Modulation of light delivery in photodynamic therapy of brain tumours

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This study was performed to determine whether modulation of light delivery could improve tumour kill in photodynamic therapy (PDT) of brain tumours, as optimal dosimetry has not been fully established. One hundred and sixty-five adult Wistar rats were treated, of which 70 had an implanted C6 cerebral glioma. Haematoporphyrin derivative (HpD) was injected at doses between 0 and 20 mg/kg, 24 h prior to irradiation with 630 nm laser light. The total energy dose was varied from 0 to 1200 J/cm², with fluence rates of 625, 3125 or 9375 mW/cm². In some studies, the light delivered at 3125 mW/cm² was divided into 10 fractions of approximately 13 s, with refractory intervals of 60 s. The most striking finding was that HpD was much more potent than previously reported. All doses greater than 1.0 mg/kg resulted in normal brain damage with light doses above 50 J/cm². However, at 1.0 mg/kg, significant normal injury was not apparent until 1200 J/cm². Failure of drug–light dose reciprocity indicated that photobleaching occurred, protecting normal tissue. Selective tumour kill was observed to 2.2 mm depth (SE ± 0.44 mm). Using lower power or fractionated light did not improve tumour kill and normal tissue injury occurred with fluence rates of 9375 mW/cm². In conclusion, the doses of HpD currently used in clinical brain tumour trials may be too high to achieve selective tumour kill. Higher light fluence rates allowed shorter intraoperative irradiation times with no loss of efficacy. Photodynamic therapy continues to demonstrate potential as an effective treatment for local control of cerebral lesions.

INTRODUCTION

The outcome for patients with high grade astrocytomas remains unsatisfactory despite advances in the conventional treatments of surgery, radiotherapy and chemotherapy, with over 80% of tumour recurrences occurring within 2 cm of the radiographic edge of the tumour.2–4 Photodynamic therapy (PDT) has been investigated as a novel therapy to improve tumour eradication in this brain adjacent to tumour region. Although some clinical trials have been performed with encouraging results,5–11 the dosimetry required to achieve the optimal effect of PDT on cerebral lesions remains unclear. A major factor limiting PDT is the depth of brain tissue which can be treated without damaging normal brain. Oxygen is a critical requirement for the photodynamic generation of cytotoxic species using most photosensitizers12–16 and reversible tissue hypoxia has been shown to occur during PDT,17,18 as the photochemical reaction consumes oxygen and tissue reperfusion is impaired because of abnormal tumour microcirculation. Several studies have suggested that such tissue hypoxia may significantly impair the efficacy of PDT.18–21 Modulation of light irradiation, including fractionation of the light delivered into several periods of ‘on’ treatment with ‘off’ periods to allow rediffusion of oxygen, has been suggested to facilitate greater production of photochemical species and improve tumour kill.19–25 Another important factor in optimizing dosimetry in PDT is the effect of photobleaching of the photosensitizer. Assuming adequate oxygen supply, the amount of reactive species produced by light energizing a photosensitizer may be expected to be proportional to the concentration of the drug and to the amount of energy delivered to generate the metastable triplets which are the basis of the photochemical reaction. This assumption of drug and light dose reciprocity has been applied to estimate the maximal safe or threshold dose of PDT which can be administered to brain tissue in animal models which in some series have found extreme normal brain sensitivity.26,27 However, although this concept of reciprocity of drug and light dose has been found to hold generally, there is an important exception when large amounts of light are delivered following low doses of photosensitizer as photodestruction, or photobleaching of the photosensizers has been shown to occur and this has been suggested as a potential mechanism for preserving normal tissue in PDT.24,29

In this study, an initial re-evaluation of the dose–response effect of haematoporphyrin derivative (HpD) mediated PDT was required to establish dosimetry which mediates selective tumour kill in the C6 glioma model. The effects of varying the power (fluence rate) and fractionation of the light delivered were then examined in an animal model, to determine which light delivery protocol gave the maximal therapeutic effect.

