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OPTIMIZATION OF BRAIN PHOTODYNAMIC THERAPY:

A study of extended-time low-fluence-rate ALA-photodynamic therapy in a rat glioma model

by

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M.D. Semmelweis University, Hungary, 2005

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in partial fulfillment of the requirements for the degree of

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in the program of

Biomedical Physics

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Hadi Karimi

ABSTRACT

Optimization of brain photodynamic therapy

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The importance of the rate of light delivery and the length of photodynamic therapy as an alternative treatment modality for brain glioma have been investigated and shown that optimized PDT regimens would have the potential to reduce the tumor recurrence and the treatment-induced morbidity. In a rat glioma model, using photosensitizer ALA-PpIX, we further investigated the effect of extended-time low-fluence-rate PDT on the number of apoptotic, necrotic, surviving, and infiltrated tumor cells, associated with three fluence rates of 0.5, 1.0, and 1.5 mW cm⁻², each at three treatment lengths of 24, 48, and 72 hours. Our results demonstrated that lowest fluence rate used in our study has an improved apoptosis to necrosis ratio; however higher fluence rates show a clear supremacy for tumor control in shorter treatment times. Therefore, low-fluence-rate brain ALA-PDT may only be more beneficial for tumor control at longer treatment periods.

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INTRODUCTION

Glioma

Malignant brain tumors cause 2% of all cancer deaths in the world (World Cancer Report 2008, WHO). The main treatment modalities are surgery and radio/chemo-therapies, but patients typically relapse, and cure is rare (CBTRUS 2005). Glioblastoma multiforme, the most common and aggressive subtype, has a median survival of 14 months after a combination of surgery, postoperative radiation therapy, and chemotherapy (Stupp *et al.* 2005). Recurrences often occur from tumor cells invading the normal brain adjacent to the bulk tumor within 2 cm from the resection margin (Wallner *et al.* 1989). These clusters of cells which have penetrated into interstitial healthy tissue surrounding the bulk tumor and have the potential of developing into bulk tumor are called microfoci infiltrates (Figure 1). Photodynamic therapy is a potential adjuvant treatment modality which is under investigation for treatment of malignant brain tumors.

Figure 1: Scheme of characterizing structure of malignant gliomas (glioblastoma multiforme). Left: T1-weighted MRI with contrast-enhancement. Right: Histological scheme of malignant gliomas, characterized by central necrosis, marginal enhancing, solidly-proliferating tumor and a broad margin of infiltrating tumor cells, which are surgically inaccessible (Comprehensive series in photochemistry and photobiology volume 6, Photodynamic therapy, T. Patrice, European Society for Photobiology 2006).

Photodynamic Therapy

Photodynamic therapy (PDT) is a promising treatment for cancer and other localized diseases, which continues to gain clinical acceptance. PDT makes use of light-induced singlet oxygen to kill cells by causing lethal oxidative damage to biologically important structures. This therapy involves the administration of a photosensitizing agent (photosensitizer) followed by exposure of the tissue to visible light. Photodynamic therapy, in its application, requires irradiation of the tumor site with non-damaging red or near-infrared light as well as selective uptake of the photosensitizer agent by the tumor to allow a proper therapeutic ratio with the surrounding tissue. However, the photosensitizers are non-toxic in the absence of light. When the photosensitizer molecule absorbs light of appropriate wavelength, it is excited. This electronically excited molecule will undergo a series of photochemical processes that cause a transient increase in the chemical reactivity of the oxygen molecule, and convert it into a relatively strong oxidizing agent known as singlet oxygen.

Photosensitizers

The knowledge of photosensitizing drugs is ancient, but it is mainly throughout the last 30 years that PDT has been developed as a treatment modality for cancer (Ackroyd *et al.* 2001). A good photosensitizer must absorb light efficiently. Molecules with chains of conjugated bonds can have electron states matching the photon energy of visible or near infrared light. The skeleton of most sensitizers used in PDT is the Porphin, which consists of four pyrrole rings joined to a ring structure with a cyclic, conjugated system of 18 π -electrons. This system has electron states matching visible light. Molecules in excited electron states may participate in chemical reactions, damaging biomolecules that are vital for the function of a cell. Generally, such reactions do not depend on the presence of oxygen, but photodynamic effect is defined as oxygen-dependent reactions exclusively (Blum 1941).

Most states excited by photoexcitation are short-lived (a few nanoseconds), and will return to the ground state by fluorescence or heat-transfer before interaction with other molecules can occur. For most molecules, both the ground state and the first excited state are so-called singlet states, with all electrons paired. But almost exclusively in excited states, molecules can also be in triplet states in which two electrons are unpaired. Excited triplet states can be sufficiently stable to allow

interaction with other molecules. Photodynamic effect depends on a high quantum yield for formation of such excited triplet states after photoexcitation. Quantum yield is the number of singlet oxygen molecules produced divided by the number of photons absorbed by the photosensitizer. For good photosensitizers this quantum yield is typically 0.2–0.9 (Redmond & Gamlin 1999).

The dominant reactions for most sensitizers used in PDT are mediated by the type II pathway, where the excited triplet state ($^3\text{PS}^*$) interacts with molecular oxygen by energy transfer. The sensitizer returns to the singlet ground state (^1PS), and oxygen is excited to the reactive species singlet oxygen ($^1\text{O}_2$) (Kawaoka *et al.* 1967). Singlet oxygen can return to the ground state by phosphorescence. This is a very slow process, so in non-gaseous environments, participation in chemical reactions is much more likely. $^1\text{O}_2$ reacts with a variety of biomolecules, and these reactions are so efficient that the intracellular lifetime of $^1\text{O}_2$ is in the nanosecond range, corresponding to a radius of action around 0.01 microns (Moan & Berg 1991, Niedre *et al.* 2002). This means that most of the initially induced damage is restricted to the subcellular compartment containing the photosensitizer. Consequently, when distributing the photosensitizer, intracellular localization may be as important as concentration. Reported estimates of the number of $^1\text{O}_2$ molecules necessary to kill a cell has varied with three orders of magnitude from 10^8 to 10^{11} in different experimental setups (Niedre *et al.* 2005). There is some uncertainty in these estimates, but the vast range reflects the fact that PDT inactivation is not necessarily a simple matter of acute lethal $^1\text{O}_2$ damage. A thorough understanding of the biology is necessary to describe the PDT-induced damage, which may be affected significantly by factors such as cell signaling pathways, programmed cell death, indirect vascular damage, and effects of the host's immune system (Dougherty *et al.* 1998).

Photochemical reactions can also be mediated by the type I pathway, where the excited triplet state interacts with a substrate by electron or proton transfer. These reactions are shown in Figure 2. This leads to generation of cytotoxic agents, including reactive oxygen species other than $^1\text{O}_2$. The prevailing hypothesis is that $^1\text{O}_2$ is the important cytotoxin in PDT (Weishaupt *et al.* 1976, Fuchs & Thiele 1998), although additional effects of type I reactions cannot be discounted. It has been reported that reactive oxygen species other than $^1\text{O}_2$ can dominate for certain sensitizers and environments (Vakrat-Haglili *et al.* 2005).

Figure 2: Pathways leading to photosensitizer action (Wainwright, Mark. Photosensitisers in biomedicine. Wiley-Blackwell 2009).

