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Interstitial5-ALA photodynamic therapy and glioblastoma: preclinical model development and preliminary results.

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Interstitial 5-ALA photodynamic therapy and glioblastoma: preclinical model development and preliminary results.

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Abstract:

Objective: Photodynamic therapy (PDT) has become a well-established modality for the treatment of many cancers. Photodynamic eradication of tumor cells depends on the presence of a photosensitizer, oxygen and light. However, oxygen depletion during PDT is a well known problem. Modulation of light delivery could address this issue by counteracting tumor hypoxia, thereby improving tumor cell killing. This preclinical study was designed to validate an animal model incorporating 5-aminolaevulinic acid (5-ALA)-PDT using U87 glioblastoma cells. We aimed to evaluate the effects of light modulation for inducing specific tumoral lesions in this model (i.e., necrosis or apoptosis).

Materials and Methods: U87 glioblastoma cells were stereotactically engrafted into the brains of male fox1 rnu/rnu rats. Light delivery was studied after 5-ALA injection (100 mg/kg i.p.). 26 J of 635 nm light was interstitially delivered to U87 tumor-bearing rats at a radiant power of either 30 mW (high fluence rate) or 4.8 mW (low fluence rate). In each group, half of the population received illumination in 2 fractions with a refractory interval of 120 seconds, whereas the other half received continuous illumination.

Results: Twenty-two animals received 5-ALA-PDT, and the level of necrosis was scored. In the high-fluence-rate group, we observed a greater degree of tumor necrosis in rats receiving fractionated delivery than in rats receiving continuous illumination. Similar differences were not observed in the low-fluence-rate group, which exhibited only sparse necrosis. Higher morbidity and mortality rates were observed in the high-fluence-rate group.

Conclusion: We have developed a reproducible and reliable rodent model for interstitial 5-ALA PDT. We found that the effects of 5-ALA-PDT are dependent on light delivery conditions. Although the low-fluence-rate treatment was better tolerated, 5-ALA-PDT induced more necrosis using fractionated delivery at a high fluence rate. These results require

confirmation with further studies involving larger populations and additional fractionation schemes.

Keywords: photodynamic therapy; high-grade glioma; 5-ALA; PpIX; U87; rodent model

Conflict of interest: none

1. Introduction

With an incidence of 3-5 cases/100,000 individuals/year, glioblastoma is the most common malignant primary tumor of the central nervous system². The standard treatment is a combination of surgery, radiotherapy and chemotherapy, and it achieves a median patient survival time of 15 months³⁷. The goal of surgery is to achieve maximum tumor cytoreduction while preserving neurological function⁴². The majority of treatment failures are due to local recurrence of the tumor, suggesting that more aggressive local therapy could be beneficial³³. Photodynamic therapy (PDT) is a local treatment based on light activation of a photosensitizer (PS) using a laser of a specific wavelength^{19,32}. In the presence of oxygen, light interacts with the PS and forms cytotoxic species. 5-Aminolevulinic acid (5-ALA) is a PS precursor. Indeed, the heme biosynthesis pathway leads to the production of 5-ALA-induced protoporphyrin IX (PpIX), an endogenous PS³². PpIX is an excellent candidate for clinical PDT because of its highly selective uptake by tumors^{23,35,39}. Furthermore, PpIX is relatively nontoxic, with a cutaneous photosensitization period limited to 1-2 days^{35,45}. These qualities are exploited during Fluoro-Guided Resection (FGR) of high-grade gliomas using 5-ALA. A randomized study demonstrated that complete resections of contrast-enhanced tumors were more frequently achieved in malignant glioma patients when 5-ALA FGR was included³⁵.

Previously, clinical studies reported promising results when employing PDT on patients suffering from recurrences of high-grade gliomas, with a median survival increase from 3-9 months to 15 months^{4,34}. Nevertheless, several issues still need to be addressed, including our ability to identify the optimal doses of light and PS, the inter-individual variability in light distribution, and PS accumulation and tissue oxygenation. In particular, oxygen is a critical factor in the efficacy of PDT, as the cytotoxic effect of PDT is mediated by reactive oxygen species^{17,32}. Thus, in glioblastoma, pre-existing hypoxia is increased by the oxygen consumption required to carry out the photochemical reaction. This reversible tissue hypoxia might significantly reduce the efficacy of PDT by decreasing the production of photochemical species^{12,17,21}. Furthermore, the vasoconstriction of abnormal tumor microcirculation induced during PDT suppresses reperfusion and potentially induces angiogenesis in the brain tissue adjacent to the tumor, subsequently contributing to tumor regrowth⁴⁹.