MATERIALS AND METHODS

Animals

A total of 165 adult Wistar rats were evaluated in these studies. Seventy of these were implanted with the C6 cerebral glioma cell line and 95 were non-tumour bearing animals. The rats were anaesthetized using inhaled vapourized Penthrane in a jar followed by injection of 1 ml per 100 g of chloral hydrate solution (3.6 g/100 ml) into the i.p. cavity.

Glioma cell line

The C6 glioma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA). The cells were grown in RPMI 1640 medium (Lif Technologies) supplemented with glutamine and 5% new born calf serum (Commonwealth Serum Laboratories, Parkville, Australia) and harvested during the log phase of growth.30

Intracerebral tumour model

C6 glioma cells were implanted into the frontal lobes of the rats to establish an intracerebral glioma, based on the technique of Kaye, Morstyn and Ashcroft.30 In 32 rats 1 × 10⁶ cells were implanted, however, the subsequent tumours were often very large with extensive areas of necrosis. Drowsiness and focal neurological signs were also evident in some animals 10 days after implantation. The number of cells implanted was, therefore, reduced to 1 × 10⁶ which resulted in slower tumour growth and a more histologically homogeneous tumour with much smaller regions of necrosis. The mean diameter of the tumours generated was 5.1 mm (SE 0.37).
**HpD preparation and administration**

HpD was prepared by the Pharmacy Department at the Queen Elizabeth Medical Centre, Adelaide, South Australia. It was made by acetylation of haematoporphyrin using the technique described by Forbes et al.\(^{31}\) The HPLC characteristics of the HpD from this source have not measurably changed since 1983 and vary little from batch to batch (G Walker and W Dollman, personal communication). The bottles containing HpD were wrapped in aluminium foil to prevent light mediated degradation and stored at 4°C. The photosensitizer was administered 10 days following tumour implantation in tumour bearing animals, via the i.v. route in doses ranging from 1 to 20 mg/kg, and diluted in sterile normal saline so that approximately 0.5 ml was injected.

**Photo-irradiation**

The delivery of light to the brain was based on the method described by Kaye and Morstyn,\(^{32}\) and this was performed 24 h following administration of HpD. Briefly, a 4 x 4 mm craniotomy was cut using a high speed dental drill, centred on the site where implantation of tumour cells occurred. The bone flap was removed and the bony defect measured. All procedures were performed using an operating microscope and in no case did either dimension vary by more than 0.5 mm. Six hundred and thirty nanometre wavelength light was generated by a variable power output rhodamine 6B dye laser (600 series Dye module set to 630 nm, quasi continuous wave, max power 3.2W, Laserscope, San Jose CA, USA) pumped by a frequency doubled Nd-YAG KTP/532 surgical laser (Laserscope, San Jose CA, USA). The light from the dye module laser was delivered via a 400 mcm inner core diameter silica quartz optic fibre, fitted with a micro lens (Miravant Inc. Santa Barbara, USA) to ensure even light distribution over the irradiation field. The output of the fibre was calibrated before each operating session using an integrating sphere coupled to a power meter. The optic fibre was then mounted vertically on an adjustable stand so that the beam of light was just contained within the dimensions of the craniotomy site. During irradiation of the brain, continuous irrigation with isotonic saline at room temperature was undertaken to avoid tissue hyperthermia.\(^{32}\)

A dose reduction series of experiments was performed initially to determine an appropriate drug and light dose for examining the effect of modulating light delivery. Delivering 400 J/cm\(^2\) of 630 nm light 24 h after i.v. injection of 1.0 mg HpD/kg resulted in tumour necrosis to an approximate depth of 1 mm. This ‘standard’ total dose of HpD and continuous light was then administered using light fluence rates of 625, 3125 and 9375 mW/cm\(^2\) (corresponding to laser outputs of 100, 500 and 1500 mW, respectively). Fractionation of light at 3125 mW/cm\(^2\) was performed by means of a simple manual technique to shield the craniectomy site from the light beam during the ‘dark’ periods. The total ‘on’ delivery time of 128 s was divided into nine fractions of 13 s and a final 11 s fraction, each separated by 60 s to give a total treatment time of 668 s (11.08 min). It was elected to administer 10 fractions to attempt to maximize any benefit gained by reoxygenation of the tissue within the time limitations of the safe period of animal anaesthesia used.