Most photosensitizers used in PDT are applied systemically by intravenous injection. Different photosensitizers, formulations and application schemes can target different tissues. Figure 3 presents a range of photosensitizers are currently approved for various clinical indications. Typically, short drug–light intervals (down to minutes) lead to vascular damage, while direct damage to tumor cells can be achieved using longer drug–light intervals (up to several days). The localization is also strongly dependant on the physiochemical properties of the drug, and in some cases of its ability to bind to specific targets in endothelial or tumor cells (Chen *et al.* 2006). The photosensitizer, the drug administration, and the drug–light interval should ideally be optimized to achieve sufficiently high accumulation of photosensitizer in the target tissue and acceptably low accumulation in the surrounding normal tissues.

As an alternative to direct administration of a photosensitizer, it is possible to make an organism photosensitive by administering drugs that are not photosensitizers themselves. Porphyrin precursors can be used as pro-drugs to induce accumulation of endogenous porphyrins that act as photosensitizers (Peng *et al.* 1987, Malik & Lugaci 1987). The porphyrins are synthesized intracellularly by the heme synthesis pathway. The rate limiting step of this pathway is the synthesis of aminolevulinic acid (ALA). If an excess of ALA is supplied exogenously, the insertion of iron

into the porphyrin ring to form heme becomes the rate limiting step. This leads to accumulation of the photosensitizer protoporphyrin IX (PpIX). Figure 4 is a schematic representation of endogenous heme synthesis and exogenous introduction of ALA as a photosensitizer.

Figure 3: Chemical structure of photosensitizers approved by medical authorities for treatment of (a) esophageal dysplasia, (b) head and neck cancer, (c and d) actinic keratosis, (d) non melanotic skin cancer, and (f) wet macular degeneration. The prodrugs (c) and (d) are synthesized into (e) intracellularly (Wainwright, Mark. Photosensitisers in biomedicine. Wiley-Blackwell 2009).

Properties of tumors that make selective photosensitizer uptake possible include: a leaky vascular system, poor lymph drainage, large extracellular volume, high amounts of collagen, decreased pH, and deviating cellular receptors and uptake mechanisms (Dougherty *et al.* 1998). Still, the selectivity achieved with systemic photosensitizers is often not better than around a factor of two (Moore *et al.* 1997). A major exception is brain tumors, where at least one order of magnitude of selectivity can be achieved for photosensitizers that to a low degree penetrate the blood-brain barrier (Chopp *et al.* 1996, Obwegeser *et al.* 1998, Lobel *et al.* 2001). Maximum selectivity is always desirable, but the degree of damage that can be tolerated in the surrounding normal tissues varies substantially. In some cases - like for m-tetrahydroxyphenylchlorin mediated PDT for head and neck cancer - severe necrosis following PDT can heal well with minimal scarring (Hopper *et al.* 2004). In other areas - like the esophagus, brain, or retina - damage to normal tissue can be dangerous.

Figure 4: The heme biosynthesis that takes place in eukaryotic cells. Excess of ALA will lead to accumulation of PpIX, the immediate precursor to heme. For several neoplastic cell types this accumulation is enhanced due to increased activity of porphobilinogen deaminase, decreased activity of ferrochelatase, or increased transport past the mitochondrial membrane due to abundant expression of the peripheral benzodiazepine receptor (Collaud *et al.* 2004).

The degree of photosensitization following application of porphyrin precursors depends on the three following steps: (1) distribution of the porphyrin precursor into the tissue, (2) cellular uptake of the porphyrin precursor and conversion into photosensitive porphyrins, and (3) relocation, degradation and clearance of porphyrins and porphyrin precursors. A wide variety of tissues are photosensitized by porphyrin precursors, but certain tissue types have a low ability to produce endogenous porphyrins. These include cases where damage would be critical such as connective, muscle, and brain tissues (Fukuda *et al.* 1992). This represents a useful selectivity enhancement, but it can also limit the efficacy of the treatment for certain tumors that have a low ability to produce endogenous porphyrins (Morgan *et al.* 2004).

It is clear from the literature that for brain tumors the distribution of the porphyrin precursor contributes to selectivity. The biodistribution studies demonstrated ten-fold selectivity versus

normal tissue and hundred-fold selectivity versus normal gray matter far from the tumor (Madsen *et al.* 2006). Hebeda *et al.* (1998) in an extensive biodistribution study involving two rat tumor models reported that there was no detectable porphyrin fluorescence in normal gray matter far from the tumor. Conversely, Stummer *et al.* (1998) and Lilge *et al.* (1998) reported significant porphyrin levels in normal brain, causing only ten-fold tumor selectivity in rat and rabbit models. They both showed that the porphyrin content in the normal brain was related to edematous flow from the tumor. Hence, the photosensitization of the normal brain is influenced by differences between the nature of the implanted tumor, and the observed porphyrin level depends on the tumor's ability to photosensitize the brain indirectly from tumor to surrounding healthy tissue than directly from blood stream to healthy tissue.

The influx rate of ALA as a porphyrin precursor has been quantified by Ennis *et al.* (2003) and shown that there is low transfer of ALA into normal brain through the blood-brain barrier. Their results agree with the plasma and brain levels in pharmacokinetic studies employing ¹⁴C-labelled ALA and doses relevant for PDT (Hua *et al.* 1995, Obwegeser *et al.* 1998). Also, preclinical data from animal models indicate that ALA compared to other sensitizers, because of its localization in mitochondria which makes it capable of causing apoptotic cell death, can lead to a much higher light fluence threshold for necrosis in normal brain tissue (Lilge *et al.* 1996, Olzowy *et al.* 2002). A pilot study of ALA mediated PDT has shown that the treatment is safe even for relatively high light doses (Beyer *et al.* 2006). It was also demonstrated that ALA PDT with interstitial light delivery can be used to ablate bulk tumor tissue in inoperable gliomas. Therefore in general, ALA is an appealing candidate for the PDT treatment of malignant brain tumors. ALA can also be of diagnostic value as a contrast agent, utilizing protoporphyrin IX's characteristic fluorescence. For malignant brain tumors the usefulness of ALA-based, fluorescence-guided resection of brain tumors has been tested extensively in Germany (Stummer *et al.* 2006). An example of application of ALA in fluorescence-guided resection is presented in Figure 5.

Figure 5: Accumulation of porphyrin fluorescence in human malignant gliomas. Left: Resection cavity under white light, showing the opened cerebral cortex with tissue of unclear status (healthy or malignant) at the base of the tumor cavity. Right: Fluorescence image of same cavity and visible red fluorescence identifying tissue as tumor (Comprehensive series in photochemistry and photobiology volume 6, Photodynamic therapy, T. Patrice, European Society for Photobiology 2006).

Light

The targeted application and limited penetration of light makes PDT a local treatment modality. Transport of light through tissue is limited by absorption and scattering. These processes result in an approximately exponential decrease in the fluence rate (i.e., the light intensity inside the tissue) as the light penetrates tissue. Light penetration depth is strongly dependent on wavelength. Most Porphin based photosensitizers have their absorption maximum in the blue region around 400 nm, but the hemoglobin found in most tissues limits the penetration depth of blue light to about a tenth of a millimeter. The photosensitizers most commonly used in PDT absorb also in the red region, where the penetration depth is 2–3 mm for most tissues (Cheong 1995). The best light penetration is found in the near infrared region from about 750 to 850 nm where penetration depths can reach 5–6 mm. The sensitizers motexafin lutetium (732 nm) and Pd-bacteriopheophorbide (TOOKAD®) (762 nm) absorb in this region and are used for experimental PDT of prostate cancer (Zhu *et al.* 2005, Weersink *et al.* 2005).

