TECHNICAL NOTE

PPS www.rsc.org/pps

Fluorescence image-guided brain tumour resection with adjuvant metronomic photodynamic therapy: pre-clinical model and technology development

Arjen Bogaards,[a] Abhay Varma,[c] Kai Zhang,[b] David Zach,[b] Stuart K. Bisland,[a] Eduardo H. Moriyama,[a] Lothar Lilge,[a] Paul J. Muller[c] and Brian C. Wilson*[a,b]

[a] *Division of Biophysics and Bioimaging, Ontario Cancer Institute/ University Health Network and University of Toronto, Toronto, Ontario, M5G 2M9, Canada*

[b] *Laboratory of Applied Biophotonics, Ontario Cancer Institute/ University Health Network and University of Toronto, Toronto, Ontario, M5G 2M9, Canada*

[c] *Division of Neurosurgery, St. Michael's Hospital, Toronto, Ontario, M5B 1W8, Canada. E-mail: Wilson@uhnres.utoronto.ca*

Received 24th September 2004, Accepted 22nd March 2005
First published as an Advance Article on the web 14th April 2005

Fluorescence-guided resection (FGR) and photodynamic therapy (PDT) have previously been investigated separately with the objectives, respectively, of increasing the extent of brain tumour resection and of selectively destroying residual tumour post-resection. Both techniques have demonstrated trends towards improved survival, pre-clinically and clinically. We hypothesize that combining these techniques will further delay tumour re-growth. In order to demonstrate technical feasibility, we here evaluate fluorescence imaging and PDT treatment techniques in a specific intracranial tumour model. The model was the VX2 carcinoma grown by injection of tumour cells into the normal rabbit brain. An operating microscope was used for white light imaging and a custom-built fluorescence imaging system with co-axial excitation and detection was used for FGR. PDT treatment light was applied by intracranially-implanted light emitting diodes (LED). The fluorescent photosensitizer used for both FGR and PDT was ALA-induced PpIX. For PDT, ALA (100 mg kg^{-1}) and low light doses (15 and 30 J) were administered over extended periods, which we refer to as metronomic PDT (mPDT). Eighteen tumour bearing rabbits were divided equally into three groups: controls (no resection); FGR; and FGR followed by mPDT. Histological whole brain sections (H&E stain) showed primary and recurrent tumours. No bacteriological infections were found by Gram staining. Selective tumour cell death through mPDT-induced apoptosis was demonstrated by TUNEL stain. These results demonstrate that the combined treatment is technically feasible and this model is a candidate to evaluate it. Further optimization of mPDT treatment parameters (drug/light dose rates) is required to improve survival.

Introduction

Protoporphyrin IX (PpIX) can be used as both a tumour marker for fluorescence-guided resection (FGR)[1] and as a photosensitizer for photodynamic therapy (PDT)[2] of brain tumours. After systemic administration of 5-aminolevulinic acid (ALA) and a suitable time interval (typically 2–6 h), ALA is selectively metabolized into protoporphyrin IX (PpIX) by certain brain tumours.[3–10] PpIX is fluorescent, with characteristic red emission peaks at 635 and 704 nm under UV/blue light excitation. When photoactivated it can also generate short-lived cytotoxic free oxygen radicals, namely singlet oxygen.[11] Hence, ALA-induced PpIX is used for a variety of both photodiagnostic and phototherapeutic applications.

In particular, PpIX fluorescence has been investigated for detection and image-guided resection of brain tumours.[1] In a preclinical model, we recently demonstrated that fluorescence guided resection (FGR) of brain tumours decreased residual tumour volume by a factor of 16 compared to white light resection,[12] while Stummer *et al.* demonstrated that FGR decreased residual tumour in glioma patients and additionally influenced survival.[13]

Extensive clinical[1,2] and preclinical[5–7] studies with other PDT photosensitizers, including hematoporphyrin derivatives, show that PDT can kill brain tumours *in situ*, but at the cost of some damage to normal brain adjacent to tumour.[6,14] Using ALA-PpIX pre-clinically *in vitro* and *in vivo*, we[6,7] and others[5] suggest that tumour selective cytotoxicity is feasible. In particular tumour-cell apoptosis can be induced by low dose ALA-PDT without inducing a necrotic response in tumour or normal brain and with negligible apoptotic cell death in the latter.[15] However, adequate and selective tumour kill likely cannot be achieved in a single treatment, so that it will be necessary to apply the photosensitizer and light at low dose rates over an extended period, a regime that we have termed metronomic PDT (mPDT).[16,17]

In summary, although both FGR and PDT have, individually, demonstrated potential benefit, it is likely that the combination will be more effective than either approach alone. It is the objective of the present work to develop and evaluate pre-clinical models and techniques required for this, for ultimate translation to clinical trials.