**Measurement of photodynamic effect**

The rats were killed 3–5 days following irradiation. The brains were removed, fixed in formalin and then histologically sectioned and stained with haematoxylin and eosin. The maximal depth of coagulative necrosis perpendicular to the cortical surface was then measured using a cross-hair graticule under light microscopy.

**Fig. 1** Normal brain necrosis following various doses of HpD given intravenously 24 h prior to irradiation with 630 nm light (n = 3–13 in each group). Note that normal injury was dramatically reduced following administration of 1 mg HpD/kg and that significant damage occurred at all light doses when greater than 2.5 mg HpD/kg was used.

**Exclusions**

Twelve rats were excluded from histological examination of their brains because of anaesthetic complications, the presence of a subdural blood collection on raising the bone flap, optical fibre dysfunction or technical problems in histological processing. The 52 rats which had 1 x 10\(^6\) glioma cells implanted were also excluded from the results. No other normal rats or rats implanted with 1 x 10\(^6\) tumour cells were excluded from evaluation and at least three normal rats and five tumour bearing animals were used in each major treatment group. The mean and standard error for each group was calculated assuming a normal distribution.

**RESULTS**

**Normal brain response to PDT**

The effect of PDT on normal brain following administration of HpD and light is shown in Fig. 1. Initially the rats were treated with 20 mg HpD/kg as the optimal dosimetric parameters found in Kaye and Morstyn’s previous study were 20 mg HpD/kg and 200 J/cm\(^2\) of 630 nm light.\(^{32}\) However, all three rats which then received a dose of 200 J/cm\(^2\) of light died within 24 h. Histological analysis of the brains of these animals revealed the presence of regions of extensive necrosis and oedema. At a dose of 5 mg/kg, the dose of HpD used in clinical trials, marked necrosis could be induced following doses of light as low as 50 J/cm\(^2\). However, following administration of 1.0 mg HpD/kg a much wider range of light doses was possible and only after delivering light at doses greater than 800 J/cm\(^2\) of light was significant normal tissue toxicity evident. In addition nine non-tumour bearing animals were irradiated without prior administration of HpD. The group receiving the highest light dose (1200 J/cm\(^2\)) at the highest fluence rate (9725 mW/cm\(^2\)) demonstrated brain necrosis to a depth of 1.37 mm, indicating a toxic effect of high energy delivery in the absence of a photosensitizer. Three rats irradiated with the same high total energy dose (1200 J/cm\(^2\)), but delivered at an intermediate fluence rate of 3125 mW/cm\(^2\), demonstrated a reduced degree of injury (0.53 mm). However, the group of rats which received the moderate total energy dose of 200 J/cm\(^2\) delivered at 625 mW/cm\(^2\) showed no evidence of normal brain necrosis.

**Failure of drug–light reciprocity**

Determining the photochemical dose, or the amount of toxic species actually generated by PDT, is complex.\(^{26}\) In terms of
dose was increased proportional to the decrease in photosensitizer dose. Photobleaching of the photosensitizer occurs at this concentration resulting in less normal tissue injury. Note the decreased photodynamic effect at 1.0 mg HpD/kg suggesting that photobleaching of the photosensitizer occurs at this concentration resulting in less normal tissue injury.