Diode lasers and light emitting diodes (LEDs) are currently the dominant light sources in PDT. Laser light with a wavelength matching the absorption maximum of the photosensitizer provide the most efficient excitation. The production of light energy with suitable wavelengths and intensity for PDT purposes is quite straightforward. However, successful PDT requires light application systems allowing for a homogenous irradiation of the target tissue. The most cost-

effective, robust and long-lasting laser systems suitable for ALA-based PDT are certainly diode laser systems. When using a laser it is also possible to couple the light into an optical fiber to illuminate sites inside organs such as bulk tumors and resection cavities. For PDT with fiber-based applicators, laser light offers the advantage of being efficiently injected into fibers.

For brain tumors, light application is performed interstitially by insertion of multiple fibers into the bulk tumor. Resection cavities can be irradiated with fiber applicators equipped with a bulb-shaped tip, dispersing the light uniformly in all directions. It is, however, not always easy to ensure a homogenous irradiation during PDT. In order to enhance homogeneity of irradiation of bulk tumor tissue, and to avoid excess heating at the fiber tip, the use of small-diameter radial diffuser tips rather than bare fibers is indicated. For proper placement of the fibers, information on the tissue optical properties is required so that we can obtain a good estimate for the light penetration depth as well as the appropriate inter-fiber distance. In extended-time PDT treatments, application of specially adapted light applicators is required. The main challenge of such applicators is mobility and stability of system for continuous extended-time irradiation.

Dosimetry

The ultimate dose measure in PDT is the distribution of the $^1\text{O}_2$ formation, which depends on the treatment variables light, photosensitizer, and oxygen. However, the distribution of $^1\text{O}_2$ is very difficult to calculate since these three factors are far from constant during the light exposure (Wilson *et al.* 1997). The PDT effect alters the blood supply and hence the oxygen supply. The oxygen concentration is also reduced as oxygen is consumed by the production of $^1\text{O}_2$. In addition, the photosensitizer concentration is in most cases significantly reduced (photobleached) by the presence of light. The change of blood supply, the oxygen consumption, and the photobleaching may depend heavily on the rate of the light delivery. To top off the complexity, the optical properties of the tissue depend strongly on blood content, blood oxygenation, and photosensitizer concentration. Nevertheless, it is possible to assess the $^1\text{O}_2$ formation directly by *in vivo* measurement of $^1\text{O}_2$ phosphorescence (Niedre *et al.* 2002). However massive technical difficulties must be overcome to introduce such measurements clinically.

Due to these difficulties the most common dose measure in PDT is a simple specification of the light delivered to the tissue. For a given clinical application there usually exists a recommended fluence or energy density, given in Joules per square centimeter (J cm^{-2}). The irradiation parameter determines the amount of light that has been successfully delivered to the tissue. This parameter is called fluence rate, irradiance, incident intensity, or power density, usually given in milliwatts per square centimeter (mW cm^{-2}). A simple relationship holds between irradiation H , irradiance E and time t :

$$H = E \times t \quad (\text{Eq. 1})$$

The fluence rate distribution for a specific treatment session depends on both the geometry and the optical properties of the tissues. It can be calculated by solving the Boltzmann transport equation, a partial differential equation describing the transport of light in turbid media, or by using Monte Carlo simulation to stochastically imitate the propagation of photons through turbid media (Star 1997). Calculations of fluence rate distributions are mostly used for general purposes, and are rarely performed for a single patient.

The photobleaching (destruction by light) of PpIX during irradiation affects light dosimetry. At first, one might think that this effect would be negative, but such is not the case. Bleaching prevents the possible overdosing of light. Once all of the ALA induced PpIX is used up, any further irradiation has no additional effect, especially on adjacent normal tissue. Of course, if the initial concentration of PpIX is too low, there will not be enough singlet-oxygen molecules produced to kill the tissue. A minimum concentration of PpIX is, therefore, required in the target tissue. The surrounding normal tissue can, however, be perfectly protected from lethal effects if the concentration of PpIX is below the threshold to produce lethal damage on prolonged irradiation. Even very high light doses would then not be harmful.

Cell death pathways

There are two different types of cell death: necrosis and apoptosis. Necrosis can be caused by a major chemical or physical assault to the cell, resulting in a loss of membrane integrity, swelling and rupture of the cell. Organelles, such as the mitochondria or the nucleus, remain intact

throughout the process. During necrosis, due to the rupture of cell membrane, the cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue. Therefore, cellular necrosis is often associated with inflammation.

In contrast, apoptosis is a highly regulated process, and is described as programmed cell death. There are characteristic molecular and biochemical events which occur in an apoptotic cell. Most notably are activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments and flipping of phosphatidylserine from the inner side of the cell membrane to the outer side. DNA condensation and degradation is one of the last steps occurring in an apoptotic cell before the membrane starts to bleb which results in the production of membrane-enclosed particles that contain intracellular material known as apoptotic bodies. The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response. The manifestations of apoptotic cells which can be recognized by stereotypical morphological changes include cell shrinkage, plasma and nuclear-membrane blebbing or budding, organelle relocation and compaction, chromatin condensation, and apoptotic bodies. These changes can be easily distinguished from the events occurring in cells that are dying from necrosis (Van Cruchten, 2002).

Apoptosis-inducing therapies are considered particularly useful in the treatment of brain tumors because of the lack of inflammatory responses and associated edema. In contrast, necrotic cell death is accompanied by inflammation and edema, which often results in post-treatment morbidity.

Clinical and preclinical brain PDT

PDT has been tested clinically for destruction of tumor cells in residual bulk tumor after standard or fluorescent guided resection surgery. The average survival gain reported in pilot studies are from only a few months at worst to a few years at best (Stylli *et al.* 2006 and Eljamel *et al.* 2007). These results indicate that PDT could have a definite potential in neurosurgery for the treatment of gliomas as an adjuvant therapy; however, as Stylli *et al.* (2005) reported, the post PDT morbidity due to PDT induced inflammation may depend on how rigorously resection of tumor has been

performed and consequently, fewer residual cells undergoing necrosis following PDT result in fewer cellular toxic products released to produce acute cerebral edema. Also, a more extensive resection would result in the presence of a larger cavity for any edematous brain to expand into after the treatment which in turn reduces the post treatment morbidity. In addition, *in vivo* studies show PDT-induced inflammatory response in the brain and subsequent edema, combined with the already expansive tumor burden, result in high rates of morbidity and, in some instances, mortality (Hirschberg H. 2006).

In most clinical trials where PDT has been performed as an adjuvant therapy, high-fluence-rate PDT (a total fluence of 500-5000 J cm⁻² delivered at treatment time less than an hour) has been used. Since PDT is almost entirely an oxygen dependent treatment modality, exposure to high fluence rate is likely the cause of rapid O₂ depletion associated with high-fluence-rate photodynamic therapy. Evidence for fluctuating tumor blood flow has been provided by numerous groups suggesting that within high- fluence-rate PDT treatment, tumor blood vessels are often temporarily occluded, resulting in acute hypoxia. Fluorescence angiography measurements showed that the increased necrosis following high fluence rate treatment is likely due to ischemia/reperfusion injury (Moriyama *et al.* 2008). Also, with limited kill rate per cell doubling it is unlikely that a single treatment would be sufficient to prevent tumor recurrence. Nevertheless, comparison PDT studies suggest that continuous low-fluence-rate PDT is more advantageous over repeated PDT (fractionated) with respect to tissue oxygen conservation (Babilas *et al.* 2003). In addition, repeated PDT treatments of brain would have the same logistical difficulties that continuous extended-time low-fluence-rate PDT has. Namely, the repeated application of light delivery to the tumor site in multiple occasions is not more convenient than application of permanent light delivery system into the brain. Hence, other approaches seem necessary to make PDT beneficial for brain tumor patients.