Hence, in this paper we demonstrate the technical feasibility of the combined treatment for the first time in a tumour-bearing animal model. This requires a relevant tumour model, a means of photosensitizer delivery and a light source/delivery system for low dose-rate photoactivation. This work is an extension of preliminary observations reported previously.[18,19]

Materials and methods

Tumour induction and study groups

This study was approved by the Animal Care Committee of St. Michael's Hospital, Toronto. Rabbits with intracranially-induced VX2 carcinoma were used as the model. We recently demonstrated this is an appropriate first model for assessing FGR,[12] since it provides tumours of reasonable size (5–8 mm),

DOI: 10.1039/b414829k

Table 1 Study groups, light delivery devices, light/ALA dose, administration time and mPDT frequency

Group	*n*	FGR	mPDT device	Light dose/J[a]	FGR/mPDT ALA dose/mg kg^{-1}[a]	Illum./admin. time / min h^{-1}	mPDT freq.
A	6	–	None	0	0/0	0	0
B	6	+	Dummy	0	0/0	0	0
$C_{1,2}$	2	+	Manual	15	20/100	42/5.5	Every other day
$C_{3,4}$	2	+	Sub-cutaneous	30	20/100	84/5	Daily[b]
$C_{5,6}$	2	+	Backpack	30	20/100	84/5	Daily

[a] Light and ALA dose is given per treatment. [b] In $C_{3,4}$ mPDT was aborted 2 days post-FGR due to discomfort of the subcutaneous timer.

which simplifies the technique compared to intracranial rat tumour models.[18] The VX2 tumour has similarities in growth characteristics compared to human malignant gliomas, including microinvasion, pseudo-palisading, growth along the blood vessels and in perivascular spaces and break down of the blood-brain barrier within the tumour and in brain adjacent to tumour.[15] However, since the VX2 is not a glioma, it may not the optimal model in terms of the biological responses to mPDT, as discussed in more detail below.

The techniques for tumour induction and FGR in this model have previously been described in detail.[12] Briefly, tumours were induced in male New Zealand White rabbits (3.3–3.8 kg, Charles River Canada, Montreal, QC, Canada). For this, 2×10^5 VX2 cells in 50 µl phosphate buffered saline (PBS) were injected through a burr hole at a depth of 2 mm beneath the dura and allowed to grow. Eighteen of 22 animals survived without complications and were divided equally into three groups shown in Table 1 as A, B and C. Group A did not undergo FGR nor mPDT. Group B underwent FGR followed by LED implant, but PDT was not applied. Group C underwent FGR followed by LED implant and received mPDT. All animals were monitored twice daily and sacrificed by *i.v.* T61 injection (Intervet Canada, Whitby, ON, Canada) upon the first signs of neurological deficit, manifested as a pronounced head tilt that we estimate could not be misssed more than once. Hence, the subjectivity in this observation was estimated to be 12 h.