Modulation of light delivery could decrease tissue hypoxia^{10,12}, and low fluence rates have been reported to improve the efficiency of PDT, leading to increased apoptosis^{3,12,27}. However, the delivery of such illumination schemes will likely require specific protocols or devices (e.g., to repeatedly administer PS, an intracranial optical-fiber-based device might be necessary for several days). In addition, the application of multiple fractions of light exposure¹² at higher fluence rates is being investigated as a means to counteract hypoxia. Fractions are defined as alternating 'on/'off' periods that allow for reoxygenation. Therefore, fractionation is expected to improve PDT because available molecular oxygen can be supplied by the capillary vasculature during the off-period. Fractionated PDT is promising because it may improve efficiency without dramatically increasing treatment time. Most studies on illumination fractionation have investigated tumors in other locations, such as prostate cancers

and skin lesions^{15,46}. Studies of high-grade gliomas failed to demonstrate any benefit of fractionated illumination in PDT using HpD or m-THPC^{9,40}. These PSs appear to be less sensitive to fractionation, as they rapidly damaged tumor vasculature⁸. No experimental studies to date have reported the effects of fractionated 5-ALA-PDT on high-grade gliomas.

Based on promising results in other areas of oncology, we investigated the effect of fractionated 5-ALA-PDT in a model of glioblastoma. Due to the importance of angiogenesis in glioblastoma, the most relevant model involves U87 gliomas engrafted into male fox1 rnu/rnu rat brains³⁸. U87 is one of the most commonly used cell lines in the field of glioblastoma research^{6,18,22,25,48}. This well-characterized cell line allows creates rapidly growing tumors with a high mitotic ratio and a satisfactory engraftment rate. Although U87 does not reproduce all the characteristics of human glioblastoma, such as spontaneous necrosis and an infiltrative pattern, these differences could be advantageous, as 1) the observed necrosis can be attributed completely to PDT, and 2) the absence of an infiltrative pattern should allow for more precise characterization of the treatment at a distance from the fiber tip.

Properly investigating the benefits of light fluence rates and of fractionation schemes requires a comprehensive experimental design including different groups of animals (e.g., high/low fluence rates, fractionation/no fractionation, and sham group with light/without light). Considering this, defining a preclinical model must be addressed before further studies can be performed. In this study, we define a proof of concept model as a basis for future studies. Thus, our main objective was to validate a preclinical model and experimental design for 5-ALA PDT with respect to treatment response, grafting procedure, histology analysis and imaging procedures. Second, we studied the influence of light fractionation and light fluence rate using this model to define preliminary trends for the treatment response. Toward this end, two fluence rates and one light fractionation scheme were evaluated to prove the utility of the procedures and outline protocols for further study.

2. Materials and methods

2.1. Cell culture

PDT experiments were conducted using the U87 human glioma cell line (ATCC HBT-14). The cell line was cryopreserved in liquid nitrogen. U87 cells were grown as monolayers in DMEM (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, Glutamax, pyruvate, and essential and nonessential amino acids. Cell culture plates were incubated at 37°C and 5% CO₂. After harvesting the U87 cells with 0.25% trypsin, they were washed with Dulbecco's phosphate-buffered saline (PBS, Gibco Life Technologies) and counted.

2.2. Experimental animals

Athymic Fox1 rnu/rnu male rats (Harlan, Gannat, France) weighing 90-100 g (8 weeks old) at the start of the study were caged in air-filtered cages. The animal holding rooms were maintained at a constant temperature and humidity on a 12-hour light and dark schedule with an air exchange rate of 18 changes per hour. Animal care and protocols were in accordance with national legislation and institutional guidelines^{16,29}. The rats were anesthetized with

isoflurane (1-2%) in oxygen during tumor implantation, photo illumination and neuroimaging.

