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Suppression of tumour growth from transplanted astrocytoma cells transfected with luciferase in mice by bioluminescence mediated, systemic, photodynamic therapy

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ABSTRACT

Background: Grade 4 astrocytomas are usually incurable due to their diffusely infiltrative nature. Photodynamic therapy (PDT) is a promising therapeutic option, but external light delivery is impractical when cancer cells infiltrate unknown areas of normal brain. Hence the search for endogenous sources to generate light at cancer cells. *In vitro,* astrocytoma cells, transfected with firefly luciferase, can be killed by bioluminescence-mediated PDT (bPDT). This study asks if bPDT can suppress tumour growth *In vivo*, when all components of treatment are administered systemically.

Methods: Transfected astrocytoma cells were injected subcutaneously or intra-cranially in athymic CD1 nu/nu mice. bPDT required ip bolus of mTHPC (photosensitiser) and delivery of the D-luciferin substrate over 7 days via an implanted osmotic pump. Control animals had no treatment, photosensitiser only or D-luciferin only. For subcutaneous tumours, size and BLI (light emitted after D-luciferin bolus) were measured before and every 2 days after PDT. For intracranial tumours, monitoring was weekly BLI.

Results: For subcutaneous tumours, there was significant suppression of the tumour growth rate ($P<0.05$), and absolute tumour size ($P<0.01$) after bPDT. Proliferation of subcutaneous and intracranial tumours (monitored by BrdU uptake) was significantly reduced in treated mice. ($P<0.001$)

Conclusions: This study reports bPDT suppression of tumour growth from luciferase transfected astrocytoma cells with all components of treatment given systemically, as required for effective management of recurrent astrocytomas in unknown sites. However, research on systemic bPDT is needed to establish whether effects on non-transfected tumours can be achieved without any *unacceptable* effects on normal tissues.

1. Introduction

Grade 4 astrocytomas are the commonest malignant brain tumours but are rarely curable due to the infiltrative nature of the disease. The use of intraoperative fluorescence to guide gross total resection following pre-operative administration of the photosensitising agent ALA (5 -aminolevulinic acid, 5-ALA) has been shown to significantly increase the progression free survival. However, this has not been associated with an improvement in overall survival, reflecting the natural history of these cancers to spread into areas where small volumes of tumour tissue cannot be detected on conventional imaging [1]. There is benefit from additional radiotherapy and chemotherapy with

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temozolamide, but very few, if any, of these cancers can be cured [2].

Photodynamic Therapy (PDT) is an alternative approach for cancer treatment that produces localised necrosis of tissue with low power red light after prior administration of a photosensitising agent and in the presence of oxygen [3]. It is attracting increasing interest in the management of gliomas [4]. Being a well localised and cold photochemical process with a relatively selective effect on tumour tissue, it is considered likely to be better for preserving surrounding normal tissues like the brain, with less potential for the long term neurocognitive effects that can be associated with radiotherapy and chemotherapy. It is repeatable and can be used on tissues that have already received the maximum tolerable dose of ionising radiation. Compared to chemotherapeutic agents, side effects are rare, and general photosensitivity is readily tolerable with appropriate precautions. It has long been recognised that there is some selectivity of effect between PDT effects on cancers and on the surrounding tissue in which the cancer has arisen. In most cases, the selectivity is relatively weak, but in early studies, the ratio of photosensitiser concentration in tumour to normal brain has been reported to be as high as 10:1 for the photosensitiser *m*THPC (temoporphin) [5], with selective necrosis described in an early mouse model of glioma [6]. In brain tissue, the breakdown of the BBB (blood-brain barrier) around a tumour enhances this selectivity [7].

PDT requires appropriate concentrations of photosensitiser, light and oxygen to be present everywhere in the target tissue. Photosensitisers for internal organs can be given systemically, but conventional PDT requires light to be delivered to all target areas from an external source. In early reports of PDT for high-grade gliomas [8], light was delivered directly into the tumour cavity intraoperatively following subtotal or gross total resection. The procedure was safe, but no definite survival advantage was shown. More recent studies have used image guided, interstitial light delivery [9]. Nevertheless, these techniques cannot work when the exact location of every focus of cancer is not known. The challenge lies in achieving targeted delivery of light to disseminated, deep seated foci of tumour cells.

1.1. Bioluminescence-mediated photodynamic therapy (bPDT)

There are several possible techniques for endogenous light generation for PDT. These include chemiluminescence and Cerenkov radiation but the one that is attracting the most attention currently is bioluminescence, the emission of light by a living organism [10]. The amount of light produced by bioluminescence is orders of magnitude less than that delivered by an external light source so the ability of bioluminescence to activate a photosensitiser cannot result solely from the radiative absorption of the photons from bioluminescence by the photosensitiser. The likely alternative mechanism is bioluminescence resonance energy transfer (BRET), which involves the non-radiative transfer of energy from a donor enzyme, the luciferase, to a suitable acceptor molecule, the photosensitiser, after administration of a substrate such as luciferin. The transfer of excited-state energy is inversely proportional to the sixth power of the distance between donor and acceptor dipoles, providing an effective range of less than 10 nm [11]. Thus for bPDT to have any prospect of success, the photosensitiser should have an absorption spectral profile that closely matches the emission profile of the bioluminescence together with a high extinction coefficient and a high quantum yield of singlet oxygen when activated. Further, the photosensitiser needs to be within about 10 nm of the luciferase and the bioluminescence substrate, D-luciferin.

In 2003, we reported that bioluminescence, generated by the addition of D-luciferin to luciferase-transfected NIH 3T3 cells, could activate the photosensitiser, Rose Bengal, to kill these cells [12]. Later work from other groups confirmed that bPDT (bioluminescence mediated PDT) can kill mammalian cells [13–15]. Recently, we reported that Grade 4 astrocytoma cells, sourced from a human glioma and transfected with firefly luciferase (U87-luc), could be killed by the addition of the substrate D-luciferin using the photosensitiser hypericin or mTHPC (Temoporfin) [16]. The present report takes our results forward to investigate the potential of bPDT *In vivo*.

2. Methods

All *In vivo* experiments were undertaken in accordance with the institutional guidelines of the Barts Institute of Cancer, Queen Mary University of London, where the experiments were undertaken, under appropriate Home Office licences.

The U87-luc transfected glioma cells from our previous study that had produced the strongest bioluminescence were transplanted subcutaneously or intracranially into 6–8 weeks old, 20–22 g athymic CD1 nu/nu mice (Charles River Laboratories, France) [17].

