Effects of Low-Fluence Rate PDT on Glioma Spheroids

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ABSTRACT

The effectiveness of low-fluence rate PDT on human glioma spheroids incubated in 5-aminolevulinic acid (ALA), or hexyl-ALA, is investigated. Spheroid survival is monitored following PDT at representative drug concentrations, light fluences and fluence rates. It is shown that, for above threshold fluences, significant PDT response can be obtained at a fluence rate of 5 mW cm^{-2}. At this fluence rate, the response of human glioma spheroids to PDT with lipophilic ester derivatives, such as hexyl-ALA, is equivalent to that obtained with ALA, however, this equivalency is observed for ester concentrations 20 times lower than the parent compound. The efficacy of PDT is significantly reduced at a sub-threshold fluence of 25 J cm^{-2}. Spheroid response appears to be somewhat fluence rate dependent at sub-threshold fluences – survival is marginally poorer at the higher fluence rate (25 mW cm^{-2}) investigated. In addition, the efficacy of PDT at 25 J cm^{-2} depends significantly on photosensitizer concentration at the lowest fluence rate investigated (5 mW cm^{-2}).

Keywords: Photodynamic Therapy, 5-Aminolevulinic Acid, hexyl-ALA, glioma spheroids, fluence rate

1. INTRODUCTION

The prognosis for patients with glioblastoma multiforme (GBM) is dismal – five-year survival is less than 5%[1]. The high mortality rate is primarily due to an inability to surgically remove the tumor – in approximately 80% of patients, the tumor recurs within 2 cm of the resection margin[2]. Clearly, a more aggressive local therapy could be of benefit to GBM patients. A number of studies suggest that photodynamic therapy (PDT) may prove useful as an adjuvant local treatment. Although PDT appears promising, the destruction of tumor cells at cm depths in the resection margin is difficult to achieve due to poor light penetration in brain tissues and inadequate photosensitizer concentrations in tumor cells.

Porphyrins such as Photofrin® and hematoporphyrin derivative, have been employed almost exclusively in clinical PDT trials of the brain. Although a number of groups have reported favorable results with Photofrin[3,4], it has a number of drawbacks that limits its effectiveness. Perhaps the most significant of these is the uncommonly long period of skin photosensitization observed following systemic administration of this drug. The slow clearance of Photofrin from cutaneous tissues has limited its use to single treatments and, in the case of brain PDT, treatments have been carried out intraoperatively. Under these conditions however, only the most superficial tissues receive threshold light fluences. ALA, a precursor of the potent photosensitizer Protoporphyrin IX (PpIX), may be a promising alternative to the sensitizers commonly used in brain PDT. The fast clearance of ALA from cutaneous tissues (24 – 48 h) makes it ideally suited to fractionated and long-term repeat PDT regimens. Another attraction of ALA is that it can be administered

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orally.

A significant shortcoming of ALA is its limited ability to cross certain biological barriers such as the stomach wall, intestine and cellular membranes as a result of its low lipid solubility[5]. This may require clinically impractical ALA drug doses in order to induce sufficient Pp IX accumulation and subsequent photodamage. In order to improve the bioavailability of ALA, prodrug derivatives are currently being synthesized and evaluated. A prodrug is a pharmacologically inactive derivative of the parent that is enzymatically converted to the parent at the site of action. It is a well known concept in pharmacology that increasing the number of carbon atoms in a carbon chain attached to an existing drug results in increased lipophilicity[6]. This process is known as esterification. The esterification of ALA changes the relative lipophilicity by three to four orders of magnitude compared to the parent compound[7]. Increased lipophilicity is expected to facilitate membrane transport and ultimately increase PpIX concentrations within tumor cells.

This study investigates the effects of low fluence rates (5 mW cm^{-2}) on human glioma spheroids incubated in ALA or hexyl-ALA. Previous studies[8] have found fluence rates of 10 mW cm^{-2} very effective at destroying spheroids incubated in ALA. The present study extends these findings to lower fluence rates and compares the efficacy of ALA to hexyl-ALA.

2. MATERIALS AND METHODS

2.1 Chemicals

Aminolevulinic acid hydrochloride was purchased from Sigma (St.Louis, MO). ALA hexyl ester was supplied by PhotoCure (Oslo, Norway). The ester was first dissolved in DMSO (100 mM) before further dilution in culture medium.