Fluorescence-guided resection and surgical LED implant

FGR was performed as described previously.[12] Briefly, 20 mg kg^{-1} ALA (Levulan, DUSA Pharmaceuticals, Valhalla, NY, USA) was diluted in PBS, buffered to pH 6.2–6.8 and injected *i.v.* within 15 min after preparation. Surgery was performed 7 days post-VX2 induction and 4 h post-ALA injection. Animals in groups B and C were anesthetized, a craniotomy was performed, the bone flap was removed, the dura was cut and subsequently the tumour was located with an operating microscope under white light illumination and resected by FGR. For this, the surgical cavity was illuminated with 405 nm (20 nm FWHM) light and red PpIX fluorescence was detected at 630 nm (20 nm FWHM, >OD 7 rejection of the blue/UV excitation light). The eyes were closed with tape during FGR, to prevent eye and skin photosensitive reactions. Biopsies were taken under FGR, fixed in formalin and saved for histopathology. Groups B and C received a permanent LED implant that was positioned inside the surgical cavity (L711SED/H, Kingbright, EMX Enterprises Ltd., Richmond Hill, ON, Canada). The LED was attached to the bone flap through an enlarged burr hole, as shown in Fig. 1a. To avoid irritation, the electric wires of the LED were isolated with medical grade silicone. To prevent light exposure to the skin, the rear side of the LED was covered with black medical grade epoxy. Electric wires were connected to a power supply in three different configurations, as described below. Hibitane antibiotic cream was applied topically on a daily basis to prevent irritation and infection. As described above, analgesic was administered. Steroids (Dexamethasone, Sabex, Boucherville, QC, Canada) were administered *i.m.* daily to all animals, starting 7 days after tumour induction, to decrease intracranial pressure and to mimic the clinical situation.

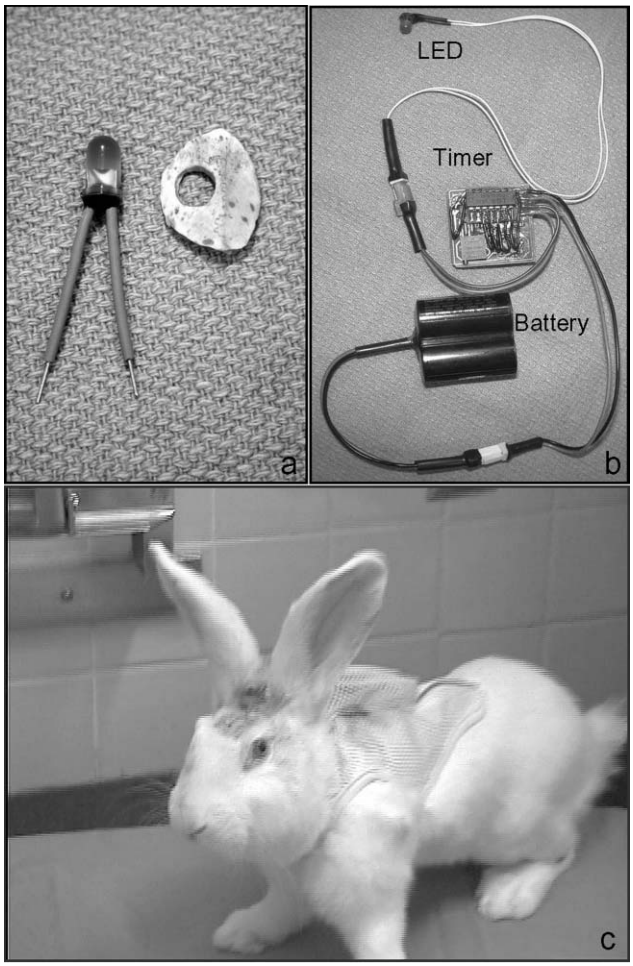

Fig. 1 Light was delivered to the brain with a surgically implanted LED that was placed into a burr hole in the bone flap, as shown in (a). Electricity was delivered to the LED in three different configurations. Manually, with the electric wires in (a) connected to an external 6 V DC power supply, or (b) with a miniature power and timer device, that was placed subcuteneously, or (c) held in a backpack.

Drug and light delivery for mPDT

For each mPDT treatment 100 mg kg^{-1} ALA was prepared and injected as described above. mPDT was started the day following FGR. For this, the animals were not anaesthetized. Animals were kept under dim light levels for the entire period after tumour resection. The output power of the LED with 5 mm diameter was 6 mW when supplying a 20 mA driving current. We assumed the hemispherical diffuse LED surface to be in contact with brain tissue, which translated to an irradiance of approximately 6 mW cm^{-2}. The center wavelength was 635 nm (30 nm FWHM), verified with a spectrometer. Because of the low power a relatively high ALA dose was used. Hence, significant