2.1. Tumour induction and bioluminescence generation

2.1.1. Subcutaneous tumour transplants

All cells were maintained using standard cell culture techniques and in complete media containing 1 mg/ml neomycin. Initially, under inhalational anaesthetic (isofluorane), a tumour cell suspension (5×10^6 cells in 100 μl) was injected subcutaneously into the dorsal flank of the mice [18]. To optimise xenograft survival and shorten the time to reach exponential growth, tumours were passaged several times and the one reaching exponential growth fastest removed for mincing, resuspension in PBS and passage into new animals. Tumour development was followed with bioluminescence imaging (BLI), undertaken at regular intervals under inhalational anaesthesia after transplantation in a Xenogen IVIS 100 cooled CCD camera system after a bolus ip (intraperitoneal) injection of 1.5 mg/g of D-luciferin (Caliper Life Sciences). The bioluminescence signal within a designated region of interest was expressed as average radiance and documented quantitatively. Imaging started 2–3 mins after injection of D-luciferin, for a total of 5 mins to ensure that peak photon emission was captured. These tumours became palpable by around 10 days after injection, so their growth could then be estimated by direct manual palpation and calliper measurement.

2.1.2. Intracranial tumour transplants

A similar procedure was followed for intracranial tumours. However, more preparation of the minced tumour was required. The U87-luc cells were further cultured in a mitogenic Dulbecco's modified Eagle's medium to form neurospheres (free floating aggregates) and harvested once deemed of sufficient size [19] Under ip ketamine and xylazine anaesthesia using a stereotactic frame and Hamilton needle, 10 μl (10^5 cells) of cell suspension in serum free media were injected into the brain parenchyma through a midline incision, 0.5 mm posterior, 2.5 mm lateral to the bregma to a depth of 3.5 mm [20] Despite the use of neurospheres, it was not possible to establish the early exponential growth rate that was achieved with the subcutaneous lesions. Measurement of the BLI was undertaken weekly using the same technique as for the subcutaneous tumours. CT imaging of the intracranial tumours was attempted but was not satisfactory. MRI was not available, so the only way of monitoring tumour progression *In vivo* was by BLI.

2.1.3. Optimising Bioluminescence for bPDT

PDT with an external light source is usually undertaken with light delivery over a relatively short time, typically up to an hour or so, and often divided into several fractions. With bPDT, the rate of light generation is very low and will depend on the amount of D-luciferin present at any given time, with a risk of luciferase saturation and metabolism/excretion of D-luciferin if given as a bolus.

In initial studies on 4 mice with intracranial tumours that had been growing for 6 weeks, BLI was monitored following a bolus ip injection of D-luciferin, at 1, 2, 3, 4, and 5 mins, then every 5 mins up to 60 mins. This showed that the light generation lasted less than an hour. Fig. 1a. It was considered unlikely that a single bolus of D-luciferin would be sufficient to give a significant bPDT effect and it would be difficult to

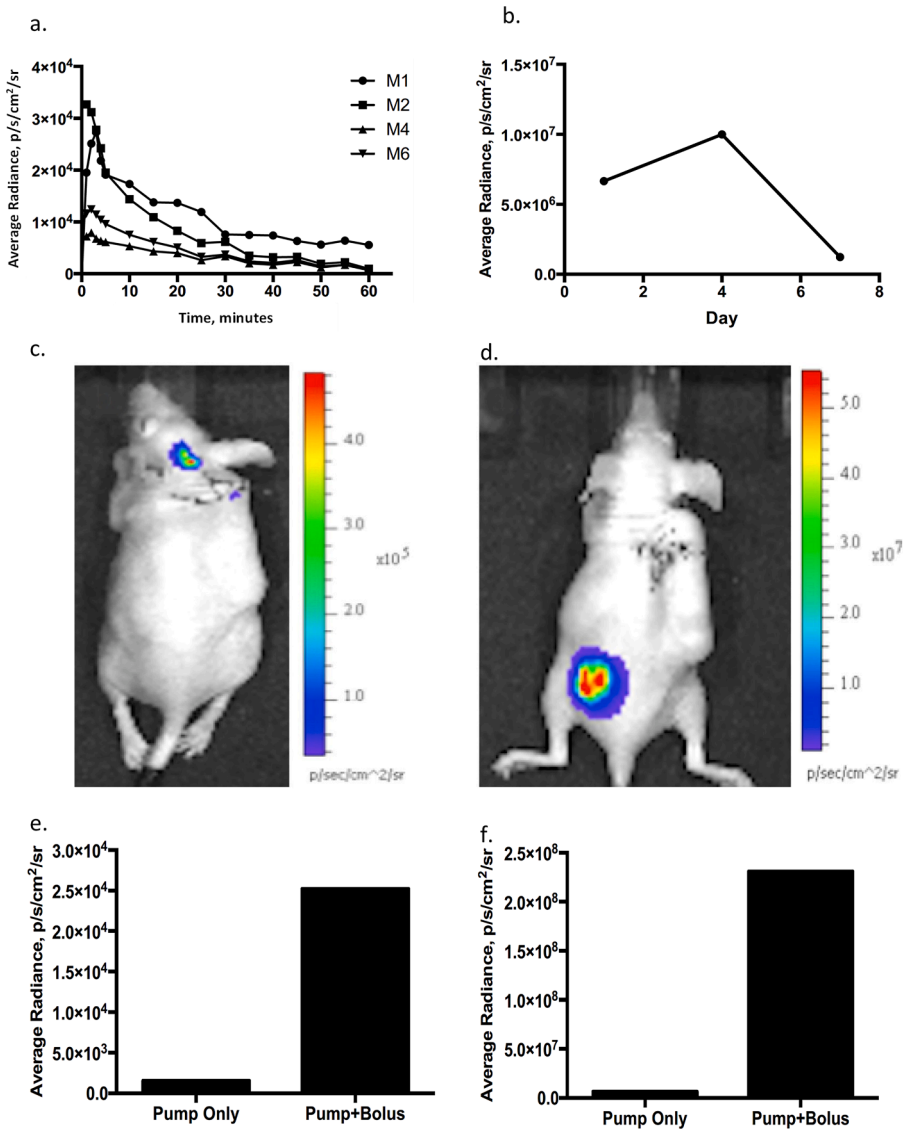

Fig. 1. Monitoring light generation: a). Peak intensity and duration of light generation in response to an intraperitoneal bolus injection of D-luciferin in 4 mice with intracranial tumours monitored over a period of 1 hour. b). Change in average light generation from D-luciferin administered by an Osmotic Pump over a period of one week in a subcutaneous tumour. c). Light generation in an intracranial tumour. d). Light generation in a subcutaneous tumour. e). Direct comparison of the average light generation over one week from an osmotic pump with the peak light intensity immediately after an additional intraperitoneal bolus of luciferin in an intracranial tumour. The dose of D-luciferin delivered by a single bolus injection was around 30 mg and the total dose given by the osmotic pump was around 10 mg over a week. f). The same comparison for a subcutaneous tumour. The light detected from intracranial tumours was considerably less than that from subcutaneous tumours.