2.2 Cell Cultures

Cells from a grade IV GBM cell line (ACBT- G. Granger, University of California, Irvine) were cultured in DMEM (Gibco, Grand Island, NY) with high glucose and supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10 % heat-inactivated fetal bovine serum (Gibco, Grand Island, NY). Cells were maintained at 37 ^{0}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 minutes. The resultant cell clusters (consisting of approximately 10 cells) were transferred to a petri dish and grown to tumor spheroids of varying sizes. Spheroids of 400 μm diameter were selected by passage through a screen mesh (Sigma, St. Louis, MO). It took approximately 20 days for the spheroids to reach a size of 400 μm. The spheroid culture medium was changed three times weekly.

2.3 PDT Treatments

Spheroids were incubated in: (1) ALA at a concentration of 0.25 or 5.0 mM, or (2) hexyl-ALA at a concentration of 0.025 or 0.25 mM. In all cases, the incubation time was approximately 4 h. Spheroids were irradiated in an incubator (Figure 1) designed to maintain physiological conditions (37 ^{o}C and ca. 5% CO_2). Since CO_2 could not be introduced into the main incubator, the spheroids were put in a sealed Lucite box that was placed into the incubator. Four petri dishes containing baking powder were also put in the Lucite box. Just prior to irradiation, acetic acid was added to the baking powder in order to produce CO_2. The box was sealed and 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 that was introduced into the incubator via an aperture. 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 3 times, a total of 48 to 72 spheroids were followed for a given set of parameters. Spheroids

were followed for up to 28 days. In the case of the fluence studies, spheroids were subjected to a light fluence of either 25, 50, or 100 J cm^{-2} delivered at a fluence rate of 5 mW/ cm^{-2}. For the fluence rate studies, spheroids were irradiated at 5 or 25 mW cm^{-2} using a sub-optimal fluence of 25 J cm^{-2}.

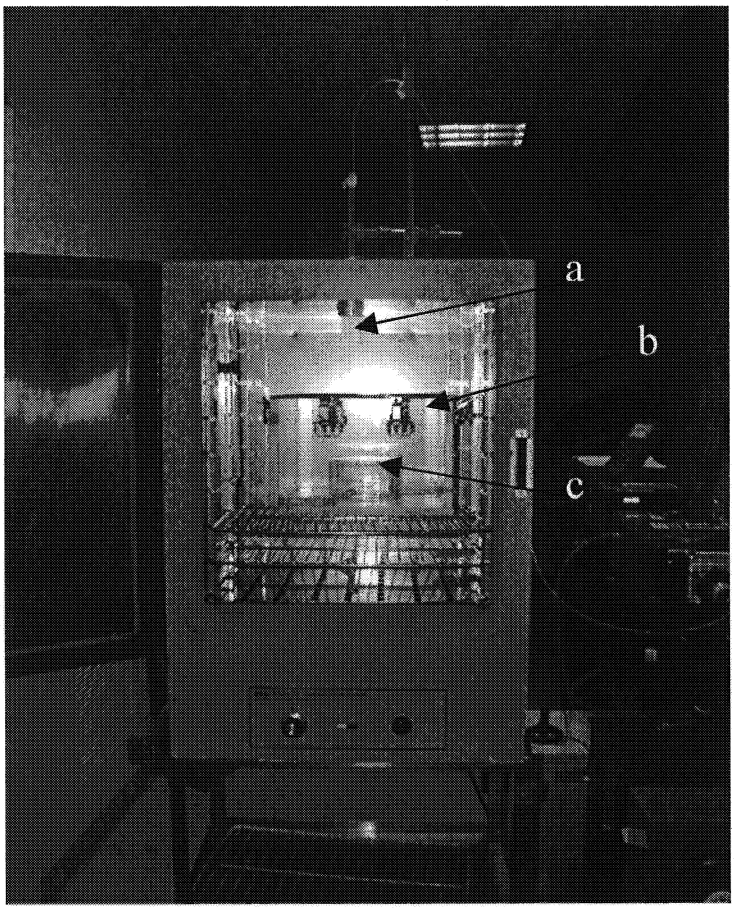

Figure 1. Photograph of incubator. (a) Source fiber, (b) Lucite box, (c) Petri dish with spheroids.