bleaching of PpIX was not expected, based on our previous FGR work where similar power levels were used for excitation of fluorescence and bleaching was not observed.[12] The temperature of the LED did not exceed 37–39 °C. For animals $C_{1,2}$ the light was delivered every other day by manually connecting the LED wires exposed on the outside of the scalp to an external 6 V DC power supply, delivering 15 J over 42 min at 5.5 h after ALA administration. With the intention to be less constraining, for animals $C_{3,4}$ the light was delivered by a miniature automatic timer switch and 6 V lithium battery (2CR5MPA/1B, 1400 mAh, Panasonic) both embedded in medical grade silicone. This timer device was developed for this study and comprised a circuit board of three stages: (1) a timer integrated circuit (IC) (LM556, Texas Instruments, TX, USA) operating in monostable mode and generating a square wave clock signal as input for the second stage, (2) a 12 bit binary counter (CD4040, Texas Instruments, TX, USA), the output of which was fed into (3) a similar timer IC operating in monostable mode to provide the 20 mA current for the LED. This device was able to switch on the LED daily for 84 min with the above battery.

In animals $C_{3,4}$, the timer and battery were placed subcutaneously on the shoulders with the electric wires of the LED passed subcutaneously from the skull, delivering 30 J over 84 min once per day at 5 h after daily ALA administration. The device shifted sideways causing discomfort, so that after two mPDT treatments the timer device and battery were surgically removed, but the LED remained in place. To avoid this problem in animals $C_{5,6}$ a similar automatic timer switch and battery were held in a backpack (NP-620076, Harvard Apparatus, Saint-Laurent, QC, Canada), as shown in Fig. 1c. These were well tolerated by the animals.

Histopathology

After sacrifice the brain was removed intact and fixed in 10% formalin for a minimum of 30 days. Sections 5 μm thick were prepared of the whole brain including the tumour regions and stained with hematoxylin and eosin (H&E). Gram stains were also performed to investigate the presence of any bacterial infection. Fluorimetric TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling) staining was performed to investigate the presence of PDT-induced apoptotic cells. This stained the DNA of apoptotic cells with green fluorescein fluorescence (Promega, Madison, WI, USA), while propidium iodine (Molecular probes, Eugene, OR, USA) stained all cell nuclei with red fluorescence. These were examined simultaneously using a confocal laser-scanning microscope (LSM510, Zeiss, Germany) with 488 nm excitation and 505–550 nm detection for fluorescein and 543 nm excitation and >585 nm detection for propidium iodine.

Results

Surgical procedure and metronomic PDT

Tissue with positive PpIX fluorescence was detected and resected in animals in group B and C. Two rabbits did not recover from anesthesia, likely as a result of anesthetic overdose. One animal did not recover well from surgery, demonstrating unresponsive behavior and had to be sacrificed 4 days post-resection. One animal scratched out the LED implant and was sacrificed, no other animal attempted to do so. The remaining 12 animals recovered well from tumour resection, showing normal responsive behavior, motor function, bowel output and food intake. *I.v.* ALA injections were performed within a ±15 min window and the timer circuits worked successfully with an accuracy of ±3 min.

Survival

All surviving animals were sacrificed between 8 and 21 days after tumour induction when demonstrating a head tilt. The average survival times were, group A: 13.3 ± 2.9 days, B: 17.0 ± 1.4 days and C: 17.7 ± 2.2 days, as shown in the scatter plot in Fig. 2. Rabbits $C_{1,2}$ were sacrificed at 15 and 16 days. These animals received 4 and 5 PDT treatments, respectively, performed every other day. Rabbits $C_{3,4}$ were sacrificed at 17 and 19 days. Both these animals, in total, received only 2 mPDT treatments during the first 2 days after FGR. The third day after FGR the timer was removed due to discomfort of the subqutaneous timer that shifted sideways, as described above. Rabbits $C_{5,6}$, in which the backpack was used, were sacrificed at 18 and 21 days. These animals received 11 and 14 PDT treatments, respectively. An independent-sample *t*-test demonstrated a significant survival increase ($p < 0.05$) in group B compared to group A, indicating a benefit of tumour resection *vs.* no resection. Group B and group C showed no significant difference ($p > 0.05$), indicating that daily mPDT of up to 30 J and 100 mg kg^{-1} ALA did not significantly influence survival.