administer multiple doses to mice. A method of generating a more sustained source of bioluminescence was thought to be more appropriate [21,22] The solution adopted was the use of a subcutaneous osmotic pump (model 2001, ALZET, Durect Corporation, Cupertino, USA). The largest that could be tolerated by these mice was 3.0 × 0.7 cm, weight 1.1 g. Under sterile conditions, the osmotic pump was filled to its maximum volume of 200 μl with 50 mg/ml of D-luciferin and under inhalational anaesthesia, an incision was made in the dorsal cervical skin, a subcutaneous pocket created by blunt dissection, the pump implanted, and the skin closed with a vertical proline mattress suture. These pumps delivered a total drug dose of around 10 mg at 1.0 μl/hr for 1 week, (about 0.05 mg D-luciferin /hr). The dose given as a bolus for each BLI monitoring measurement was around 30 mg. The intensities and duration of light generation were compared between a bolus injection, when light generation was complete within an hour, and using an osmotic pump, when the light was emitted over a week. Fig. 1. The decision was made to use the osmotic pump for all *In vivo* bPDT studies.

2.1.4. *Choice of photosensitiser*

The photosensitisers studied *In vitro* were hypericin and mTHPC. mTHPC was chosen for *In vivo* studies using the osmotic pump for slow delivery of D-luciferin for 4 reasons:

1. There are many more promising clinical reports of PDT for gliomas and other tumours using mTHPC than there are for hypericin.
2. In rodents, mTHPC reaches peak tissue levels in 36–48 hr after administration and only falls to about half after a week, with persistent levels in the body and in targeted tissues for well over a week [5],
3. The absorption spectrum for mTHPC, although not ideal, was considered to be a better match than that for hypericin to the emission spectrum for firefly luciferase. The spectra for mTHPC are shown in Fig. 2 [16]
4. Light for PDT delivered over an extended period produces a greater effect than the same dose delivered over a shorter period [22].

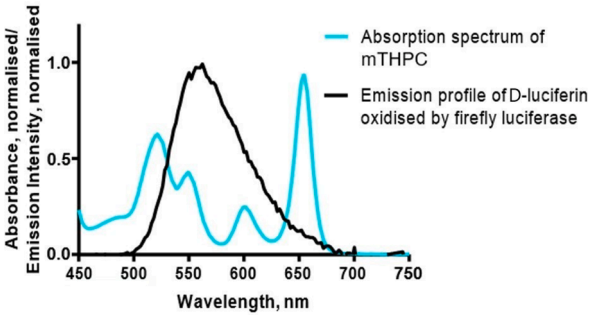

Fig. 2. Absorption Spectrum of mTHPC and Emission Spectrum of D-Luciferin Oxidised by Firefly Luciferase.

3. Statistics

Statistical analysis was conducted using GraphPad Prism software. For survival analysis (trial 1), Kaplan-Meier curves were generated for each of the experimental groups and log-rank analysis using a Bonferroni correction threshold for multiple comparisons was used to determine if there was a significant difference in survival between the groups.

For tumour volume and growth rate, bioluminescent signal and proportion of BrdU positive cells, results were analysed using each measurement on individual animals. A 1-way or 2-way ANOVA analysis was used for comparison between multiple groups. A 2-tailed Student t-test was used when only 2 groups were compared, particularly when all treated animals as a group were compared with a combined group including all controls (Trial 3, BrdU uptake). Statistical significance is denoted by asterisks (*, $P<0.05$, **$P<0.01$, ***, $P<0.001$. The mean and SEM are presented in the figures where appropriate.

4. Bioluminescence-Mediated photodynamic therapy (bPDT)

The first quantitative studies were undertaken on subcutaneous tumours as it was easier to quantify the results compared with intracranial lesions.

4.1. Trial 1. bPDT suppression of growth in subcutaneous tumours

4.1.1. Tumour implantation and treatment

U87-*luc* cell lines were passaged 4 times then implanted into the left flank in 15 mice (100 μl cell suspension). BLI imaging was undertaken on day 4 following a diagnostic bolus of D-luciferin and the mice divided into 4 groups: 3 control groups: C (3 mice): no treatment, D (3 mice): photosensitiser only (mTHPC bolus 0.3 mg/kg), L (4 mice): D-luciferin only (30 mg in 200 μl), and one treatment group DL (5 mice): photosensitiser + D-luciferin. Groups were chosen so the average peak intensity of light emission for mice in each group on day 4 was the same. On day 5, an osmotic pump was inserted in each animal and remained in place for 7 days. mTHPC (groups D and DL) was given on day 5, the day after the bolus of D-luciferin (by which time the level of D-luciferin from the bolus would have fallen to close to zero, (Fig. 1a)). Thus the only prolonged source of D-luciferin available for a bPDT effect was that from the osmotic pump. On day 13, all mice underwent BLI and calliper measurement of tumour size. These measurements were subsequently repeated every 2 days. Mice were culled if they appeared unwell, showed signs of distress or the tumour size exceeded 15 mm. The tumours were undetectable manually at 5 days. All mice had a regular bolus of D-luciferin for monitoring, but only group DL had therapeutic levels of light and photosensitiser present at the same time.

4.1.2. Tumour growth results

One control group C mouse (C3) was culled on day 13 due to tumour ulceration together with a matching treated mouse (DL1) for histological comparison. The other 13 mice reached the criteria for culling on day 17, apart from 2 longer survivors (24 and 26 days, both in the treated group). All 9 controls and 2 of 4 treated tumours grew between days 13 and 17. 2 of 4 treated tumours did not. Fig. 3a. These reductions in growth rate in treated animals compared with controls were statistically significant ($P<0.05$) for both group C alone and groups C + D + *L* together compared with DL (ANOVA). Analysis of covariance (ANCOVA, using SAS v9.3) showed a significant difference in absolute tumour size between the control and active treatment groups at day 17 ($P<0.01$).

The BLI in all tumours increased between days 13 and 15, but for most, it was stable or fell between days 15 and 17, despite the continuing growth in tumour size. Fig. 3b. Nevertheless, the average increase in BLI in the control groups between days 13 and 17 was almost double that in treated mice (not significant).