2.3. 5-ALA preparation and administration

5-ALA was obtained as a hydrochloride powder. For intraperitoneal administration, 5-ALA was dissolved in PBS immediately before injection. All injections were intraperitoneal.

2.4. Rat glioma model

U87 glioblastoma cells (5×10^5 in $5 \mu\text{l}$) were stereotactically implanted into the right putamen using Paxinos atlas coordinates to induce intracerebral glioblastomas³¹. The anesthetized rats were fixed in a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). The skin was incised under local anesthesia (lidocaine hydrochloride), and a burr hole was drilled at the following coordinates: 0.5 mm anterior and 2.7 mm to the right of the bregma. The needle was introduced through the burr hole and into the brain at a depth of 4.4 mm, and $5 \mu\text{l}$ of culture medium was injected. A delay of 10 min was observed between needle insertion into the brain and the onset of cell injection. The needle was left in place for 15 min after completing the injection to prevent the spread of tumor cells during retraction. The cells were injected at a rate of $0.5 \mu\text{l} \cdot \text{min}^{-1}$ via an n°30-G needle (RN G30 PST3 51 mm, Hamilton Company, Reno, NV, USA) fixed to a $10 \mu\text{l}$ microsyringe (1700 model 1701-RN, Hamilton Company) controlled by an electrical pump (KDS 310; KD Scientific, Holliston, MA, USA). Prior to needle introduction, an anchor²⁶ was erected to preserve a consistent position for grafting and treating. This anchor consisted of a cranial plate, a cannula projecting from the upper face of the plate and a prepositioning clip projecting from the bottom face of the plate. The cannula, the plate and the clip formed a hollow tube that guided the fiber perpendicular to the plate. The clip was positioned over the burr hole and then the anchor was positioned and fixed with biological tissue adhesive (Dermabond®, Johnson & Johnson/Ethicon, Somerville, NJ, USA). Finally, closure was performed around the cannula of the anchor using Vicryl 4/0 (Ethicon, Somerville, NJ, USA).

2.5. Magnetic resonance imaging (MRI)

After engraftment, U87 tumor-bearing rats were followed-up periodically using a small-animal 7T MRI system (Bruker BioSpec, Ettlingen, Germany). Following anesthesia, animals were subjected to T2-weighted (T2W) fast spin echo pulse sequences (TR=5000 ms, TE=77 ms, slice thickness=850 μm) and T1-weighted (T1W) fast spin echo pulse sequences (TR=400 ms, TE=9 ms, slice thickness=850 μm) with and without gadolinium (T1WGd)-based contrast (MultiHence, 0.4 ml, i.p.). In the post-contrast studies, images were acquired 10 minutes after gadolinium administration. Tumor volumes were evaluated using standard viewing and reformatting software (*OsiriX 4.1.2*, 32-bit). The tumors were identified from their pathological contrast enhancement and the T2 signal. T2W sequences were also later used to assess perifocal edema. Tumor volume was computed by manual delineation based on each T2W image slice. The presence of an intratumoral hypointense signal was noted. MRI studies were performed at 7 and 14 days following engraftment and 48-72 hours after 5-ALA-PDT administration.

2.6.5-ALA-PDT of gross tumors

To determine the effects of 5-ALA-PDT on the tumors, 22 animals were subjected to treatment 14 days following inoculation with U87 cells. In all cases, animals received i.p. injections of 100 mg/kg 5-ALA³⁶, and 5-ALA-PDT was performed 5 hours later. This time interval was chosen in accordance with previous experiments^{30,36}. Prior to illumination, animals were anesthetized and positioned in the stereotactic frame. A 350- μm , bare, flat-end quartz fiber with a 0.29 numerical aperture was introduced through the anchor and into the brain at a depth of 4.4 mm. The fiber was tightened to the anchor, and the fiber was introduced into the tumor under the guidance of MRI. Light was delivered from a 635-nm diode laser. The animals were randomized into four treatment protocols. Animals in the continuous treatment mode group were subjected to a radiant energy of 26 J at a radiant power of either 4.8 mW (low-fluence-rate group, n=5) or 30 mW (high-fluence-rate group, n=6). For the fractionated regime, rats were exposed to 26 J at 4.8 mW (n=5) or 26 J at 30 mW (n=6); in these two groups, the light dose was paused after 5 J for a duration of 120 seconds before delivering the remaining light dose (21 J). The rats were euthanized 72 hours after 5-ALA-PDT and subjected to a final MRI. Their brains were then removed and prepared for histopathology.