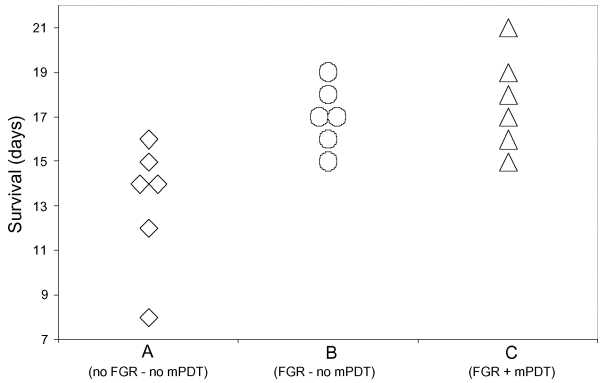

Fig. 2 Scatter plot of survival times for groups A, B and C. A significant difference ($p < 0.05$) was found between groups A and B, but not between B and C ($p > 0.05$).

Histopathology

H&E stained sections of the biopsies taken during FGR revealed regions of infiltrative tumour under microscopy, similar to our previous findings.[12] When removing the brain in groups A, B and C, the brains were swollen and the tumour was visible with the naked eye. Coronal sections demonstrated large primary tumours in group A and large recurrent tumours mostly refilling the surgical cavity made with FGR in groups B and C, as illustrated in Fig. 3. No necrosis was observed in the proximity of the LED. Gram stains were negative for all animals, demonstrating the sterility of the resection and LED

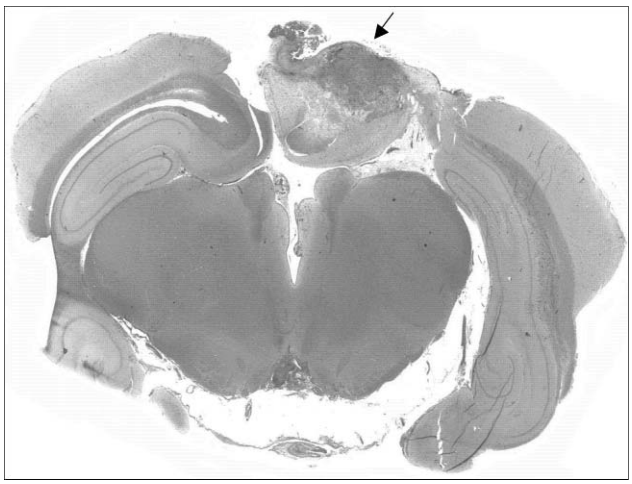

Fig. 3 H&E stained coronal section of the rabbit brain with the arrow pointing towards a recurrent VX2 tumour re-filling the resection cavity previously made by FGR.