Selective tumour kill

Figure 3 shows the effect of photodynamic therapy on the implanted cerebral gliomas, using 1.0 mg HpD/kg 24 h prior to irradiation with 630 nm light. Selective tumour kill is demonstrated with administration of light doses between 400 and 800 J/cm². The mean depth of tumour necrosis following 800 J/cm² of light was 2.2 mm (SE 0.44, n = 6) and the maximal necrosis obtained in any tumour was 3.4 mm. At higher doses of light significant normal tissue injury became apparent. The histological pattern of both normal brain and tumour necrosis which resulted from PDT was that of coagulative necrosis with a well defined margin, as previously described.26-27,32 Four tumour bearing animals had 'sham' operations, in which no laser light was administered. Three showed no evidence of necrosis, but one had a very narrow finger of necrosis consistent with the needle tract made during tumour implantation, indicating that the necrosis seen following PDT was the result of treatment, not the surgical procedure alone.

Variation of fluence rate

Figure 4 shows the depth of necrosis in the Wistar rat model following light delivery at various fluence rates. There was a trend towards increased depth of tumour necrosis with increased rather than decreased fluence rates, although the differences did not reach statistical significance. This trend was contrary to the hypothesis that slower light delivery would increase tumour kill by allowing greater production of free oxygen radicals. Normal tissue injury again became evident when light fluence rates of 9725 mW/cm² were used to give a total of 400 J/cm². This effect is important to consider in maximizing the rate of light energy delivered during PDT. For this experimental system, 3125 mW/cm² was the maximal safe light fluence rate. There no benefit evident in reducing the rate of energy delivery.

Fractionation of the light dose

Figure 5 shows the depth of necrosis in tumour and normal tissue following PDT using continuous and fractionated delivery of the light. There was actually a trend towards poorer tumour kill using the fractionated schedule (mean depth of 0.46 mm, SE 0.25, n = 5) compared to using continuous light (mean 1.21 mm, SE 0.27, n = 9), although the difference found was not statistically significant. Allowing time for oxygen diffusion to replenish that consumed by the photochemical reaction was not a major limiting factor in the photodynamic reaction.

DISCUSSION

In order to establish a role in multimodal treatment for brain tumours, it is essential that PDT achieves selective tumour kill. This has been demonstrated in a limited number of studies using animal models26-31 and this series further contributes to the evidence that this is possible. Observing the effect of modulating light delivery, rather than obtaining the maximal possible depth of selective tumour kill, was the focus of this study, however, selective cytotoxicity was demonstrated to 2.2 mm. The maximal tissue necrosis seen in this series was 7.5 mm, suggesting that a treatment field of at least this depth is possible.
Photobleaching of the photosensitizer in normal tissues appeared to be essential in protecting normal brain from the photodynamic effect. Some animal model series have found extreme normal brain sensitivity when drug and light dose reciprocity has been assumed to estimate the maximal safe or threshold dose of PDT. However, photodestruction of photosensitizers by light appeared to be an important mechanism for preserving normal tissue during PDT in this study. When the HpD dose was reduced to 1.0 mg/kg a dramatic and disproportionate decrease in the cytotoxic effect was seen, which allowed a much wider range of light doses to be delivered safely. The higher levels of HpD found in tumour tissue may then allow a selective cytotoxic effect to occur (Fig. 3).

The 1 mg/kg dose of HpD required for protection of normal brain in this series is much less than the approximate equivalent photosensitizer dose used in other studies on animal brain, which may explain the frequent failure to elicit a selective tumour response in some series. The baseline dosimetry presented here, however, also conflicts with previous studies showing selective tumour kill by several orders of magnitude.

Despite important advances in light delivery, including more efficient and reliable lasers and the use of microdoses to evenly distribute light, this component of PDT is accurately measured and major unexpected changes are unlikely. Furthermore, the dose of light delivered after 20 mg/kg of HpD would have to have been far less than the previously recommended 400 J/cm² to prevent normal brain necrosis.

The HpD used for photosensitization is less easily monitored using laboratory assays. Prior to 1989 HpD was stored frozen awaiting orders, which may subtly alter its chemistry. It is also possible that some variation in raw materials and production methods has occurred, as HpD, like Photofrin, is a complex mixture of porphyrins and the precise measurements of the levels of active drug administered or found in target tissues is not possible. It is, therefore, imperative that the biological activity of these photosensitizers is monitored in appropriate models. In clinical use, imaging of anatomical and physiological properties of the target region, such as MRI and magnetic resonance spectroscopy, may complement histological animal studies.

