Photodynamic therapy of human glioma spheroids: a comparative study of the effectiveness of 5-aminolevulinic acid and its esters

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ABSTRACT

Although 5-aminolevulinic acid (ALA) has several advantages over other photosensitizers, its hydrophilic nature gives rise to relatively poor transport across cell membranes. Esterification of ALA is a commonly used strategy to improve the effectiveness of ALA. In this study, the effectiveness of photodynamic therapy (PDT) in human glioma spheroids incubated in ALA, or ALA esters, is investigated. Spheroid survival and growth are monitored following PDT at representative drug concentrations and light fluences. It is shown 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 observed for ester concentrations 10 to 20 times lower than the parent compound.

Keywords: Photodynamic therapy, ALA, ALA esters, glioma spheroids, brain tumor, light fluence

1. INTRODUCTION

ALA induced protoporphyrin IX (PpIX) has been used in PDT and fluorescence detection in a variety of cancerous and precancerous lesions[1]. The advantages of ALA-mediated PDT include: rapid clearance from cutaneous tissues, easy administration (oral and/or topical), and the possibility of repeated treatment. Unfortunately, due to its hydrophilic nature, ALA does not easily penetrate cellular membranes. As a result, therapeutic levels of PpIX often require high ALA concentrations and long application times. A number of suggestions have been proposed to improve the penetrance of ALA in biological tissues. These include: (1) the use of transport promoters, such as DMSO, or liposome packaging[2], (2) iontophoresis[3], (3) modulation of the biosynthetic pathway by addition of iron chelators[4], and (4) synthesis of more lipophilic ALA esters[5].

The esterification of active substances to obtain a more lipophilic prodrug, which upon entering a cell is hydrolized by esterases, is a well-known pharmacological concept[6]. In the case of ALA prodrugs, conversion into original ALA, at the site of action, results in PpIX synthesis in the same way as administration of ALA itself. Thus, from a photochemical point of view, there is no difference between administration of ALA prodrugs and ALA. The use of ALA esters has been shown to induce comparable PpIX production at much lower drug doses compared to ALA[7]. ALA esters also demonstrate increased spatial confinement when applied to skin and exhibit greater tissue penetrance relative to ALA[8]. Finally, the results of both *in vitro*[9] and *in vivo*[10] 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 and drug combinations. Spheroid survival was monitored as a function of

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drug concentration and light fluence.

2. MATERIALS AND METHODS

2.1 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. 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[7]. The esterfication of ALA changes the relative lipophilicity by three to four orders of magnitude compared to the parent compound[7].

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 concentrations ranging from 0.025 to 5.0 mM, or (2) ALA ester derivatives at concentrations of 0.025 to 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 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 6,12,25 or 50 J cm^{-2} delivered at a fluence rate of 25 mW/ cm^{-2}.

3. RESULTS

The effects of PDT at drug concentrations ranging from 0.025 to 0.5 mM are shown in Figure 1. Each data point is the mean of 3 experiments (approximately 64 spheroids) irradiated to a fluence of 25 J cm^{-2} (fluence rate = 25 mW cm^{-2}) and evaluated after four weeks in culture. As illustrated in Figure 1, all spheroids survived treatment at the two lowest ALA concentrations. In contrast, both ALA ester 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. In an initial set of experiments, the response of spheroids to PDT with m-ALA was found to be identical to that of the parent compound (data not shown). This is not surprising since both compounds are water-soluble. As a result, m-ALA was excluded from further evaluation.

Spheroid growth kinetics at various concentrations of ALA or h-ALA are shown in Figure 2. In all cases, spheroids were irradiated to 50 J cm^{-2} at a fluence rate of 25 mW cm^{-2}. As illustrated in Figure 2, 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 noted that ALA concentrations were 10 times higher compared to h-ALA.

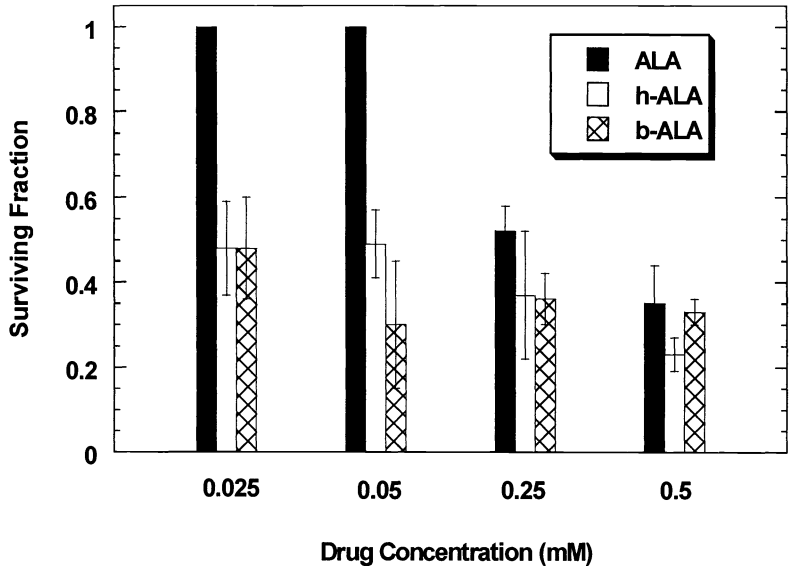

Figure 1. Effects of PDT on spheroid survival at various drug concentrations. Spheroids were exposed to a fluence of 25 J cm^{-2} delivered at a fluence rate of 25 mW cm^{-2}.