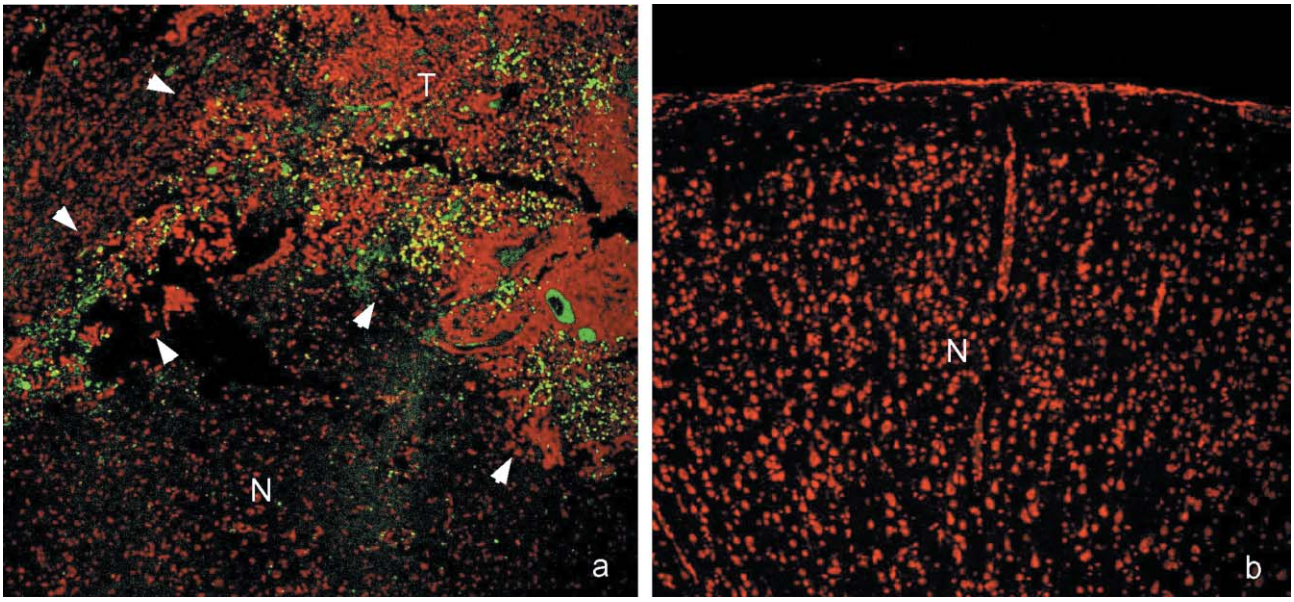

Fig. 4 Fluorescence confocal microscopy image (10× objective, NA 0.5) of fluorimetric TUNEL assay of rabbit brain sections. (a) The infiltrative tumour margin (T) and normal brain (N) 12 h after 14 daily mPDT treatments. TUNEL positive (apoptotic) cells are stained green and all cells nuclei are labelled red (propidium iodine). The tumour margin can be identified, since the tumour has a larger nuclear density in comparison with the normal brain: arrows point towards the tumour margin (reproduced from ref. 17 with the permission of the American Society of Photobiology). (b) Image of contralateral normal brain.

implant surgical procedures. PDT-induced apoptosis is likely maximum in the 12–24 h time interval after the last mPDT treatment,[20] making it difficult to quantify apoptosis here, since most animals were sacrificed at different time points, according to neurological signs: $C_{1,2}$ at 24–48 h, $C_{3,4}$ at >48 h (because here the subcutenous timer was removed) and $C_{5,6}$ at 12 and 24 h after the last mPDT treatment. TUNEL staining of group A (no mPDT) demonstrated only small percentages of apoptosis, in the range of 1–2% in the tumour, with no detectable apoptosis in the contralateral normal brain. Similar results were found for animals $C_{1\text{-}4}$. By contrast, some sections of animals $C_{5,6}$ demonstrated areas with a higher amounts of tumour-selective apoptosis, in particular the animal sacrificed at 12 h post-mPDT, as shown in Fig. 4a. Note that the apoptosis is selectively present in the tumour and to a much lesser extent in the brain adjacent to the tumour. The normal contralateral brain shows no apoptosis as seen in Fig. 4b. H&E staining of adjacent sections demonstrated cell shrinkage and pyknosis, which are associated with apoptotic cells, at similar locations and rates of incidence.[21]

Discussion

These results demonstrate that a new treatment strategy for malignant gliomas, comprising fluorescence guided resection (FGR) with adjuvant metronomic PDT (mPDT), is technically feasible and that the intracranial VX2 model in the rabbit is a candidate to evaluate this combined treatment.

Tissue morphology of group B demonstrated recurrent brain tumours approximately 10 days after FGR, refilling the entire surgical cavity. This is likely the result of small tumour nests that were undetected by FGR,[12] but that can be detected using high sensitivity bioluminescence imaging with luciferace-transfected tumour cells.[18] This is similar to gliomas, where recurrence is also often within close proximity of the resected margin.[22,23] This observation further validates the need to follow FGR by an adjuvant therapy such as mPDT.

The presence of apoptotic cell death with high tumour selectivity demonstrates that the concept of mPDT is valid in this model. However, FGR followed by mPDT with daily light and drug doses of up to 30 J and 100 mg kg^{-1}, for up to 14 days, did not influence survival compared to the FGR-only group. This indicates further optimization of the mPDT treatment parameters (drug/light dose rates and application times) is required to improve survival, and dose escalation studies are in progress.