The development of newer second generation photosensitizers with longer wavelength light, higher tumour to normal tissue uptake ratios, more easy photobleaching, or less vascular uptake may in future enable a more effective and specific cytotoxic effect to be demonstrated. Drugs which can be readily measured and monitored would allow much more precise and reliable dosimetric parameters, which is an important step for the progression of PDT into clinical use.

Modulation of light delivery has been proposed as an alternative means of improving PDT efficacy. Following the observation that lower power and fractionated light delivery significantly delayed tumour growth in a mouse mammary carcinoma model, Foster et al. examined the oxygen consumption estimated to occur during the Type 2 photochemical reaction and found that oxygen consumption rates at relevant light intensities were large enough to decrease the radius of oxygenated cells around an isolated capillary and create hypoxic areas which are resistant to PDT. This effect is expected to be more pronounced at higher power densities, and ‘dark intervals’ of 5–45 s to allow rediffusion of oxygen were suggested to increase the amount of tumour tissue sensitive to PDT destruction. This mathematical model is supported by findings using transcutaneous oxygen electrodes which documented reversible tissue hypoxia during PDT. Henning further developed this concept using a transient one-dimensional mathematical model to estimate that the time required to deplete oxygen supplies was less than 2 s, indicating that an unequal on/off interval is required for optimal light delivery. As well as allowing tissue reoxygenation during treatment, fractionation of the light dose has other theoretical benefits such as reducing the vascular effects of PDT, allowing time for increased oxygen delivery to deep hypoxic sites as more superficial cells become damaged, and allowing better repair of sublethal injury in normal cells compared to neoplastic cells. Subcellular redistribution of photosensitizer following initial light exposure, which has been noted to occur, may also have an impact on the cytotoxicity of the treatment. Several pre-clinical studies have suggested an improved effect with light modulation. When using EMT6 sarcoma spheroids, Foster et al. found that the fraction of surviving cells following PDT had a strong dependence on the fluence rate. In animal studies some groups have also supported using a lower photoradiation rate to improve efficacy; others however, have found the opposite effect. In CNS series, Kaye et al. found no difference between PDT with laser outputs between 200 and 1200 mW in their preliminary study using a cerebral model and neither did Chen et al. in a study of normal brains of rats varying the light fluence rate from 10–200 mW/cm². Fractionation of the light dose, with one or more interruptions in its delivery, has also been advocated in some studies of non-CNS tissues. Not all studies have been consistent with a hypoxic theory however. For example, Messman found similar results with a single interruption of between 10–90 s compared with a number of fractions. Interestingly, van Geel found no improvement with fractionating light delivery following Photofrin sensitization, but noted benefit following administration of an alternate sensitizer mTHPC. In the only study reported on brain tissue, Chen et al. found no difference in the lesions produced using 30 s on/off protocol with a Photofrin dose of 12.5 mg/kg.

The results presented here for fractionated light delivery, in combination with the trend towards poorer efficacy seen with lower power light delivery, indicate that reversible tumour tissue hypoxia is not a major limiting factor in PDT of cerebral lesions using HpD. Leaching or sub-cellular redistribution of activated factors from the target tissue during the ‘off’ time may be a factor in reducing the treatment efficacy. The reduced cytotoxicity with fractionated light may also be consistent with a predominantly vascular mechanism of tumour necrosis in which rapid activation and necrosis of vascular structures leads to thrombosis of tumour vasculature with subsequent tissue necrosis.

![](image)
20–500 mW/cm². The results of this study suggest that the total dose of light could be delivered more quickly, up to 3125 mW/cm², without loss of efficacy. This might be achieved by using more powerful lasers or multiple light sources and would dramatically reduce the intraoperative time required for PDT. However, careful consideration must also be given to the different physical properties, particularly the thermal effects, of irradiating a relatively large cerebral cavity in determining a maximal safe rate of light delivery.