3. RESULTS

Effects of fluence on spheroids incubated in ALA or hexyl-ALA are illustrated in Figure 2. It is shown that, even at the low fluence rate investigated (5 mW cm^{-2}), significant spheroid response is observed at fluences of 50 J cm^{-2} and higher. Although there is no difference in response between the two compounds, the hexyl-ALA data are obtained at a concentration 20 times lower than the parent drug.

The effects of fluence rate on spheroids subjected to a sub-optimal fluence of 25 J cm^{-2} are shown in Figure 3. The lower fluence rate seems particularly ineffective at evoking a response. This is especially true at the lower ALA and hexyl-ALA concentrations where PDT has no effect, i.e., 100% survival. Increasing the fluence rate to 25 mW cm^{-2} results in significant improvement in response of spheroids incubated in the lowest concentration of ALA (0.25 mM) or hexyl-ALA (0.025 mM). There are only slight variations in response among the four groups of spheroids subjected to the higher fluence rate – spheroids incubated in 0.25 mM hexyl-ALA seem to have a marginally better response than the others.

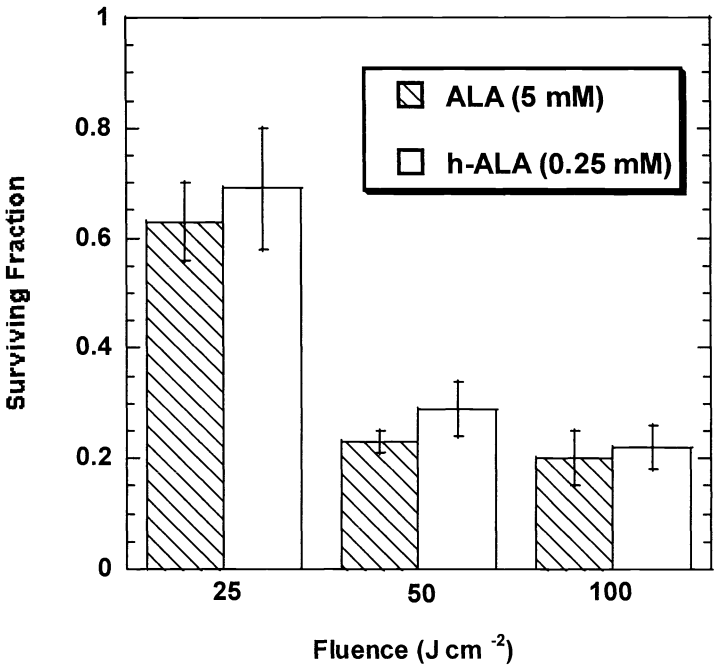

Figure 2. Spheroid response as a function of fluence. In all cases, spheroids were irradiated at a fluence rate of 5 mW cm^{-2}.

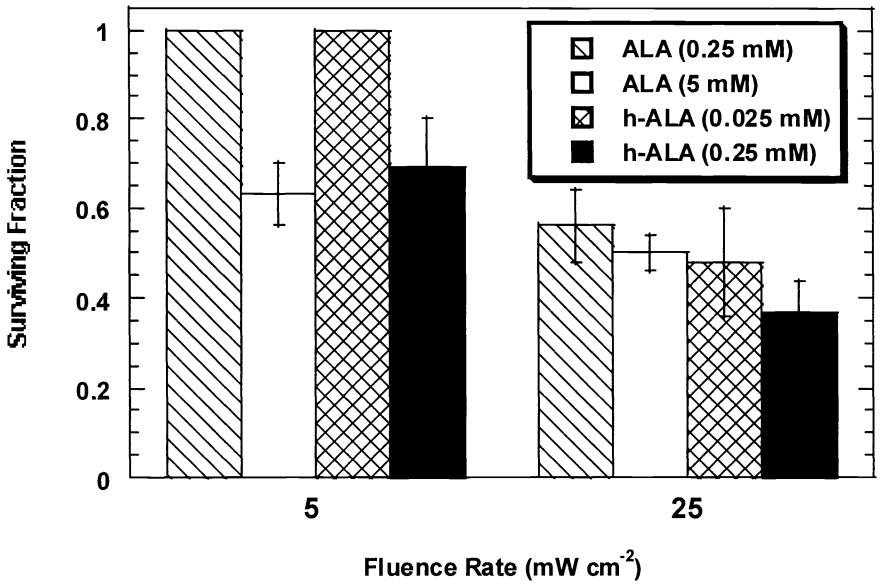

Figure 3. Spheroid response as a function of fluence rate. In all cases, spheroids were subjected to light fluences of 25 J cm^{-2}.