It seems reasonable that a reduction in treatment-induced mortality might be achieved through a judicious choice of PDT regimen that minimizes inflammatory response. In that context, the results of Henderson *et al.* (1998) are relevant because they demonstrated low inflammatory responses following low-fluence-rate ALA-PDT treatment. In this case, the low inflammatory response was attributed to a combination of the high apoptotic fraction associated with low-fluence-rate PDT. Lilge *et al.* (2000) reported that PDT-induced apoptosis has a threshold behavior. In other words, apoptosis is seen beyond the necrotic boundary in the PDT treated

tumor bearing animals. Therefore, from the light propagation pattern of this treatment we can assume that the threshold doses are lower than those for necrosis. Following the same principles, Bisland *et al.* (2004) introduced the metronomic PDT (mPDT) concept as an alternative PDT treatment. In mPDT the same total fluence which is used for high-fluence rate PDT is deposited into the tissue through low-fluence-rate light irradiation over an extended period of time. They showed that metronomic PDT is capable of producing a considerable amount of tumor cell death through apoptosis. Building on this work, our study was performed to investigate the effect of low-fluence-rate at extended treatment periods on the PDT-induced tumoricidal pathway (apoptosis and necrosis) as well as tumor growth control.

Hypothesis

The intention of this study is then to examine if we can reduce the risk of treatment induced inflammation by reducing the necrotic tumoricidal effect of PDT treatment through low-fluence-rate PDT, and also eliminate microfoci infiltrate through continuous PDT treatment for an extended period of time which is thought to reduce the risk of tumor regrowth.. It is likely that low-fluence-rate treatment might serve optimally as an adjunct to other modalities aimed at debulking the tumor and may prevent tumor regrowth.

To evaluate this hypothesis, we initiated the following study in a brain tumor model consisting of parental CNS-1 glioma cells in rats and delivered low fluence light as long as 3 days by a backpack laser light source. In this study we investigated the importance of the rate of light delivery on the effects of low-fluence-rate ALA-PDT on tumor-bearing rats. Apoptotic fraction and microfoci infiltrates associated with different low-fluence-rate PDT regimens were examined.

METHODOLOGY

CNS-1 glioma cell line

CNS-1 cells were grown as monolayers in air-filtered 175 cm² Nunclon flasks (Fisher Scientific, Ottawa, Ontario, Canada) containing Dulbecco's medium (GIBCO BRL, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS), penicillin (100 pg/mL) and streptomycin (100 pg/mL) in 5% CO₂ at 37°C in a humidified incubator.

***In vivo* tumor induction**

The *in vivo* PDT response was investigated using CNS-1 cells grown as intracranial solid tumors as CNS-1 rat GBM syngeneic model. CNS-1 cells were implanted intracranially in the striatum of syngeneic Lewis rats (220–250 g). For tumor induction, animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (20 mg/kg) and the head was immobilized in a stereotaxic frame. A 15 mm long incision was made along the midline of the skull, and the skin was pushed back. Using a high-speed drill, a 2 mm bur hole was made unilaterally in the left hemisphere, anterior to the coronal fissure (coordinates: 1 mm forward and 2 mm lateral from the bregma). 5 µL of PBS solution containing 10⁵ CNS-1 cells was injected into the parietal cortex, 2 mm beneath the dura mater using a 26G-10 µL Hamilton syringe fixed to the stereotactic frame, over a period of 5 min to avoid mechanical or pressure damage. The needle was slowly retracted, the bur hole was covered by bone wax and the skin sutured closed. Saline solution (3 mL) was administrated subcutaneously to prevent dehydration. For the surgical procedures, animals received subcutaneous injection of 0.05 mg/kg Buprenorphine as painkiller following the surgery. Animals were allowed to recover and their health status was closely monitored. This procedure led to formation of tumors of 1-2 mm diameter after 14 days of growth. Untreated tumor bearing rats had a median survival of 20 days.

Animal Care

Rodents were caged in pairs and housed in a specific pathogen free environment, and were closely monitored. Animals were housed in a humidity and temperature controlled vivarium on a 12:12 hours light/dark cycle (lights on 07:00) with free access to food and water. Tumor bearing rats were weighed daily and examined for symptoms of increased intracranial pressure caused by tumor growth. Animals were terminated when they showed a weight loss of 10% and/or clinical signs of severe stress because of intracranial tumor growth. The rats were terminated by intracardiac administration of 1 mg/kg Euthanyl under deep anesthesia. All experiments were approved and carried out in accordance with the Animal Care Committee of Ontario Cancer Institute, University Health Network, Toronto, Canada.

PDT protocols

A total of 50 animals were evaluated. 30 animals were randomly assigned to the different treatment groups according to the different protocols. Animals were divided in 3 groups exposed to light at fluence rates of 0.5, 1.0, and 1.5 mW cm⁻² respectively. Each group was divided in three subgroups exposed to light for time periods of 24, 48 or 72 hours respectively, resulting in 9 treatment protocols of between 40 to 360 J cm⁻² radiant exposure. The total fluence for each subgroup as well as the number of treated animals for each protocol is shown in Table 1. The light fluence rate was kept constant in each group through the entire treatment period. Animals were kept under dim ambient light levels for the entire period of treatment.

Control animals were divided into two groups of light only control and ALA only control. As light only control, 20 tumor bearing animals were used 14 days post tumor implantation. For each subgroup (9 subgroups), two control animals were assigned. The control animals were exposed to the same fluence rate light as related subgroup without introduction of photosensitizer. For example, for subgroup A-24, two tumor bearing animals (14 days post tumor implantation) received 0.5 mW cm⁻² light for 24 hours without any ALA.

The effect of photosensitizer alone on tumor bearing animals has been investigated previously (Bisland *et al.* 2004). However, in order to assess possible effects of light source (backpack) and light delivery application on animals (ALA only control), two animals were given ALA alone at the

same dose as the rest of treated animals (100 mg per day). The backpack light source with its feeding fiber to the brain was implemented through the same procedure as treated animals for 3 days but light source was kept off at all time.

Groups	A	B	C
Fluence rate Treatment time	0.5 mW cm ⁻²	1 mW cm ⁻²	1.5 mW cm ⁻²
24 h	A-24 (n=4); 40 J cm ⁻²	B-24 (n=3); 80 J cm ⁻²	C-24 (n=4); 120 J cm ⁻²
48 h	A-48 (n=3); 80 J cm ⁻²	B-48 (n=4); 160 J cm ⁻²	C-48 (n=3); 240 J cm ⁻²
72 h	A-72 (n=3); 120 J cm ⁻²	B-72 (n=3); 240 J cm ⁻²	C-72 (n=3); 360 J cm ⁻²

Table 1: Total deposited energy (fluence) for each subgroup is presented. Three main groups of A, B, and C are differing in applied fluence rate. Each group is divided to three subgroups differing in treatment lengths of 24, 48, and 72 hours. (n) presents the number of treated animals in each subgroup.