Although the VX2 model has similarities in growth characteristics compared to gliomas, as discussed above, it is likely not the optimal tumour model in terms of the biological response to mPDT, since it is a carcinoma rather than a glioma. The murine 9L gliosarcoma, human U87 glioma or the rat CNS-1 glioma models are more relevant, so that we are extending this work to these models. Since the rat brain is smaller, this is substantially more challenging, both to achieve a high degree of tumour resection by FGR and to apply the mPDT light.

The methods used here to assess tumour response, namely the onset of neurological deficit and post-mortem histopathology, provide information at only one time point and require sacrifice of the animal. It would be preferable to obtain tumour response data over multiple time points in single animals. This may be feasible with techniques such as MR imaging, but this is costly and measures only the gross tissue response. As mentioned above, an alternative is the use of bioluminescence imaging. We have recently demonstrated the feasibility of bioluminescence monitoring of (non-metronomic) PDT response *in vivo*.[24] We plan, therefore, to use bioluminescence imaging to monitor completeness of FGR resection, tumour re-growth after resection and tumour response to mPDT.[18]

Acknowledgements

This investigation was supported by grant CA 43892 DHHS/NIH/NCI and by the Canadian Institutes of Health Research. DUSA Pharmaceuticals NY kindly provided ALA. Microscopes and image processing software for histopathological analysis were provided by the Advanced Optical Microscope Facility (AOMF) at the Ontario Cancer Institute, Toronto, supported by the Canadian Foundation for Innovation.

References

1 S. A. Friesen, G. O. Hjortland, S. J. Madsen, H. Hirschberg, O. Engebraten, J. M. Nesland and Q. Peng, 5-Aminolevulinic acid–based photodynamic detection and therapy of brain tumors (review), *Int. J. Oncol.*, 2002, **21**, 577–582.