Excluding the treated mouse culled on day 13 (DL1) to match the histology on C3, the average survival time for untreated animals was 16.6 days compared with 21 days for treated mice. A Kaplan-Meier curve showed there was no significant difference in survival time between any of the control groups and the treated group. Throughout this study, the only symptoms exhibited by any of the mice were those that could be directly attributed to tumour growth. No systemic effects were seen.

4.2. Trial 2 early response to bPDT in subcutaneous tumours

4.2.1. Tumour implantation and treatment

This trial was undertaken in a similar way to trial 1. 12 mice were injected subcutaneously in the flank using 100 μl of a suspension of cells prepared from a tumour in a known exponential growth phase (multiple passages of tumour from trial 1). 2 days after tumour implantation, BLI was measured, and the mice randomised into 4 groups of 3 animals as in trial one. 3 days after tumour implantation, an osmotic pump was implanted in all animals. After 7 days of treatment (active or sham), 10 days after tumour implantation, the BLI measurements were repeated, and calliper measurements made to estimate the tumour volume. The animals were culled the same day.

4.2.2. Tumour growth results

Before and after culling, it was difficult to measure the tumour size as these lesions were poorly formed without distinct margins and some could only be harvested in several pieces. No tumour was detected in one control mouse. In the other 11, the BLI increased during treatment by a factor of between 4 and 83 (average 26) for controls and between 9 and 21 (average 15) for treated animals (not significant). Data for individual mice are not shown.

4.3. Histology and immunohistochemistry of subcutaneous tumours

4.3.1. Histology

The mice were culled with CO2 and then cervical fracture, following institutional guidelines. The tumours were dissected, fixed in formalin, and sectioned. Stained with haematoxylin and eosin (H&E), these showed that tumours from treated and control groups were composed of several adjacent but relatively discreet nodules, often with a higher concentration of cells around the edges of nodules than in the centre.

In trial 1 (mice culled 13–27 days after tumour injection), all sections (9 control and 5 treated tumours) were composed essentially of solid tumour without evidence of necrosis with no difference in the findings between control and treated tumours. Fig. 4a,b. However, there was one notable and focal exception in a treated mouse. Fig. 4c,d. In this small, circular nodule, the central area was composed of the shells of dead cells, surrounded by a thin rim of proliferating tumour cells. BrdU showed almost no uptake in this area Fig. 4e. This animal was one of the longest survivors, being culled 24 days after transplantation and 11 days after completion of one week of PDT. It was one of the only two mice, both treated, in which there was no increase in tumour size between

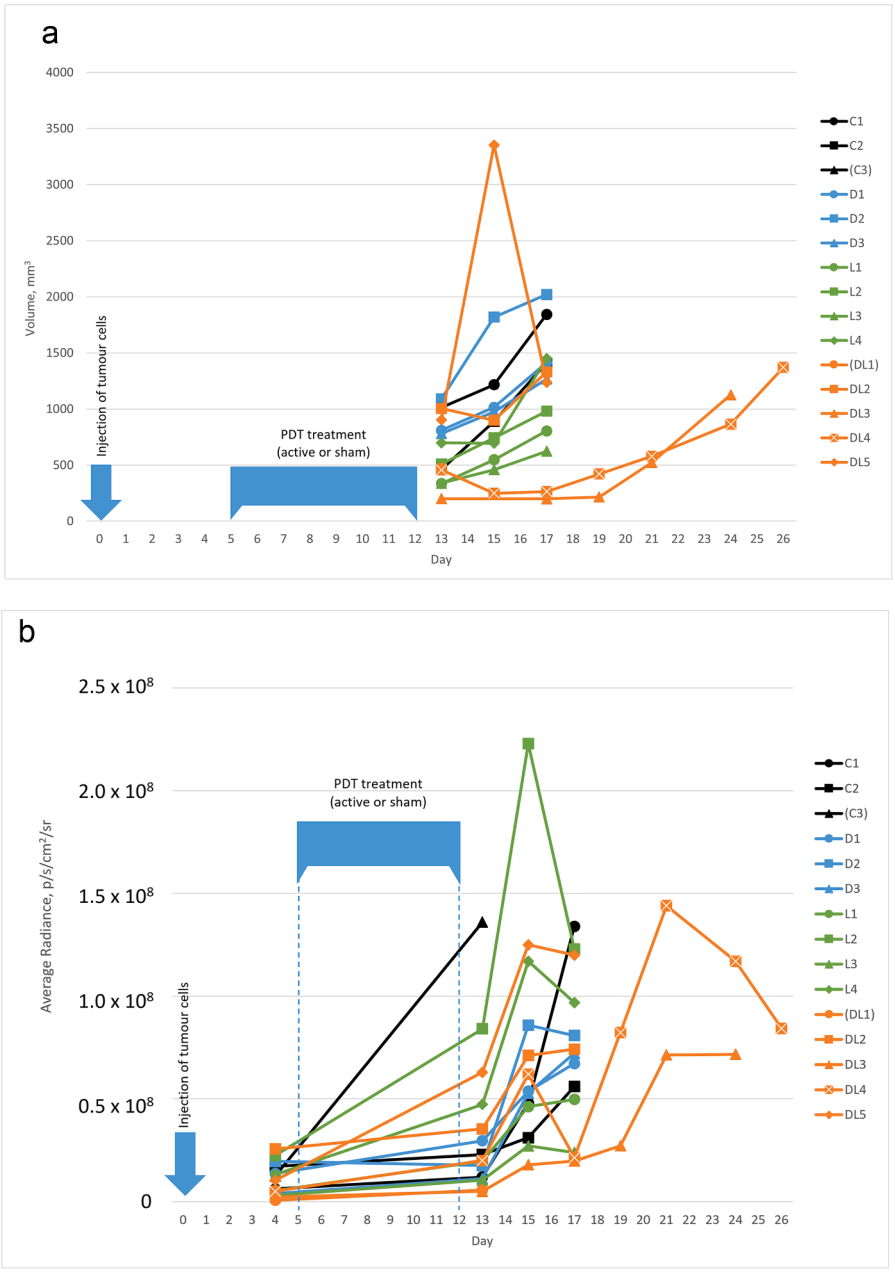

Fig. 3. Trial 1. bPDT for subcutaneous tumours. Results are shown for individual mice. a) Tumour volume, measured by calliper. Prior to treatment (PDT or sham), the tumours were too small to be measured by calliper. Treatment groups: Black: Group C (no treatment), Blue: Group D (photosensitiser only), Green: Group L (light only): Orange: Group DL (photosensitiser plus light). Groups C, D and L are controls, Group DL is active treatment. 2 mice were culled on day 13: C3 (vol 1013mm^3, culled due to animal distress) and DL1 (vol 472mm^3, culled for histology to match the time of culling for C3). For clarity, these are not included on graph 2a as there is only one point for each mouse. For correlation between Figs. 2a and 2b, their numbers are put in brackets. b) BLI (light emitted) following an ip (intraperitoneal bolus) of D-luciferin. All mice had regular diagnostic boluses of ip D-luciferin. mTHPC (groups D and DL) was given the day after the diagnostic bolus of D-luciferin (by which time the level of D-luciferin from the bolus would have fallen to close to zero, (Fig. 1a)). This was the day the osmotic pump was inserted, so the only prolonged source of D-luciferin available for a bPDT effect was that from the osmotic pump. For the 2 mice culled on day 13 (C3 and DL1), data is only available for days 4 and 13. The black line for C3 is clear. The orange line for DL1 almost exactly overlays the orange line for DL4 and is difficult to see.

days 13 and 17.(Fig. 3a).