Photosensitizer application

ALA in hydrochloride form was dissolved in physiologic saline (20 mg/ml) with the pH adjusted to 6.4-6.7 by (1 M) NaOH and injected intraperitoneally at a dose of 50 mg/kg every 12 hours (to achieve total dose of 100 mg/kg/day), starting 12 hours prior to the start of light exposure and continuing to the end of treatment. This is the optimal dose for ALA as the photosensitizer for brain PDT of rats recorded by Bisland *et al.* (2004).

Light exposure

Animals were irradiated using a laser with wavelength of 635 nm which is within the peak of the PpIX excitation spectrum. The light was delivered interstitially from bare optic fiber tip. The light distribution in a rat brain model for photodynamic therapy using a similar light delivery system as we use (bare end optical fiber with the same diameter) has been computed through Monte Carlo stimulation (Angell-Petersen *et al.* 2007). In this study, the ratio of interstitial fluence rate to radiant power as a function of vertical distance and lateral distance was plotted and shown that for vertical distance of 3-4 mm and lateral distance of 0-2 mm from the source fiber this ratio is 1. Since the tumor location of our study corresponds to this region, the output power of the light sources set

to 0.5, 1.0, or 1.5 mW in order to achieve the equivalent fluence rates. 14 days post tumor implantation, before light exposure, the animals were anesthetized and secured in the stereotactic frame. The skin incision was opened and the bone wax removed from the bur hole. A 2 mm diameter 3 mm long cylindrical stainless steel hollow screw was anchored into the bur hole of the animal skull. A 2 mm thick, 5 cm long plastic tube was fixed to the exposed end of screw. A bare flat-end 200 μm core optical fiber was run through the plastic tube and the hole within the screw into the skull to just below the dura. The fiber inside the plastic tube and the plastic tube were both fixed and coupled to a 635 nm diode laser light source assembled in a backpack, and strapped to the back of animal by a jacket worn by the animal (Figure 6). The miniature laser light source weighs 30 g in total and is powered by a 3 V lithium battery which produces the output power of up to 2 mW. Batteries were replaced every 12 hours. The temperature of the backpack did not exceed 37 °C and was well tolerated by the animals.

Figure 6: Backpack laser light source and its application on animal.

Histology Preparation

Animals were terminated immediately after PDT treatment by intracardiac administration of 1 mg/kg Euthanyl under deep anesthesia. The entire brain was removed from the skull and fixed in 10% formaldehyde for 48 hours (Figure 7). The fixed brain was halved coronally along the tumor injection trace or fiber imprint and both anterior and posterior halves were dehydrated and embedded in paraffin. Tissue paraffin blocks were sectioned using a microtome. Sections of 10 μ m thickness were cut every 100 micron from each half which were mounted onto slides, deparaffinized using xylene and stained with hematoxylin and eosin (H&E) for histopathological assessment.

Figure 7: Extracted brain post PDT. Note: arrow points to optic fiber's entrance impression into animal's brain. The dark spot above the fiber impression is a clotted superficial vessel.

Histopathological evaluation

A variety of techniques have been developed to assess two types of cell death, most of which are based on the loss of plasma membrane integrity and penetration of stains into the cell. However, during apoptosis, the cell membrane remains intact for a relatively long time, and therefore some of these assays cannot detect the early stages of apoptosis. Alternatively, assays that detect all types of cell death do not discriminate between apoptosis and necrosis. Therefore, a combination of several methods is required to distinguish between the two separate types. However, combination methods, such as PI+TUNEL, owing to their relatively low specificity are usually not used for quantitative analysis, rather they are usually used for qualitative analysis. Thus, in order to evaluate

our result we decided that it seems more reliable to choose a technique which relies on specific morphological changes associated with these two processes so that we utilized light microscopy of the H&E-stained sections and used distinct morphological hallmarks of both types of cell death which were specific to their own to differentiate between different types of cell death. In addition, in order to minimize bias, our method of cell evaluation was solely based on a standard observational criteria and not the observer's point of view (Van Cruchten, 2002). The morphological features specific to apoptosis and necrosis which we used to distinguish between two types of cells are summarized in table 2.

Apoptosis	Necrosis
<p>Cell shrinkage</p> <p>Bubble-like blebs on cell surface</p> <p>Chromatin condensation, fragmentation, and margination at the nuclear periphery</p> <p>Disintegration of nuclear envelope</p> <p>Formation of membrane-bound apoptotic bodies</p>	<p>Cell swelling</p> <p>Nuclear swelling</p> <p>Chromatin flocculation</p> <p>Loss of nuclear basophilia</p> <p>Loss of cell membrane integrity</p>

Table 2: Morphological features of apoptosis and necrosis.

Among all prepared slides for each brain sample, the slide containing the tissue section bearing the largest tumor size was chosen to be evaluated. As it is presented in Figure 8, for each evaluated slide, four separate $100 \times 100 \mu\text{m}$ windows were selected inside the bulk tumor in four separate and different locations with regard to the optical fiber tip or light illumination pattern. Each window was evaluated visually and separately. The cells of interest were apoptotic, necrotic, and surviving tumor cells. Evaluated cells were counted and scored visually (Figure 9). The scoring procedure of each window of interest was repeated three times, blinded to the treatment protocol. The amount of apoptosis and necrosis were also measured in the brain tissue section (excluding tumor) of selected slides of control animals. In order to compare the effect of different PDT protocols on the risk of developing PDT induced inflammation, apoptosis-necrosis ratio was calculated for each protocol as well as each main group. A lower ratio reflects the higher chance of PDT induced inflammation.

Figure 8: A diagram of window selection. H&E stained sections of rat brain. a, 1.5× magnification of tumor bearing hemisphere of animal brain. Entire tumor is seen in the black box. Red arrow presents the optical fiber position and direction of light irradiation. b, 3× magnification of tumor. Dashed boxes present the selected windows. c, 20× magnification of a selected window for cell type evaluation.

Figure 9: Cell evaluation after PDT. H&E stained 40 $\times$ magnified sections of different regions of interest to be scored. In H&E staining, hematoxylin colors basophilic structures such as nucleic acid rich structures (nucleous) with blue-purple hue. Eosin colors acidophilic structures such as protein or cytoplasm with bright pink. Note: cells of interest are scored according to their presenting morphological characteristics. In all images, bold arrow points to a necrotic cell presenting swelling cell and nucleus with loss of basophilia. Dashed arrows point to apoptotic cells presenting condensed, fragmented, and periphery marginated chromatin plus shrinking blebbing cell membrane and loss of nucleus integrity. In image a, star points to an apoptotic body presenting membrane bound condensed chromatin fragment (stained dark blue-purple). In image b, S points to a surviving tumor cell presenting large intact nucleus and cytoplasm with distinct nucleolus and noticeable ribosome concentration in cytoplasm. R points to a red blood cell featuring bright pink color without nucleus. In image d, L points to a late stage apoptosis presenting condensed chromatin bodies with disintegrated nucleus and cytoplasm.

Statistical analysis

In our experiment, results represent mean $\pm$ standard error for n = number of animals in each subgroup $(3-4) \times$ number of selected windows for each animal $(4) \times$ number of observations (3) . In our study, the sample size was very low, limited to the number of experimental animals. Also, the value to be considered as significantly different was practically unknown; therefore we should conclude that our statistical analysis is rather descriptive analysis than stochastic. Statistical analysis was conducted using Microsoft Excel 2007 software.

To evaluate the effect of light exposure pattern on four selected windows located in different positions of tumor with respect to the optical fiber entrance on each slide, we used one-way analysis of variance (ANOVA). The null hypothesis stated that the number of each cell type is the same for all four windows in a selected slide for each treatment protocol. Each window is scored three times therefore we have four groups each containing three values. These four groups of values were compared with probability values $P < 0.05$ considered significant ($\alpha=0.05$, 5% significance level), meaning the null hypothesis would be rejected if the p-value is less than 0.05.