4. DISCUSSION

The effects of fluence rates have been investigated in a variety of *in vitro* and *in vivo* systems. In a previous study[8], it was shown that low-fluence rate PDT can elicit a significant response in human glioma spheroids – near total spheroid kill was observed at fluence rates of 10 mW cm^{-2} (total fluence = 50 J cm^{-2}). Studies using lower fluence rates are difficult to perform due to the long irradiation times required to deliver threshold fluences. For example, it takes approximately 3 h to deliver a fluence of 50 J cm^{-2} at a fluence rate of 5 mW cm^{-2}. A potential solution to this problem is to perform the irradiation in an incubator where the cells will be maintained under physiological conditions throughout the irradiation period. The efficacy of low-fluence rate PDT is particularly relevant for GBM patients since tumor cells in the resection margin will be exposed to very low fluence rates. For example, calculations show that, for an input power of 1 W, fluence rates at 1.5 cm depth in typical brain tissue range from 1 to 3 mW cm^{-2}, depending on the irradiation geometry[9].

The results presented in Figure 2 show that significant spheroid response can be obtained at fluence rates of 5 mW cm^{-2}, as long as the fluence threshold is exceeded. The exact value of this threshold cannot be determined from this study, however, it appears that a fluence of 50 J cm^{-2} is sufficient. The results also suggest that, on a per molar basis, hexyl-ALA is more effective than the parent compound. At sub-threshold fluences (Figure 3), higher fluence rates appear to be slightly more effective; this is especially true at the lowest concentrations of ALA (0.25 mM) and hexyl-ALA (0.025 mM) where all spheroids survived treatment. These results are not surprising since PDT dose depends on the product of photsensitizer concentration and fluence rate[10]. PDT dose increases with increasing drug concentration and/or fluence rate, as long as the fluence rate is sufficiently low such that photobleaching is minimized.

Although ALA has several features that make it a useful photosensitizer, its relatively high hydrophilicity limits its penetration in biological tissues. This is a potential problem in brain PDT since the drug must be capable of diffusing to tumor cells located deep in the resection margin following topical application. One strategy to improve drug penetration is to increase its lipophilicity. The lipophilic ALA derivatives presently being evaluated for use in PDT are produced by esterification of ALA with aliphatic alcohols. The production of PpIX induced by ALA esters is related to the aliphatic length of the alcohol. Hexyl-ALA was investigated in this study since the results of others[7,11] indicate that the highest PpIX concentrations *in vitro* are obtained by esterification with hexanol. It should be pointed out however, that the enhancement ratio induced by esterification of ALA on PpIX accumulation is somewhat cell line dependent[11]. This is partly due to variations in cellular esterase activity among different cell lines. The results presented here suggest that there is sufficient esterase activity in human glioma cells for significant PDT response when using ALA esters.

The observation that hexyl-ALA is more effective (on a per molar basis) than the parent compound is clinically relevant since it suggests that much lower concentrations may be administered. This is important since oral administration of ALA is limited to approximately 60 mg/kg[12] due to systemic toxicity. Furthermore, due to its higher lipophilicity, higher concentrations of hexyl-ALA are expected to pass through the blood-brain-barrier compared to ALA. It is important to note that the feasibility of using ester derivatives in systemic applications is unknown. It may be the case that the improved transport properties of the esters result in higher build-up of PpIX in normal tissues and, hence, to greater systemic toxicity. This is currently being investigated in animal models. For topical applications however, the results of this study confirm the findings of other investigations, namely that the use of lipophilic ALA esters is likely to result in greater PpIX concentrations in biological tissues at depth.

5. CONCLUSIONS

The results of this study show that low-fluence rate PDT causes a significant reduction in spheroid survival when using above threshold light fluences. Although equivalent responses were observed for ALA and hexyl-ALA, the lipophilic ester derivative was effective at concentrations 20 times lower than the parent compound. The efficacy of PDT is significantly lower when spheroids are exposed to a sub-threshold light fluence. In this case, spheroid response depends critically on fluence rate and photosensitizer concentration.

From a clinical point of view, the results reinforce the importance of adequate light and drug delivery during PDT.

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