Trial 2 (9 control, 3 treated tumours) used the faster growing tumour. In one control mouse, no tumour was established. Spontaneous necrosis of variable extent was seen in at least one nodule in 5 of 8 control and 2 of 3 treated animals, both macroscopically and on H&E staining, so this could not be a bPDT effect. In one treated mouse, an area of dead cells similar to that seen in trial one, within an area of spontaneous tumour necrosis was seen. This tumour had the lowest BLI at the end of PDT (28, compared with an average of 92, range 43–203 for the other 10 tumours in the trial) and the smallest estimated tumour volume (93mm^3 compared to an average 250mm^3, range 120–439mm^3 for the other 10). No lesions comparable to these 2 small areas were seen in any of the control or other treated animals in either trial. This raises the possibility of a connection with the bPDT, but these observations could only be explored further in new experiments.

4.3.2. *Immunohistochemistry*

2h before culling, all mice received an ip injection of 5-bromo-2′-deoxyuridine (BrdU) for subsequent identification of cells that were actively replicating their DNA [23]. Sections from mice in both trials

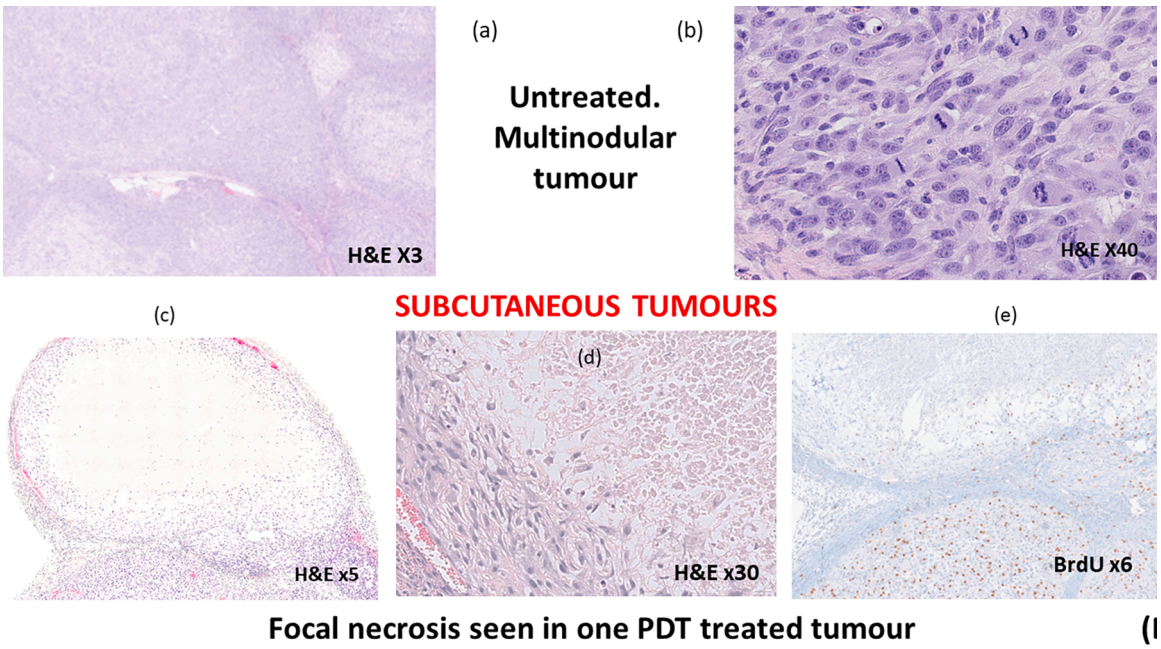

Fig. 4. Trial 1. bPDT for Subcutaneous tumours. Histology and immunohistochemistry. a), b) Typical appearance of an untreated tumour (C1): a) low power, b) high power. All these tumours were composed of several adjacent but relatively discreet nodules, often with an apparent higher concentration of cells around the edges of nodules than in the centre. No necrosis could be detected in treated or untreated tumours, with one exception: c), d), e) one small nodule in a treated mouse (DL3) showed an area of confluent necrosis adjacent to a viable nodule. c) at low power, d) at high power, the central area could be seen to be composed of the shells of dead cells, surrounded by a thin rim of proliferating viable tumour cells. e) BrdU staining (brown) showing almost no uptake in the area of necrosed cells compared with high uptake in the adjacent nodule of viable tumour.

were stained with antibody to BrdU. BrdU positive and negative tumour cells were counted in 4 randomly picked locations at x 40 magnification using Image-Pro-Plus 50 software in each mouse. Areas of spontaneous necrosis in trial 2 slides were avoided. The number of BrdU positive cells was expressed as the percentage of the total tumour cell count in the same area and averaged for each group. Uptake of antibody in small intestine crypts was confirmed as a positive control in all animals. For both trials 1 and 2, there was a significant reduction in the percentage of BrdU positive cells in the treated tumours. Fig. 5a,b.

4.4. Trial 3 suppression of growth in intracranial tumours

The first group of mice transplanted intracranially with neurospheres was used to assess the rate of production of light after administration of a bolus of D -luciferin as described earlier (Fig. 1a).

4.4.1. Tumour implantation and treatment

18 mice were implanted by injecting 10^5 cells in 10 μl of PBS of U87-l*uc* cells that had been grown to form neurospheres, through a midline incision into the brain parenchyma as described earlier. The mice were randomly designated into 4 groups, as for trials 1 and 2: (C, $n = 4$), (D, $n = 4$), (L, $n = 4$), and (DL, $n = 6$). The day after tumour implantation, osmotic pumps were inserted for 1 week, and treatment (active or sham) delivered as appropriate for each group. BLI was measured on day 8 and weekly thereafter for 8 weeks, at which time the mice were culled.