In order to evaluate the data acquired from different animals which have received the same treatment protocol, we again used one-way analysis of variance (ANOVA). The null hypothesis stated that the number of each cell type is the same for different animals that have undergone the same treatment protocol. For example, we had 4 animals receiving 0.5 mW cm^{-2} for 24 hours, each of these animals was represented by a selected slide. On each slide there are four windows and for each window we have 3 observational values for each cell type. Therefore in our analysis, there are four main groups representing animals and each group contains 12 values $(4 \text{ window} \times 3 \text{ observations})$. These four groups of values were compared with probability values $P < 0.05$ considered significant ($\alpha=0.05$, 5% significance level), meaning the null hypothesis would be rejected if the p-value is less than 0.05.

RESULTS

The result of our experiments in which multiple treatment protocols were used demonstrated that CNS-1 tumor cells were generally susceptible to PDT. In control and treated animals, despite several small perivascular hemorrhages, no significant necrotic area or extensive central tumor necrosis was noticed. Increased neutrophil infiltration after PDT and presence of white matter tracts indicated abnormal presence of plasma fluid and is interpreted as cerebral edema (Figure 10). In general, tumor bearing animals treated with higher fluence rates tolerated longer treatment periods and their body weight loss, dehydration, and depressed activity signs were less noticeable. No animals died as a result of or during the treatment procedure. However, due to the technical difficulties of long term light delivery, of the 90 animals which we originally ordered for our experiment, only 30 treated and 20 control animals resulted in valid data. Although, we used 20 animals in the beginning of our experiment to improve the stability of light source and light delivery system into the animal brain, 20 more animals had to be terminated within the treatment because of light delivery failure. The majority of such incidences involved the fiber displacement, breakage, or uncoupling from the light source. Animals under treatment had the same degree of mobility as before treatment which consequently increased the risk of extended-time light delivery failure.

Figure 10: 10× magnification of an animal brain section. Note: animal brain's white matter tracts indicate abnormal presence of plasma fluid and is interpreted as cerebral edema (20× magnification).

All control groups with light or photosensitizer alone, regardless of light fluence rate used, showed an average of 1.7 ($\pm$ 0.4 SE) apoptotic cells and no noticeable necrosis for the healthy areas of brain tissue section in the selected slides. Histopathological comparison of four selected windows under investigation in each slide located in different positions of gross tumor with respect to the optical fiber entrance, showed no significant differences in number of cells of interest to treatment response ($p < 0.05$). Histopathological examination revealed no evident normal tissue damage in the treated brain adjacent to the tumor.

According to graph-a in Figure 11, the group A, which received PDT at a fluence rate of 0.5 mW cm⁻² showed a steady increase in number of apoptotic cells as the treatment time lengthened. However, a relatively small and steady number of encountered necrotic cells were scored at the end of different treatment periods (Figure 11 graph-b). This group had the best average apoptosis-to-necrosis ratio of 3.5 ($\pm$ 0.4 SE) for all three treatment time periods measurements. As demonstrated in graph a and b of Figure 12, the total number of surviving tumor cells and microfoci infiltration decreased to ¼ of control but were not eradicated completely and their presence at the end of 72 hours treatment were noticeable.

In group B which received 1.0 mW cm⁻² of light, the number of surviving tumor cells dropped sharply from 60% after 24 hours to 25% after 48 hours and 5% after 72 hours of PDT treatment. Microfoci infiltrates followed a sharp decreasing pattern as well to almost no countable infiltrate tumor clusters in brain adjacent to tumor after 72 hours of treatment. In contrast, the amount of apoptotic cells after 24 hours scored almost double that of group which received 0.5 mW cm⁻² and reached its highest value after 48 hours of PDT treatment. Nevertheless, after 72 hours the number of cells that have undergone apoptosis dropped to almost half. In this group, necrosis is shown to be almost double the 0.5 mW cm⁻² treated group yet presenting the same steady pattern for different treatment periods. This group shows an average apoptosis-to-necrosis ratio of 1.7 ($\pm$ 0.3 SE). In addition, at the end of each treatment period, the amount of necrosis is high enough to trigger an inflammatory response. Figure 13, 14, and 15 present different histology images of untreated and PDT treated tumors.

The highest fluence rate PDT investigated (1.5 mW cm⁻²) was group C. In this group, the amount of surviving tumor cells showed a sudden drop after 48 hours of treatment. Furthermore, after 72 hours, we did not encounter any observable surviving tumor cells or any microfoci infiltration.

Histopathology showed inflammatory cell infiltration in place of diminished tumor cells. This group recorded the highest total amount of apoptosis of all treatment periods as well as the highest amount of measured necrosis. Noticeably, after 72 hours of PDT treatment the necrosis amount which seemed steady in all other groups, dropped to almost half of what was measured for 48 hours. Finally, this group showed the average apoptosis-to-necrosis ratio of 1.5 (± 0.3 SE). Table 3 is a summary of all acquired data including cells of interest such as apoptotic, necrotic, surviving tumor cells, and microfoci infiltrate for our 3 main groups and 9 subgroups treated with different protocols.

Figure 11: a, Number of apoptotic cells after PDT treatment. b, Number of necrotic cells after PDT treatment. C=Control.

a

b

Figure 12: a, Number of surviving tumor cells after PDT treatment. b, Number of total tumor microfoci infiltrates into brain adjacent to healthy tissue after PDT treatment. C=Control.

Figure 13: H&E stained histology images of tumor bearing rat brain. a, 1.5 $\times$ magnification of implanted tumor 14 days post tumor implantation. Note: arrow points to the implanted tumor and also represents the direction of optical fiber irradiating light to the tumor. b, 1.5 $\times$ magnification of implanted tumor after 48 hours of PDT treatment at 0.5 mW cm⁻². Note: tumor is located above the lateral ventricle (hollow space within the brain). c, 5 $\times$ magnification of an untreated tumor, Note: penetrating tumor cells into adjacent healthy tissue on the margin of tumor. Arrow points to the margin of tumor. C: center of tumor, N: normal brain tissue. d, 10 $\times$ magnification of an infiltrated microfocus into the healthy brain tissue adjacent to tumor. Note; arrow points to a blood vessel in the center of microfocus.

Figure 14: H&E stained histology images of PDT treated tumor bearing rat brain. a, 20 $\times$ magnification of a selected window, PDT treated at 0.5 mW cm⁻² for 24 hours. b, 20 $\times$ magnification of a selected window, PDT treated at 0.5 mW cm⁻² for 48 hours. c, 20 $\times$ magnification of a selected window, PDT treated at 0.5 mW cm⁻² for 72 hours. Note: gradual decrease in number of surviving tumor cells presented by lower concentration of dark blue/purple cells. Gradual increase in hollow spaces presents cleared dead tumor cells suggesting cell death mainly through apoptosis because of lack of infiltrated inflammatory cells in these spaces. Increase in presence of periphery margined fragments of chromatin with disintegrated nucleus suggesting increased apoptosis. d, 20 $\times$ magnification of a section of PDT treated tumor presenting aggregation of inflammatory cells as well as red blood cells in hollow spaces suggesting slight regional necrosis.