2.7. Histological studies

The rodent brains were removed and fixed in formaldehyde following sacrifice (48-72 hours after PDT) or premature death. Brains were cut in the coronal plane (along the tumor injection trace) and subjected to HES staining. Tumor necrosis was scored by a senior neuropathologist who was blinded to the treatment modalities based on the number of HE-stained sections throughout the tumors as none, sparse, or extensive. Macrophagic invasion and apoptotic reactions were also recorded in the sections.

3. Results

Across all of the experiments, a tumor engraftment rate of 82% was achieved.

3.1. Magnetic resonance imaging

A total of 22 animals were treated using interstitial PDT (iPDT). Within two days of treatment, 7 of the animals died (*Table 1*). The remaining 15 animals were investigated by MRI 48-72 hours after treatment. The tumors were observed on T2W as high-intensity signal areas at the sites of cell injection. No spread of tumor cells to other brain areas was detected. T2W images also revealed edema in the brain adjacent to the tumor within a 2 mm radius. No necrotic or cystic regions were observed in these tumors.

The median tumor volume was 1.9 mm³ (0.35-5.1) on day 7 and 10 mm³ (0.84-55.2) on day 14 following implantation. In particular, in terms of the fluence-rate groups, the median volume at 14 days was 15.8 mm³ (1.44-55.2 mm³) in the high-fluence-rate group and 4.2 mm³ (0.84-11.9 mm³) in the low-fluence-rate group. Following 5-ALA-PDT, the median tumor volume was 19.3 mm³ (3.6-50.9 mm³). For the post-treatment MRI, no significant

changes were visible using conventional sequences, including T2W and T1W Gd images.

Table 1. Assignment of animals to various illumination groups.

3.2. Histology: variations in fluence rate and fractionation of light delivery

In total, 22 animals subjected to treatment were analyzed. Seven animals from the high-fluence-rate group died within 24 hours of treatment (4 in the fractionated group and 3 in the continuous group). The remaining 15 animals were sacrificed following post-treatment MRI at 48-72 h. Representative sections from the treated tumors are presented in *Fig. 1*, where the fiber tip path is shown (path estimated according to MRI slice, *Fig. 1 (a)*) on the internal margin of the tumor.

Fig 1. Histological sections

- (a) Fiber path viewed by MRI. (b) Low-magnification (x10) microphotographs show sparse necrosis with an intact tumor; fiber path is indicated with a dashed arrow. (c) Extensive tumor necrosis. (d) High-magnification microphotographs (x20) showing the tumor border in a normal brain (B), necrosis (N), and an intact tumor core (T). (e) Necrosis and apoptotic bodies at higher magnification.**

The levels of 5-ALA-PDT-induced necrosis in the HES-stained sections are shown in *Table 2*. Necrosis was observed in both the high- and low-fluence-rate groups. In most cases, the necrosis was sparse and localized around the fiber tip. In the high-fluence-rate group, increased necrosis was observed after fractionated illumination compared with the continuous illumination group. By contrast, necrosis was sparse in the low-fluence-rate group even after fractionated treatment.

Table 2. Tumor necrosis in HE-stained sections after 5-ALA-PDT treatment with 26 J of light delivered at high or low fluence rates with fractionated or continuous illumination.

Macrophage infiltration and apoptotic reactions were also observed in the necrotic and intact tumor areas, and no 5-ALA-PDT-induced necrosis was detected in the normal brain. However, edema was observed in the brain tissue adjacent to the tumor, particularly after high-fluence rate illumination.

Fig 2. Edema observed on T2w MRI.

3.3. Morbidity of interstitial PDT

Interstitial 5-ALA-PDT delivered at a high fluence rate 14 days after tumor induction resulted in clinical signs of elevated Intra Cranial Pressure (ICP) and was fatal in 58% (7/12) of the animals in these groups. Over the course of our experiments, corticoids were used to counteract unexpected animal death when applying high fluence rates. Consequently, 4 of these 7 rats were not administered methylprednisolone following treatment. Therefore, the

steroids were expected to increase treatment tolerance.