In conjunction with surgery, radiotherapy and newer biological methods to control the proliferation of malignant cells, PDT continues to hold promise in improving the control of high grade astrocytomas through selective local eradication of tumour in the brain adjacent to tumour region. The careful application of PDT may help to overcome the perennial problem of achieving therapeutic efficacy against cerebral tumours without causing unacceptable harm to the surrounding normally functioning brain.

REFERENCES


Sequential changes and recoveries of the motor evoked potential in experimental acute intracranial hypertension

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Summary The D- and I-waves of the motor evoked potential (MEP) were investigated as a monitor for acute intracranial hypertension in 20 dogs. Intracranial pressure (ICP) was raised by inflation of an extradural balloon. The MEP elicited by electrical transcortical stimulation were recorded during inflation and deflation of the balloon. The D-waves were linearly suppressed according to the ICP (mmHg), however, the I-waves and the ICP level did not correlate. Each wave disappeared in the animals kept about 50 mmHg or more, whose pupils were dilated. In the animals kept under 60 mmHg, the amplitude of the D-wave recovered proportionate to the period during which the amplitude was suppressed less than 50%. The changes of the MEP have some relation to histopathological changes. The results demonstrate that the D-wave of MEP is a useful monitor for intracranial hypertension.

MATERIALS AND METHODS

Twenty adult dogs, of both sexes with a weight range of 10-15 kg, were used. The experiments complied with institutional guidelines for animal research. Anaesthesia was induced with 50 mg ketamine hydrochloride. Two cannulas were placed, one was in the femoral artery to monitor the blood pressure and arterial blood gases. The animals were intubated under a steep dose of thiopental sodium maintenance dose of 2-3 mg/kg and were ventilated using continuously infused pancuronium bromide and a small amount of thiopental sodium. Their blood gases were maintained as follows: pO₂ 70-100 mmHg, pCO₂ 30-35 mmHg and pH 7.3-7.4. The body temperature was maintained at 37-38°C and heart rate was monitored with an electrocardiograph. A latex balloon was inserted into the left posterior temporal extradural region and a subdural ICP sensor catheter maintained at 37-38°C and heart rate was monitored with an electrocardiograph. A latex balloon was inserted into the left posterior temporal extradural region and a subdural ICP sensor catheter was placed contralateral to the side of the balloon, through small burr holes. Each burr hole for the balloon, ICP monitor and the electrodes was scaled with bone wax (Fig. 1).

Motor evoked potential

A Neuropack-8 recording system (Nihon Kohden Ltd.) was used for stimulation and recording of the evoked potentials. For transcortical stimulation two small burr holes occupying an area of 5 mm in the left frontal bone, ipsilateral to the side of the balloon, were made just beside the frontal sinus, which is over the motor cortex as described by Redding. The cortex was stimulated by two silver ball electrodes placed on the dura through these burr holes. The MEP elicited by electrical transcortical stimulation is thought to assess pyramidal pathway function more directly.16-21 Patton and Amassian30 characterized the responses evoked by direct stimulation of the motor cortex of cats and the recording of responses using microelectrodes located at both the bulbary pyramids and their lateral funiculus of the cervical spinal cord. The MEP consisted of an early positive deflection (D-wave) travelling at about 60 m/s, and a series of later positive deflections (I-waves) following the D-wave with a time period of 1 msec or more.21,22 The D-wave might have resulted from the excitation of the initial segments of pyramidal neurons being conducted in the fast axons, whereas the I-waves might result from an indirect or relayed excitation of the pyramidal neurons through the cortical interneurons.21,22 It has been reported that the number of I-waves was several.

We observed the sequential changes of the D-waves and I-waves elicited by direct cortical electrical stimulation during the acute progressive elevation of intracranial pressure (ICP), and investigated whether they were effective or not as a monitor for intracranial hypertension.