Figure 15: H&E stained histology images of PDT treated tumor bearing rat brain. a, 20 $\times$ magnification of a selected window, PDT treated at 1.0 mW cm⁻² for 24 hours. b, 20 $\times$ magnification of a selected window, PDT treated at 1.0 mW cm⁻² for 48 hours. c, 20 $\times$ magnification of a selected window, PDT treated at 1.0 mW cm⁻² for 72 hours. Note: gradual decrease in number of surviving tumor cells presented by lower concentration of dark blue/purple cells. The presence of swelling necrotic cells as well as hollow spaces is more prominent than 0.5 mW cm⁻² treated animals. The presence of surviving tumor cells decreases noticeably after 48 hours of treatment. There are increased number of inflammatory cells specially around hollow spaces suggesting the presence of inflammation and increased number of necrosis. d, 4 $\times$ magnification of entire tumor region, PDT treated at 1.5 mW cm⁻² for 72 hours. Note: pronounced precens of hollow spaces with a few surviving tumor cells suggests the elimination of bulk tumor. presence of inflammatory cells is considerably high. No noticeable microfocus is seen in the adjacent healthy brain tissue and presence of small hollow spaces in the healthy brain tissue adjacent to tumor suggests the elimination of penetrated microfoci due to PDT treatment.

Group	Treatment time (hours)	Fluence rate (mW cm ⁻²)	Total Fluence (J cm ⁻²)	Apoptosis ± SE		Necrosis ± SE		Surviving tumor cells ± SE		Microfoci infiltrate		Apoptosis/Necrosis ratio		Average Apoptosis/Necrosis ratio	
				Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control
A	24	0.5	40	23 ± 1	10 ± 1	11 ± 1	6 ± 1	157 ± 7	223 ± 5	8 ± 3	9 ± 1	2.0 ± 0.2	1.6 ± 0.1	3.5 ± 0.4	1.6 ± 0.1
	48		80	52 ± 2	13 ± 2	16 ± 2	8 ± 2	103 ± 2	207 ± 2	5 ± 2	12 ± 2	3.2 ± 0.6	1.6 ± 0.2		
	72		120	76 ± 3	15 ± 2	14 ± 1	9 ± 1	47 ± 4	216 ± 4	2 ± 1	13 ± 2	5.4 ± 0.5	1.6 ± 0.1		
B	24	1.0	80	47 ± 1	9 ± 1	25 ± 2	4 ± 1	123 ± 8	218 ± 4	6 ± 2	7 ± 1	1.8 ± 0.2	2.2 ± 0.3	1.7 ± 0.3	1.9 ± 0.1
	48		160	84 ± 4	12 ± 1	36 ± 6	7 ± 1	52 ± 5	231 ± 6	3 ± 1	10 ± 2	2.3 ± 0.6	1.7 ± 0.1		
	72		240	33 ± 2	16 ± 2	28 ± 7	8 ± 2	8 ± 1	206 ± 3	0 ± 0	12 ± 2	1.1 ± 0.7	2 ± 0.2		
C	24	1.5	120	70 ± 7	8 ± 1	40 ± 6	5 ± 1	84 ± 7	228 ± 5	5 ± 1	8 ± 1	1.7 ± 0.7	1.6 ± 0.1	1.5 ± 0.3	1.6 ± 0.1
	48		240	94 ± 9	11 ± 1	48 ± 3	6 ± 1	24 ± 5	215 ± 3	1 ± 0.8	11 ± 1	1.9 ± 0.6	1.8 ± 0.1		
	72		360	25 ± 3	14 ± 2	21 ± 5	9 ± 2	0 ± 0	224 ± 4	0 ± 0	12 ± 2	1.1 ± 0.8	1.5 ± 0.1		

Table 3: A summary of all acquired data (SE: standard error).

DISCUSSION

Wilson *et al.* (2006) showed 0.5 mW cm^{-2} as the minimum effective fluence rate to induce considerable tumor destruction in an animal tumor model relatively similar to the one in our study. Thus, we chose 0.5 mW cm^{-2} as our lowest fluence rate and increased by 0.5 mW cm^{-2} in a stepwise manner to maximum of 1.5 mW cm^{-2} . The minimum treatment time period of 24 hours was chosen to detect the late stage apoptosis induced by PDT treatment. The maximum of 72 hours as our longest treatment time and our maximum fluence rate were chosen because in our animal model we were limited by the size of tumor and the output power of our light source as well as its stability for extended-time use.

One of the major obstacles in extended-time low-fluence-rate PDT of experimental models is the difficulty of long-term light delivery system. The miniature light source which is used for rat model should be limited in size and weight and should deliver light through an optical fiber in order to impose minimum stress or mobility limitation on animal. Nevertheless, such system would be very fragile for long-term treatments. In our experiment, once the animal recovered from anesthesia and were fully active and mobile, it became obvious that the animal did not easily tolerate the backpack or the optical fiber inserted into the brain. Since the fiber was limited to bending angles of less than 20 degrees, extreme movements of the animal head would cause the fiber to break at the point where it was attached to the backpack or to detach from the skull. Keeping the fiber secured into the animal brain while animal was fully active was our major challenge which was addressed to some extent by implementing a plastic tube covering the optical fiber fixed by a screw to the animal skull.

Another obstacle that made us limit our treatment time to maximum of 72 hours was the poor animal compliance with the backpack and the light delivery system mentioned above. Animals showed signs of severe stress, dehydration and weight loss for treatments over 72 hours although we minimized weight of our backpack to 30 g. Hence, any extended-time low-fluence-rate PDT for longer treatment times would definitely require an improved light source and light delivery

system which is smaller in size, lighter in weight and imposes less stress on animal through a less invasive way of light delivery and fiber implantation to the animal brain.

One type of cells of interest to be evaluated in our study was apoptotic cells. Apoptosis is not a synchronous process (Majno and Joris, 1995), this process can take from hours up to days (6-72 hours) (Willingham *et al.* 1999) to occur so that cells at different stages of apoptotic process will coexist in the same tissue section. For example, an apoptotic cell at its late stage of apoptosis process could be scored as an apoptosis in a 72 h treated section while this cell has undergone apoptosis as a result of treatment after just 24 h and early stage apoptotic cells have already been scored in 24 h treated sections. Hence, our measurements may overestimate the total number of cells that undergo apoptosis in response to PDT damage and the degree of this overestimation is larger at longer treatment periods. Conversely, since our animals were terminated immediately after PDT treatment, tumor cells which have been affected during the last hours of treatment might have not been scored at all. Clearly, these are important issues in terms of assessing the true significance of PDT-induced apoptosis in the brain. Necrosis is a much faster process than apoptosis and it is less likely to remain, appear, or be detected in H&E sections. Therefore our measurements will underestimate the total number of cells that have undergone necrosis. However, in contrast to our measurements for apoptosis, the degree of underestimation is the same at all treatment time periods.

Although, before our experiment we believed that our method of cell evaluation could be more reliable as well as more feasible for quantification studies than others, the extreme variation in morphological features of apoptotic and necrotic cells, similar morphological presentations of apoptotic and necrotic cells at different stages of their process, and exhausting scoring procedure (in order to minimize observational error) made us conclude that our method of cell death evaluation may not be a potential candidate for future apoptosis and necrosis quantification studies.

Since, the healthy areas of brain tissue in selected slides of both the control animals and the PDT-treated animals showed background levels of apoptosis, we can believe that the increased level of apoptosis seen in the treated tissue is attributable to the photodynamic effect. Also, since there were no observed consistent correlation between necrosis and apoptosis, it suggests that the latter is not simply a secondary response to the presence of necrotic cells.