All animals receiving 5-ALA-PDT at a low fluence rate survived the treatment and did not exhibit the severe symptoms of increased ICP after light exposure.

The observed post-treatment morbidity resulted from a combination of the 5-ALA-PDT response, the tumor volume and, most likely, hydrocephalus. Indeed, MRI images indicated several cases of hydrocephalus related to trapping of the ipsilateral horn of the lateral ventricle, which could have increased ICP. Two animals with hematomas caused by introduction of the optical fiber were also noted; one resulted in the death of the animal. Concerning the administration of 5-ALA, no adverse effect was observed.

4. Discussion

Based on our results, 5-ALA-induced PpIX appears to be a valuable PS based on several criteria. First, good clinical tolerance was observed,³⁵ and previous experimental and clinical studies demonstrated a high degree of selectivity in malignant glioma tissues^{3,23,36,41}

The main factor limiting the efficacy of 5-ALA-PDT in the treatment of malignant glioma is tumor hypoxia, either pre-existing in the tumor or as a result of oxygen depletion during PDT. This hypoxia limits photochemical reactions that depend on oxygen and can consequently cause cellular destruction. Moreover, as a side effect, tumor hypoxia can induce angiogenesis and potentiate tumor regrowth.

In our study, we first aimed to validate a preclinical model and experimental protocol for 5-ALA PDT with respect to treatment response, grafting procedure, histology analysis and imaging procedures. These preliminary experiments enabled us to evaluate the effects of light modulation in this system. Indeed, previous studies suggested that the modulation of light delivery could enhance the efficiency of 5-ALA-PDT^{3,5,20,27}. For a given total light dose, low fluence rates were reported to be more effective compared with higher rates,^{3,5,27} most likely because stronger and faster O₂ depletion was associated with higher fluence rates¹². In our experiments, necrosis was observed at both low and high fluence rates. Nevertheless, the efficiency of the high fluence rate conflicted with prior results reported in the literature^{3,5,30}. It is possible that the type of tumor used in our experiments could explain this discrepancy, as U87 tumors are homogeneous, hypervascularized, and lack necrosis⁷. Furthermore, we observed a large range of tumor volumes, which is a significant drawback of this investigation. In addition to randomizing the animals, this issue could have been avoided by waiting until MRI showed a minimum preset volume. In addition to necrosis, the neuropathologist also qualitatively observed macrophage infiltration and apoptotic reactions in the necrotic and intact tumor areas, although this observation requires quantitative investigation. Future studies involving specific apoptosis analyses should be performed using immunohistochemistry with the TUNEL method (Apoptag[®] Plus Peroxidase In Situ, Millipore, USA).

The second part of our experimental work was designed to assess the enhanced efficacy of 5-ALA-PDT using light fractionation. The number of tumor-bearing animals available in this

preliminary study was limited, but the data strongly indicated that light fractionation enhanced the effect of 5-ALA-PDT on tumor tissue. However, the effect of light fractionation was critically dependent on the treatment conditions. At the high fluence rate, our experiment revealed that the area of PDT necrosis increased when a single pause was introduced after delivering 5 J compared with continuous illumination. Although we did not find a similar difference at the low fluence rate, our results generally agreed with those of previous studies^{11,14,15}, whose authors postulated three processes to explain the efficacy of fractionated PDT. First, light exposure initially caused temporary vascular occlusion followed by hypoxia when the consumption of oxygen surpasses the flow supplied by abnormal blood vessels. The dark period enabled reoxygenation of the tumor, making the subsequent light delivery more efficient. The two other processes involve reperfusion injury and relocalization of the PS during the dark interval. Some studies have suggested that the effects are lessened when a low fluence rate is applied because the consumption of oxygen is slower^{3,5,20}. Moreover, the benefits of fractionation was shown to be greater for 5-ALA than for certain other photosensitizers, based on its intracellular effect^{14,43}. PDT with THPC or Photofrin® (which exhibits a vascular effect) as the PS was not enhanced by fractionation of the light delivery^{9,40}.