4.4.2. Tumour growth results

One control animal was excluded from analysis as the BLI just faded away over time implying no establishment of a tumour. In the other 17, a variable BLI signal was seen, mostly increasing, throughout the scheduled 8 week follow up. As the CT scans were unsuccessful, MRI was not available, and direct assessment of tumour size was not feasible; even at post mortem, tumour progression could only be monitored by

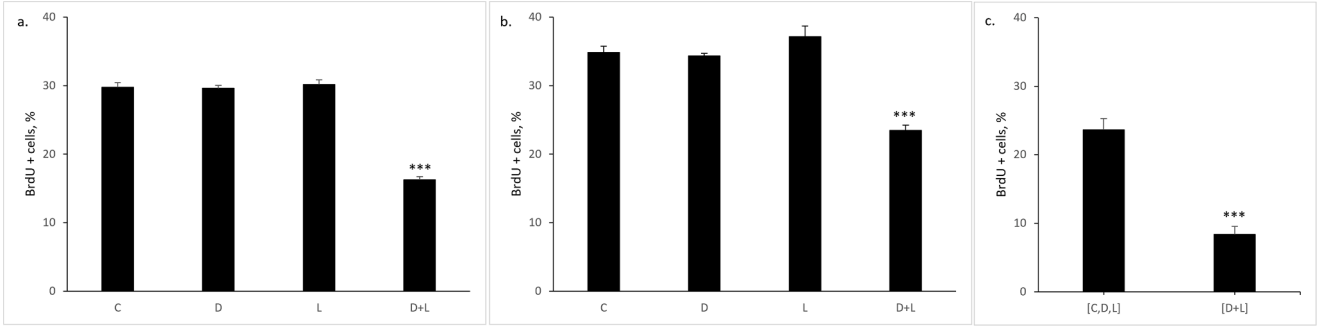

Fig. 5. Tumour cell proliferation monitored by uptake of BrdU for each trial. The results are expressed as the percentage of tumour cells taking up BrdU after PDT and compared with the uptake in cells in untreated tumours. For trials 1 and 2, the groups are the same as in Fig. 2. C; control, D: Drug only, L: light only, $D + L$: active treatment. In trial 3, the results for all control groups have been combined as data was only available from 5 mice. There were no significant differences between control groups in any trial. Compared with the controls, the value for $D + L$ (active treatment) is significantly reduced for all 3 trials *** $P<0.001$.

measurements of the BLI signal. At post mortem, the brains were harvested, fixed and sectioned for H&E staining. Tumours could only be identified histologically in 6 mice (4 controls, 2 treated), all of which were growing at least partially extra-parenchymally, and these mice had considerably higher BLI levels than those not detected on histology. The latter were presumed to be small lesions not detected in the tissue levels examined or located elsewhere in the main body of the brain. In view of these differences, the BLI results for individual animals are shown separately for those with and without histology in Figs. 6a,b.

4.4.3. *Histology of intracranial tumours*

On H&E staining the tumours were seen to be hypercellular and to consist of a monomorphic population of cells on a fibrillary background. Mitoses, as well as areas of vascular endothelial proliferation were evident, characteristic of a grade 4 astrocytoma. Fig. 7a,b. The mouse with the first treated intracranial tumour (pilot study, outside the main group) was culled immediately after completion of one week of PDT. No necrosis could be seen. Fig. 7b. As with the subcutaneous tumours in trial 1, no necrosis was seen in any of the mice with intracranial tumours, either treated or untreated.

4.4.4. *Immunohistochemistry of intracranial tumours*

Mice were injected with BrdU 2h prior to culling. Sections were stained with anti-BrdU antibody. Quantification of tumour cell proliferation was assessed as in trials 1 and 2, in the 5 of the 6 tumours detected histologically that took up the BrdU stain. An average of 4 areas were chosen randomly in each tumour for counting the percentage of tumour cells taking up the stain (a total of 13 sites in the 3 control mice and 7 in the 2 treated mice). As the average percentage of stained cells was similar in all 3 tumours in control animals (21 %, 25 %, 26 %, mean 23.6 %), these were combined into one group which was compared with the data from the treated tumours (*t*-test). There was significantly less uptake in treated tumours (8.4+/-3.0 %) compared with controls (24+/-6.0 %) $P<0.001$. Fig. 5c. BrdU data was not available for the mice with intracranial tumours not detected histologically, but 4 of 7 control tumours in this group grew significantly through the duration of the study, compared with only one treated tumour and that was only in the last week Fig. 6b. These results are comparable to the results in Fig. 6a. Only 3 of the intracranial tumours, all controls, showed any evidence of exponential growth.