2 H. Kostron, A. Obwegeser and R. Jakober, Photodynamic therapy in neurosurgery: a review, *J. Photochem. Photobiol. B: Biol.*, 1996, **36**, 157–168.
3 K. M. Hebeda, A. E. Saarnak, M. Olivo, H. J. Sterenborg and J. G. Wolbers, 5-Aminolevulinic acid induced endogenous porphyrin fluorescence in 9L and C6 brain tumours and in the normal rat brain, *Acta. Neurochir.*, 1998, **140**, 503–512.
4 B. Olzowy, C. S. Hundt, S. Stocker, K. Bise, H. J. Reulen and W. Stummer, Photoirradiation therapy of experimental malignant glioma with 5-aminolevulinic acid, *J. Neurosurg.*, 2002, **97**, 970–976.
5 W. Stummer, S. R. Ennis, A. L. Betz and R. F. Keep, *In vitro* and *in vivo* porphyrin accumulation by C6 glioma cells after exposure to 5-aminolevulinic acid, *Cereb. Blood Flow Metab.*, 1999, **19**, 79–86.
6 L. Lilge, M. C. Olivo, S. W. Schatz, J. A. MaGuire, M. S. Patterson and B. C. Wilson, The sensitivity of normal brain and intracranially implanted VX2 tumour to interstitial photodynamic therapy, *Br. J. Cancer*, 1996, **73**, 332–343.
7 L. Lilge and B. C. Wilson, Photodynamic therapy of intracranial tissues: a preclinical comparative study of four different photosensitizers, *J. Clin. Laser Med. Surg.*, 1998, **16**, 81–91.
8 J. Moan, L. W. Ma, A. Juzeniene, V. Iani, P. Juzenas, F. Apricena and Q. Peng, Pharmacology of protoporphyrin IX in nude mice after application of ALA and ALA esters, *Int. J. Cancer*, 2003, **103**, 132–135.
9 S. R. Ennis, A. Novotny, J. Xiang, P. Shakui, T. Masada, W. Stummer, D. E. Smith and R. F. Keep, Transport of 5-aminolevulinic acid between blood and brain, *Brain Res.*, 2003, **959**, 226–234.
10 M. Olivo and B. C. Wilson, Mapping ALA-induced PPIX fluorescence in normal brain and brain tumour using confocal fluorescence microscopy, *Int. J. Oncol.*, 2004, **25**, 37–45.
11 M. J. Niedre, A. J. Secord, M. S. Patterson and B. C. Wilson, *In vitro* tests of the validity of singlet oxygen luminescence measurements as a dose metric in photodynamic therapy, *Cancer Res.*, 2003, **63**, 7986–7994.
12 A. Bogaards, A. Varma, S. P. Collens, A. Lin, A. Giles, V. X. D. Yang, J. M. Bilbao, L. D. Lilge, P. J. Muller and B. C. Wilson, Increased brain tumor resection using fluorescence guidance in a preclinical model, *Lasers Surg. Med.*, 2004, **35**, 181–190.
13 W. Stummer, H. Stepp, G. Moller, A. Ehrhardt, M. Leonhard and H. J. Reulen, Technical principles for protoporphyrin-IX-fluorescence guided microsurgical resection of malignant glioma tissue, *Acta Neurochir.*, 1998, **140**, 995–1000.
14 P. J. Muller and B. C. Wilson, Photodynamic therapy of malignant primary brain tumours: Clinical effects, post-operative ICP, and light penetration of the brain, *Photochem. Photobiol.*, 1987, **46**, 929–935.
15 L. Lilge, M. Portnoy and B. C. Wilson, Apoptosis induced *in vivo* by photodynamic therapy in normal brain and intracranial tumour tissue, *Br. J. Cancer*, 2000, **83**, 1110–1117.
16 B. C. Wilson, S. K. Bisland, A. Bogaards, A. Lin, E. H. Moriyama, K. Zhang and L. D. Lilge, Metronomic photodynamic therapy (mPDT): concepts and technical feasibility in brain tumor, *Proc. SPIE*, 2003, **4952**, 23–31.
17 S. K. Bisland, L. Lilge, A. Lin, R. Rusnov, A. Bogaards and B. C. Wilson, Metronomic Photodynamic Therapy as a New Paradigm for Photodynamic Therapy: Rationale and Preclinical Evaluation of Technical Feasibility for Treating Malignant Brain Tumors, *Photochem. Photobiol.*, 2004, **80**, 22–30.
18 A. Bogaards, A. Varma, E. H. Moriyama, A. Lin, A. Giles, S. K. Bisland, L. D. Lilge, J. M. Bilbao, P. J. Muller and B. C. Wilson, Fluorescence-guided resection of intracranial VX2 tumor in a preclinical model using 5-aminolevulinic acid (ALA): preliminary results, *Proc. SPIE*, 2003, **4952**, 91–96.
19 A. Bogaards, A. Varma, A. Lin, A. Giles, S. K. Bisland, L. Lilge, J. M. Bilbao, P. J. Muller, and B. C. Wilson, Preclinical feasibility of fluorescence-guided tumor resection followed by metronomic photodynamic therapy (mPDT) using 5-aminolevulinic acid induced protoporphyrin IX: Preliminary results, *SPIE Photonics North*, abstract, 2003.
20 L. D. Lilge, K. Menzies, S. K. Bisland, A. Lin and B. C. Wilson, PDT-induced apoptosis: investigations using two malignant brain tumor models, *Proc. SPIE*, 2002, **4612**, 136–142.
21 Z. Darzynkiewics, X. Li and J. Gong, Discrimination of cells dying by apoptosis, *Methods Cell Biol.*, 1994, **41**, 15–38.
22 K. E. Wallner, J. H. Galicich, G. Krol, E. Arbit, M. G. Malkin and S. F. Wallner, Patterns of failure following treatment for glioblastoma multiforme and anaplastic astrocytoma. Evidence that inhibition of erythropoiesis is important in the anemia of chronic renal failure, *Int. J. Rad. Oncol., Biol., Phys.*, 1989, **16**, 1405–1409.
23 W. R. Shapiro, L. S. Ashby and E. A. Obbens, Current therapy for brain tumors: back to the future brain tumors, *Arch. Neurol.*, 1999, **56**, 429–432.
24 E. H. Moriyama, S. K. Bisland, L. Lilge and B. C. Wilson, Bioluminescence imaging of the response of rat gliosarcoma to ALA–PpIX mediated photodynamic therapy, *Photochem. Photobiol.*, 2004, **80**, 242–249.