In our study, we used a fractionation scheme described by Curnow et al. in the normal rat colon in which 5 of the total 25 J was delivered in the first fraction¹⁴. Curnow et al. demonstrated the importance of timing the light delivery before the dark period to avoid destroying the microcirculation necessary for further reoxygenation¹³. Another important feature of the dark period duration is that the O₂ recovery phase is likely to be tumor-type dependent owing to variations in vessel density, flow rate and oxygenation. It has therefore proven difficult thus far to define the optimal fractionation scheme. This change in oxygenation could potentially be monitored by measuring the partial pressure of oxygen in the glioma and adjusting the duration of the on/off sequences²⁴.

Clinical tolerance was another factor affecting PDT efficiency. The observed post-treatment morbidity was likely due to increased ICP. These results suggested that a low fluence rate was better tolerated; however, it should be noted that, despite randomization, the average tumor volume in the high-fluence-rate group was higher than that in the low-fluence-rate group: 15.8 mm³ (1.44-55.2) versus 4.2 mm³ (0.84-11.9), respectively. Thus, PDT-induced edema contributed to the ICP and, in some cases, caused mortality when the tumor volume was high^{4,28}.

This 5-ALA-PDT-induced edema is a common complication following PDT of brain tumors. It is typically addressed by administering steroids that improve clinical tolerance^{3,4,28}. However, in our experiments, the effects of this adjuvant treatment were impaired when treating a high-volume tumor. Furthermore, tumor volume is known to be a limiting factor during interstitial treatment⁴.

MRI is the most important tool for assessing the management of cerebral gliomas, although in our study, PDT efficacy was not properly evaluated using T1W, T1WGd and T2W sequences. These results are not consistent with those reported by Beck et al. in a clinical study, which

demonstrated a partial or complete resolution of tumor contrast enhancement within 24 hours after 5-ALA-PDT⁴. Based on these preliminary experiments, we did not find any MRI evidence to corroborate our pathological findings. However, some groups have reported the use of diffusion-weighted and perfusion MRI to evaluate the effects of vascular-targeted interstitial PDT^{1,44}. Therefore, the use of multiparametric MRI to assess PDT efficacy, without the need for histological examination, should be validated with comprehensive experimental designs, including low and high fluence rates, fractionation and continuous lighting assessed by sophisticated pathological analyses (e.g., apoptosis and necrosis).

Finally, the dose of 100mg/kg used in this study was based on a former study by Stummer et al.³⁶. The optimal dose has to be evaluated to optimize the PDT effects. A fluorescence microscopy study might be achieved for different doses to assess the optimal dose level.

1. Conclusion

In this study, we report the use of interstitial 5-ALA-PDT in a U87 glioblastoma rodent model. As angiogenesis is generally associated with glioblastoma, rats bearing U87 tumors rather than C6 tumors were chosen for these experiments^{36,47}. We established a reproducible and reliable rodent model for 5-ALA interstitial PDT. This new approach toward investigating 5-ALA-PDT allowed us to observe that its effect was critically dependent on the light delivery conditions. We therefore focused this study on assessing 1) the effects of interstitial PDT at high versus low fluence rates of delivery and 2) the effect of fractionated light delivery. Our results suggest that fractionated PDT was more effective compared with continuous light delivery. Furthermore, we noted that the efficacy of fractionated delivery also depended on the rate of light delivery, with stronger effects at the high fluence rate. Indeed, fractionated light exposure at a radiant power of 30 mW induced more necrosis compared with that observed at 4.8 mW (either by fractionated or continuous PDT). However, low-fluence-rate 5-ALA-PDT was still able to induce significant tumor necrosis. Finally, we show proof-of-concept experiments using the U87 glioblastoma rodent model to assess the effects of PDT. Future experiments should be performed to validate the effect of fluence rate and fractionation on pathology and to identify MRI markers for these treatment effects. Toward this end, we are currently testing a significantly larger group of animals receiving 25 J at a rate of 30 mW using two different fractionation schemes (compared with a sham group) to evaluate morbidity and side effects. Specific apoptosis analyses using immunohistochemistry with the TUNEL method are also planned.

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