The spatial distribution of apoptotic cells in the tissue was quite homogenous, so that treatment response may not coincide exactly with the location and profile of light illumination, and methods of determining the geometric centre of the distribution seem somewhat arbitrary. Nevertheless, the strong scattering effect of brain tissue, the small size of implanted tumor, as well as small animal skull which may act as a light trapping sphere may result in a homogenous light distribution pattern, so that the tumor response looks homogenous. In addition, the fraction of the brain exposed during PDT in rats will generally be much larger than in the human brain; therefore a stronger correlation between the fluence distribution and the radial apoptotic cell density may be presented in human brain.

Our data demonstrate that, for our experimental PDT treatment protocols, the rate of PDT-induced apoptosis is generally proportional to the fluence rate except at 72 hours. This is consistent with previous findings for apoptosis (Inoue et al, 2007). Because our animal tumor model was small in size and our CNS-1 cell doubling time was long (20 h), we suggest that limited number of surviving tumor cells under treatment may have caused this discrepancy in proportionality of apoptosis with our two higher fluence rates (1.0 and 1.5 mW cm⁻²) after 72 hours, so that we encounter a sudden drop in scored apoptosis cells. In other words, after 48 hours of treatment we are running out of tumor cells to be treated with PDT. It is possible that with larger tumor sizes we could have had the same apoptosis pattern at 72 hours as for the 24 and 48 hours of treatment.

Necrosis shows the same pattern of proportionality to the fluence rate as apoptosis but on a smaller scale. The amount of necrosis for each fluence rate after different treatment times seems to be the same and there are no significant differences between these counts. Therefore, when comparing apoptosis with necrosis - keeping in mind the gradual overestimation of apoptosis over time - it is evident that our lowest fluence rate of 0.5 mW cm⁻² has a better apoptosis to necrosis ratio compare to higher fluence rates. Nevertheless, when comparing apoptosis with the total number of tumor cells surviving after treatment, we can conclude that higher fluence rates act more aggressively to affect tumor cells and consequently have a faster effect on diminishing tumor. Also, higher fluence rates have a better and faster effect on microfoci infiltrates into the brain adjacent to tumor. However, it is possible that with longer treatment periods at lower fluence rates, we could result in more effective reduction in the number of microfoci infiltration into the brain adjacent to tumor.

In general, PDT delivered at higher fluence rate increases both the amount of apoptosis and necrosis, but the apoptosis-to-necrosis ratio is reduced from 3.5 for the lowest fluence rate to 1.5 for the highest fluence rate used. Since even a slight amount of necrosis in the brain can cause inflammatory response, we can suggest that lower fluence rates are more beneficial in ALA-PDT treatment of brain glioma with regard to less treatment-induced inflammation.

We concluded that the lower fluence rate PDT treatment was more effective in increasing the apoptosis-to-necrosis ratio in gross tumor but the crucial determining question is whether or not extended-time PDT treatment at low fluence rate is effective to produce an acceptable tumor growth control by treating the invading part of the tumor. Although treatment of gross tumor can be valuable for inoperable patients, in most cases of malignant glioma the core is necrotic and the tumor re-growth mainly takes place in the border region where microfoci infiltrate (Preusser *et al.* 2006). Addressing this question, our results indicate that, although higher fluence rate PDT has a definite effect on the tumor border region and the brain adjacent to tumor within the first 48 hours of PDT treatment, we can speculate that performing lower fluence rate PDT treatment for longer than 72 hours may result in the same tumor growth control as higher fluence rates.

Another important point to note from our data (table 2) is that we used 9 different protocols differing in fluence rate and treatment time. Nevertheless the total deposited fluence at the end of treatment for some of the subgroups were similar (A-48 and B-24 received 80 J cm^{-2} , A-72 and C-24 received 120 J cm^{-2} , B-72 and C-48 received 240 J cm^{-2}). Comparing the result of those subgroup pairs which differed in the fluence rate protocol yet had the same total fluence after treatment, showed little systemic trend. In other words, one total fluence applied in two fluence rates, produced two different cytotoxic effects in a manner that the protocol with longer treatment time showed less necrotic cells.

There might be multiple factors responsible for this effect but we can suggest that the amount of singlet oxygen produced in time indicates the fate of the tumor cell to either undergo apoptosis or necrosis. Furthermore, as treatment time is extended the newly developed tumor cells or those who survived from the earlier treatments may be affected by further PDT and consequently produce a more thorough response. Therefore, we may speculate that the total fluence is not directly a decisive main factor that influences the photodynamic cytotoxic effect, but it is the length of exposure or the fluence rate which are the main factors. It is evident that low-fluence-rate

ALA-PDT may have a potential to treat glioma at extended treatment time. However, the small size and limited volume of the rat's intracranial space lead to a lack of informative data on treatment periods longer than 48 hours, and light distribution pattern. Also, increased intracranial pressure following PDT, as a result of cerebral edema may cause considerable limitations related to edema and side effects. These differences between small animals and humans are important for the interpretation of treatment outcome and morbidity in clinical practice. For example, no severe side effects were observed among 41 patients participating in a pilot study of ALA-PDT for malignant glioma (Beyer *et al.* 2006). This strongly suggests that the severity of the side effects observed in the rat model is not clinically relevant.

CONCLUSIONS AND FUTURE WORK

PDT is a promising local adjuvant therapy after surgery for patients with malignant gliomas. High-fluence-rate PDT, although used effectively for the treatment of several types of solid tumors, shows to be not effective enough for therapy of brain malignant tumors. Post treatment morbidity and weak tumor growth control associated with high-fluence-rate PDT suggests that the current treatment variables used in clinical settings may not be optimal. Generally, PDT relies on empirically derived treatment variables that include photosensitizer and fluence rate. However, for their optimization, a full understanding of the different components that determine PDT dosimetry and effectiveness is necessary.

However, this is a difficult task because of the enormous complexity created by the interplay of the factors that determine tumor destruction and the heterogeneity in the tissue of these factors, especially the availability of oxygen required for the formation of the cytotoxic product. The duration of photodynamic exposure, which is a function of fluence rate and fluence, has been defined as an important independent factor influencing treatment outcome. It has been shown that treatment duration and fluence rate affect the response of tumor cells and treatment outcome. The reason for this phenomenon may lie in the intermittent nature of acute hypoxia in tumors. The results of our study as well as those of others indicate that the rate of light delivery is a critical parameter for the optimization of PDT protocols.

Consequently, in our study we tested the hypothesis that low-fluence-rate PDT treatment may avoid necrosis and extended-time therapy improves elimination of microfoci infiltrate. The data presented here suggest that low-fluence-rate ALA-PDT treatments are capable of reducing treatment induced inflammation and morbidity by reducing the amount of necrosis. In addition, extended-time PDT treatment provides a desirable tumor growth control and it has a strong effect on infiltrated microfoci which cause tumor relapse. Our results suggest that longer PDT treatments may be required to achieve an optimal tumor growth control with minimum inflammatory response.

Future work in the area of brain photodynamic therapy would be first to develop a better glioma model which resembles the human glioma more closely as well as developing a light source which is more stable for longer treatment periods. The key to any form of extended-time low-fluence rate PDT in a clinical setting is the ability to gain access to portable and/or implantable reliable and stable light sources. Combination of extended-time low-fluence-rate PDT treatment with fluorescent guided resection (FGR) with optimized parameters suitable for human glioma in human trials would be the ultimate goal of this work.

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