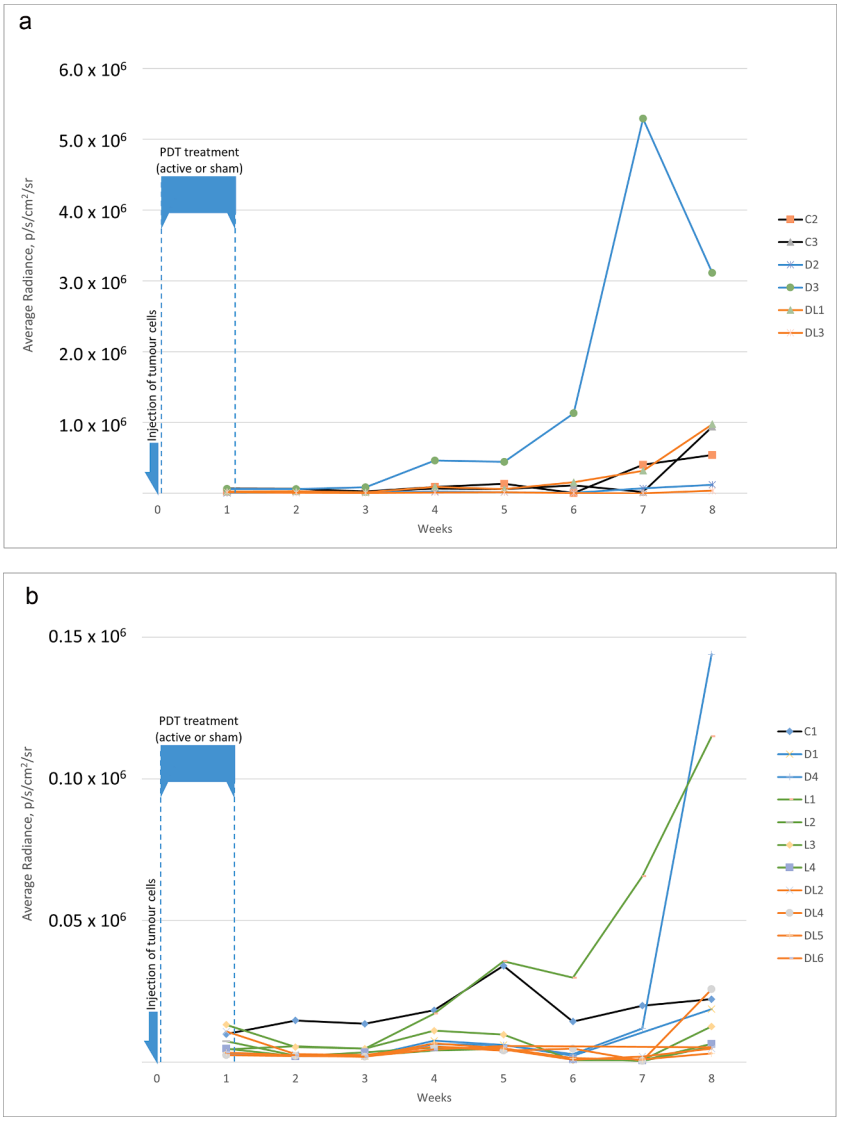

Fig. 6. Trial 3. bPDT for intracranial tumours. BLI results for individual mice. The tumour dimensions could not be measured in these mice. The figures shown are the peak light intensity (BLI) after a bolus of D-luciferin (1.5 mg/g) at each time point. a) Mice with histologically detectable tumour (6 mice, 2 treated, scale up to 6,000,000). b) Mice in which tumours could not be identified histologically (11 mice, 4 treated, scale up to 160,000). Treatment groups as in Fig. 3. Control groups: C (black, no treatment) D (blue, mTHPC only), L (Green, D-luciferin only); Active treatment: DL (orange, mTHPC + D-luciferin).

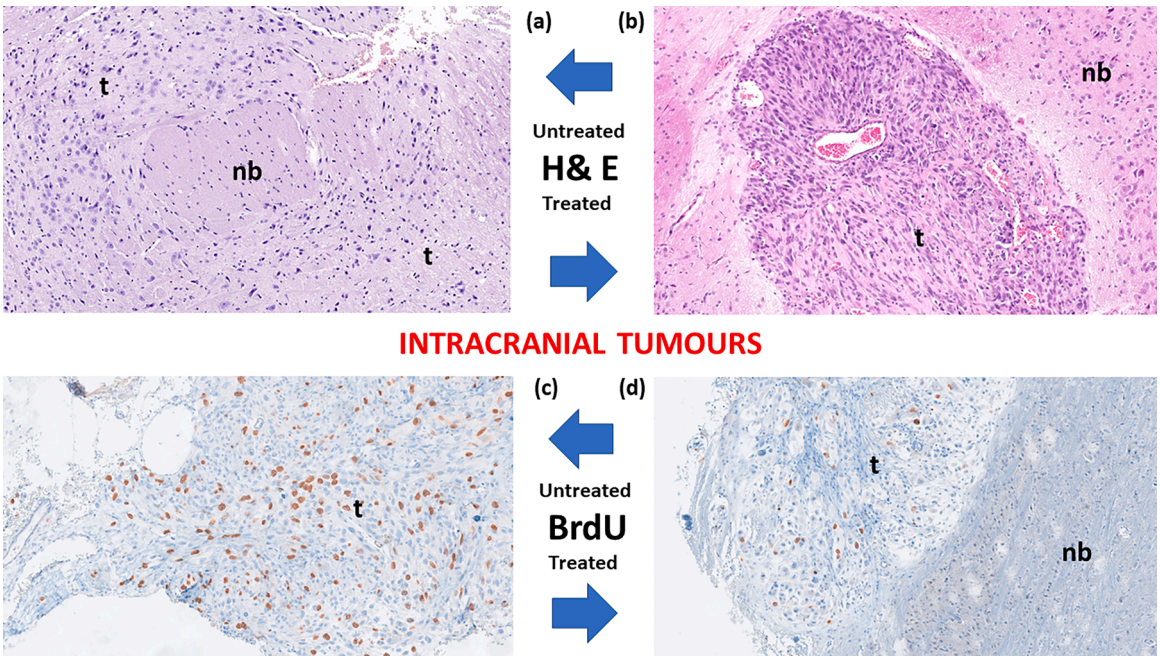

Fig. 7. Intracranial tumours. Histology and immunohistochemistry. t: tumour, nb:normal brain. a), Untreated tumour surrounding an area of normal brain. b), Pilot study: intraparenchymal tumour culled immediately after 1 week of PDT (H&E) with no evidence of necrosis. c), d) BrdU stains of tumours detected on histology,. c) untreated (23.4+/-6 % of cells stained), d) treated (8.4+/- 3 % of cells stained). There was no uptake of BrdU in normal areas of brain.

5. Discussion

There is increasing interest in Photodynamic Therapy in the treatment of a wide range of cancers, both as primary management, mostly for relatively small cancers and pre-cancers, and as an adjuvant to other treatments [3]. If surgery is unable to remove a cancer in its entirety, PDT may be able to safely ablate areas unsuitable for resection and so turn a palliative into a potentially curative procedure. Likewise, if small areas of tumour remain or recur after maximum doses of radiotherapy, PDT may be able to restore disease control. Grade 4 astrocytomas are a prime example as there are no effective treatments for cancers that recur after maximum surgery, radiotherapy and chemotherapy. The problem is particularly difficult as the precise location of the persistent cancer cells is not known until they present as recurrent disease. Bioluminescence has long been used experimentally for imaging specific cells. Now, bioluminescence mediated PDT (bPDT) is being explored for treatment.