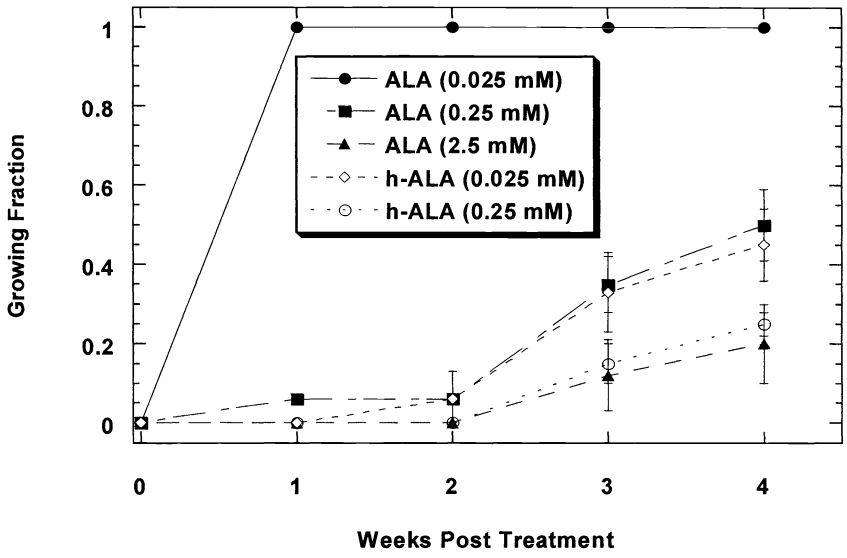

Figure 2. Growth kinetics of spheroids incubated in various concentrations of ALA or h-ALA. PDT was performed using a fluence of 50 J cm^{-2} (fluence rate of 25 mW cm^{-2}).

The effects of total fluence on spheroids incubated in ALA or h-ALA are shown in Figure 3. 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.

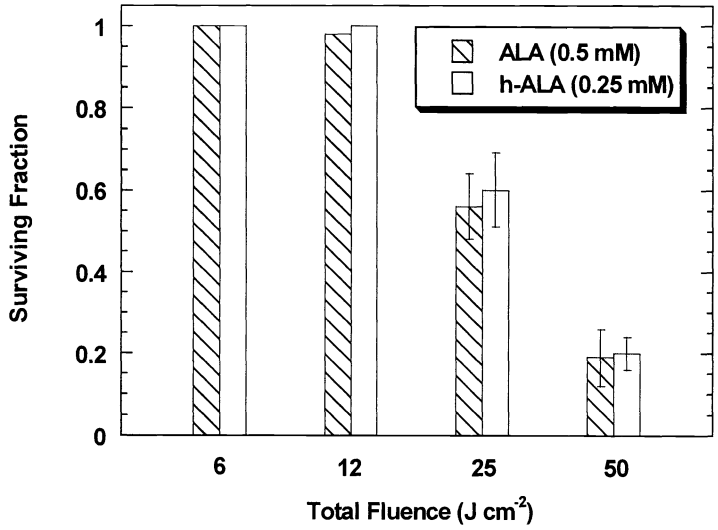

Figure 3. Effects of fluence on ALA- or h-ALA-incubated spheroids. A fluence rate of 25 mW cm^{-2} was used in all cases.

4. DISCUSSION

A number of *in vitro* and *in vivo* studies have shown that the use of ALA ester derivatives results in higher PpIX levels than free ALA[11]. Long-chain esters, such as h-ALA, seem to be particularly effective compounds[12]. For example, equivalent PpIX levels in human cells have been observed at h-ALA concentrations 100-fold lower than ALA[11]. Although the exact mechanism of uptake of ALA and its derivatives in human cells is unknown, results of recent studies support the hypothesis that ALA enters cells primarily through transporters rather than by passive diffusion. Increasing the lipophilicity of ALA may increase cellular uptake due to the increased likelihood of passive diffusion across cell membranes. It should be noted however that, although cellular uptake may be enhanced with lipophilic esters, this may not necessarily result in enhanced PpIX production since ALA esters must first be cleaved by cellular enzymes. Thus, the efficacy of ALA esters depends critically on intracellular esterase activity. The present study suggests that these enzymes are present in the human glioma spheroid model used in this investigation.

As shown in Figures 1 and 2, lipophilic ALA esters are approximately 10 to 20 times more effective (on a per molar basis) than the parent compound. It is possible that the effectiveness of the esters is even greater than the present results suggest. For example, spheroid survival and/or growth suppression may be affected at lower concentrations than those shown in Figures 1 and 2. The present findings are in qualitative agreement with those of Bigelow et al[9] who found that comparable levels of PpIX fluorescence could be achieved throughout EMT6 spheroids with 100-fold lower h-ALA concentrations compared to ALA. Although effective at much lower concentrations, the ester derivatives do not significantly increase the

maximum PDT effect in human glioma spheroids (Figure 3). Thus, the previously demonstrated light energy threshold of approximately 50 J cm^{-2} for this model[15] is independent of the type of prodrug used.

The observation that lipophilic esters are more effective at producing PpIX than free ALA is clinically relevant since PDT treatments could presumably be carried out at significantly reduced ester concentrations. The high lipophilicity 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[8]. This is relevant for PDT treatments of high-grade gliomas since the effects of direct *in situ* application of h-ALA in the resection cavity following tumor resection would offer the advantages of lower systemic side effects and high tissue concentrations compared to other modes of administration. Animal experiments are currently in progress to explore this possibility.

5. CONCLUSIONS

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 to 20 times lower. The enhanced efficacy of the esters is, in all likelihood, due to their increased membrane penetrance. In the *in vitro* system investigated, no increase in 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 fluences studied. The ester derivatives thus do not significantly increase the maximum PDT treatment effect in human glioma spheroids.

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