Trial 1 in this study has provided evidence that bPDT can slow the growth of induced subcutaneous tumours. The effect is limited both in scale and duration, and the number of animals studied was relatively small, but the benefits did reach significance using the osmotic pump to give low level endogenous light delivery over a prolonged period. The significant reduction in the percentage of tumour cells taking up BrdU in treated animals in all 3 trials compared with controls is strong evidence that tumour cell proliferation has been slowed, even though necrosis was not seen on histology. These results are consistent with those of several other groups who have reported successful bPDT *In vivo*, using different protocols. Hsu et al. [14] transplanted A549 (lung cancer) cells to create subcutaneous tumours in mice. They used QD-Rluc8 conjugates, injected directly into the tumours and CTZ (the substrate coelenterazine) given intravenously with mTHPC loaded micelles as the photosensitiser. Paraffin fixed tumour sections showed a significant reduction in PCNA (proliferating cell nuclear antigen) and CD31 (PECAM-1) expression. After a single treatment, the results were similar to those in the present study with initial suppression of tumour growth and later recurrent growth. Yu et al. [24] transfected Hela cells with firefly luciferase and demonstrated cell kill using hypericin as the photosensitiser as well as developing an effective drug delivery system. In similar studies, Kim YR et al. [13] grew subcutaneous tumours from CT26 (colorectal cancer) cells and treated them with systemic Ce6 (chlorin e6) followed 4h later by direct intra-tumour injection of Luc-QD (a conjugate of Rluc8 (Renilla immobilised luciferase) and a self-illuminating quantum dot), then CTZ. They extended their project to study the effects of multiple treatments and showed that 9 treatment sessions resulted in near complete inhibition of tumour growth. Thus in future studies, the effects may be enhanced by prolonging or repeating treatment.

The main difference between these earlier reports and the present study is that all the agents used in the present study were administered systemically whereas for the other teams, at least one of the agents had to be delivered directly to the site of the tumours to be treated. There is no natural luciferase in mammalian cells, so systemic administration of D-luciferin as in our study could only lead to light generation in the cancer cells.

In the clinical situation, getting the luciferase to the malignant cells *In vivo* is a fundamental problem that is yet to be adequately addressed. bPDT as used in this study will have no value unless it can be applied to astrocytoma cells that have not been transfected. The challenge is to get all the necessary components for bPDT (luciferase, D-luciferin and photosensitiser, mTHPC) in the right place at the right concentration at the same time, without knowing exactly where the right place is and without the full combination causing any *unacceptable* effects in any other parts of the body.

It has been recognised for many years that although PDT can affect many normal tissues, in most cases, under appropriate conditions, these lesions can heal safely without any short or long term *unacceptable* effects on structure or function [3,25]. Further, side effects of bPDT are likely to be milder and shorter lived than those associated with chemotherapy or radiotherapy [3]. The first challenge in the current situation is to understand how a whole animal responds when all components of PDT are given systemically. This will require new, detailed experiments on normal animals, most conveniently on small rodents such as rats. It has been reported that the photosensitiser Rose Bengal can be conjugated to luciferase for singlet oxygen generation by BRET [15]. Thus it should be possible to create a conjugate of luciferase with

mTHPC. The animals should be sensitised with this conjugate and subsequently activated by administration of D-luciferin. The effects should be closely monitored *In vivo* and at post mortem for evidence of PDT effects in any organ. Detailed pharmacokinetic and dosimetry measurements will be required to establish just how much systemic bPDT therapy normal tissues can tolerate and under what conditions. The liver in particular is known to take up high levels of photosensitiser. Focal PDT necrosis has been studied in the liver, and is known to heal safely [26]. However, it is not known what the response will be when the whole liver is exposed to treatment, as would be the case with systemic bPDT.

When these results are available, the next step will be to look for ways of getting high enough levels of photosensitiser, luciferase and D-luciferin to transplanted, non-transfected astrocytoma cells to kill them without exceeding what the whole animal can tolerate. Most of the selectivity of conventional PDT depends more on focal delivery of light from an external source rather than the preferential uptake of the photosensitiser. mTHPC is known to be taken up with some degree of selectivity in astrocytomas, [4,5] thought to be related to the breakdown of the blood brain barrier [7]. The most obvious approach would be to link the photosensitiser/luciferase conjugate to an antibody specific for astrocytoma cells. Monoclonal antibodies such as cetuximab dendrimer conjugates have been used to selectively deliver chemotherapeutic agents such as methotrexate to treat grade 4 astrocytomas [27]. A monoclonal antibody has been raised against the protein resulting from the most common *IDH1* mutation [28]. An anti-carcinoembryonic antigen (CEA) antibody fragment, anti-CEA diabody, has been fused to luciferase from Renilla reniformis to generate a novel optical imaging probe that when injected intravenously successfully localizes to subcutaneously transplanted CEA expressing cells [29]. These antibodies could, of course, potentially also be used for simpler approaches than bPDT such as conjugation to other therapeutic agents, but none of these approaches is likely to be 100 % selective.

The limiting factor will probably be the uptake of photosensitiser in the liver. There are several potential ways of alleviating this. Pharmacodynamic studies have shown that the ratio of mTHPC concentration between the liver and a transplanted tumour changes dramatically with the time after administration and at long times (a few days), there is more drug in the tumour than in the liver [30]. Thus it may help to have a long drug-light interval. Slow and/or intermittent light delivery (metronomic PDT) has been shown to be more effective than short duration, bolus doses [21]. Effects from intermittent, small doses in a very metabolically active organ like the liver may resolve between doses but could be cumulative in less metabolically active areas like clusters of astrocytoma cells.

This would be a major laboratory programme with no guarantee of success, although the first part, understanding the effects of systemically delivered bPDT on normal animals, would be relatively straightforward.

Several other technologies could enhance bPDT. It is known that the products of tumours treated with low dose PDT can stimulate an immunological response more strongly than the original untreated tumour, like a tumour vaccine. This could complement bPDT [31]. Shramova et al. [32] have proposed the concept of genetically encoded BRET-activated PDT, which combines an internal light source and a photosensitizer in a single-genetic construct, which can be delivered to tumours seated virtually anywhere and then triggered by the injection of a substrate to initiate treatment. Another possible mechanism has been reported, in which advanced human prostate cancer lesions were visualised in living mice by a targeted gene transfer vector and optical imaging [33].

In conclusion, this study is the first to show that bioluminescence mediated PDT (bPDT) can suppress the growth of subcutaneous and intracranial tumours in mice, without the need to know exactly where the tumour cells are located. However, this was only feasible because the astrocytoma cells were transfected with the firefly luciferase gene. Possible ways of developing this approach for treating astrocytoma cells that have not been transfected are described in this paper. If successful, bPDT has potential as an adjuvant treatment for recurrent tumour after conventional management of grade 4 astrocytomas or as a prophylactic to reduce the risk of recurrent disease from residual astrocytoma cells that have infiltrated beyond the primary tumour area. This programme, if successful, has the potential to open a range of bPDT treatments for small volumes of malignant tissue in unknown sites.

CRediT authorship contribution statement

Jane Ng: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Nico Henriquez:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Neil Kitchen:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Norman Williams:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Marco Novelli:** Writing – review & editing, Validation, Formal analysis, Data curation. **Dahmane Oukrif:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Alexander MacRobert:** Writing – review & editing, Formal analysis. **Stephen Bown:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interests

None.

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