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# Bioluminescence-activated photodynamic therapy for luciferase transfected, grade 4 astrocytoma cells in vitro

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ABSTRACT

*Background:* . Grade 4 astrocytoma is incurable due to the diffusely infiltrative nature of the disease. Photodynamic therapy (PDT) is a promising therapeutic option, but external light delivery is not feasible when cancer cells infiltrate unknown areas of normal brain. Hence the search for endogenous sources such as bioluminescence that can generate light at cancer cells. This requires a substrate (a luciferin) and an enabling enzyme (a luciferase), neither seen in mammalian cells.
*Methods:* . Preliminary studies confirmed that U87 cells (derived from a human grade 4 astrocytoma) could be killed by conventional PDT using the photosensitizers hypericin or mTHPC. U87 cells were then transfected with firefly and other luciferases and light generating cell lines (U87-luc, U87-hRluc, U87-CBG68luc) identified using the appropriate substrate. Reagent doses and conditions were optimized and U87-luc cells incubated with hypericin or mTHPC with d-luciferin added to initiate bioluminescence activated PDT (bPDT). Cell survival was assessed by MTT assay, haemocytometry and growth assay. Control groups included U87-luc cells with no added active reagents, substrate only, photosensitizer only and non-transfected U87 cells. Results were expressed as a percentage of surviving cells compared with untreated U87-luc controls.
*Results:* . There was no bPDT effect on non-transfected cells. The mean survival of treated transfected cells was 36%, ($P<0.001$) using hypericin and 35% ($P<0.001$) using mTHPC, compared with untreated U87-luc cells. bPDT effects were suppressed by the anti-oxidant, lycopene.
*Conclusions:* . bPDT can kill Grade 4 astrocytoma cells transfected with luciferase in vitro. This justifies progression to in vivo studies.

## 1. Introduction

For many years, to the current day, the standard of care for grade 4 astrocytoma, the commonest primary brain tumor, is surgical gross total resection, radiation, and temozolamide chemotherapy. [1] The use of fluorescence (5-aminolaevulinic acid, 5-ALA) to guide gross total resection, fluorescence-guided resection, has been shown to significantly improve progression free survival but not overall survival, reflecting the diffusely infiltrative nature of the disease which is not discernible on imaging. [2]

Photodynamic Therapy (PDT) produces localized necrosis of tissue with low power light after prior administration of a photosensitizing agent and in the presence of oxygen. [3] Being a well localized and cold photochemical process with a relatively selective effect on tumor tissue, so preserving surrounding normal tissues like the brain, it leaves less potential for the long term neurocognitive effects that can be associated with radiotherapy. It is repeatable and can be used on tissues that have already received the maximum tolerable dose of ionizing radiation.

*Abbreviations:* mTHPC, meta-tetrahydroxyphenyl chlorin,.

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Compared to chemotherapeutic agents, side effects are rare and photosensitivity is readily tolerable with appropriate precautions. Most effects on normal tissue heal well. Photosensitizers are taken up and retained with some degree of selectivity by neoplastic tissue in many organs. In brain tissue, where the absence of the BBB (Blood Brain Barrier) around the tumor enhances this selectivity, selective necrosis has been described in a mouse model of glioma [4]. The ratio of the concentration in tumor to normal brain has been reported to be as high as 10:1 for the photosensitizer *m*THPC (meta-tetrahydroxyphenyl chlorin, temoporphin) [5]. In animal models, PDT has been shown to initially cause break down of the blood brain barrier, swelling of astrocytes and neurones, and after 24 h, some coagulation necrosis in the brain surrounding a tumor [6]. However, this is of very limited extent, depending on the photosensitizer and its concentration, the time interval between sensitization and light exposure, and the light distribution and intensity.

In early reports of PDT for high-grade gliomas, [7] light was delivered directly into the tumor cavity intraoperatively following subtotal or gross total resection. No definite survival advantage was shown, but PDT was found to be safe and worthy of further study. One small study showed some increase in survival compared with matched controls, but the benefits were limited. The current situation has been reviewed recently [8].

Light can only penetrate a few mm into tissue. Interstitial, image guided PDT has addressed this problem for defined lesions in solid organs like the prostate [9], but external light sources cannot reach deep, infiltrative disease when the exact location of every focus of cancer is not known. The challenge for PDT for gliomas lies in achieving targeted delivery of light to disseminated, deep seated foci of tumor cells. As photosensitizers are taken up preferentially by tumor cells in the brain, targetable activation by an endogenous light source in the tumor cell itself, may allow areas of deep infiltration to be treated. One possible source of endogenous light for applications like this is bioluminescence [10].

## 2. Bioluminescence-Mediated Photodynamic Therapy (bPDT)

Bioluminescence is the production and emission of light by a living organism. It results from a chemical reaction during which chemical energy is converted into light energy. The reaction requires a substrate, generically known as a luciferin, oxygen, and an enzyme, generically known as a luciferase. 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 photosensitizer cannot result solely from the radiative absorption of the photons from bioluminescence by the photosensitizer. The likely alternative mechanism is bioluminescence resonance energy transfer (BRET). BRET involves the non-radiative transfer of energy from a donor enzyme, the luciferase, to a suitable acceptor molecule, the photosensitizer, 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 photosensitizer 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 photosensitizer needs to be within about 10nm of the bioluminescent substrate, oxyluciferin.

In 2003, we reported that bioluminescence, generated by the addition of D-luciferin to luciferase-transfected NIH 3T3 cells, could activate the photosensitizer, Rose Bengal, to kill these cells [12]. In one subsequent publication, neuroblastoma and rat glioma cells were transfected with firefly luciferase and shown to generate light on addition of D-luciferin. However, a bioluminescence activated PDT effect could not be demonstrated on these cells using a combination of D-luciferin with Rose Bengal or hypericin. The reason for the different results is not clear but may be related to the different concentrations of D-luciferin and hypericin used [13]. Later work from other groups confirmed that bPDT can kill mammalian cells [14,15,16].

The goal of the present study is to provide proof-of-principle that cells from a grade 4 astrocytoma transfected with luciferase can be killed by bioluminescence activated PDT (bPDT) *in vitro,* prior to *in vivo* studies.

## 3. Methods

Two photosensitizers were chosen, hypericin and *m*THPC, both already associated with research into glioma treatment [17,18]. Preliminary studies were undertaken to ensure that these cells could be killed by conventional PDT before proceeding to bPDT. Stock solutions of hypericin (purchased from Planta Natural Products) were prepared in DMSO (dimethyl sulphoxide); mTHPC formulated as Foscan was provided by Biolitec AG.

## 4. Cell choice and preparation

The cell line chosen for detailed study was U87, originally sourced from a human grade 4 astrocytoma [19]. For each series of *in vitro* experiments, sub-confluent cells were harvested and plated at a density of $1 \times 10^4$ cells per well of a 96 well plate (Corning). After 24 h incubation, the media was aspirated and cells were washed twice with PBS. All cell lines were sensitive to serum starvation so subsequent experiments required cells to be incubated in complete media, although drugs and reagents were constituted in serum free solutions. In all experiments, each combination of variables was repeated 3 times. For the initial studies on conventional PDT, cytotoxicity after treatment was assessed using the MTT assay, in which a yellow dye is reduced to purple formazan in the mitochondria of living cells and the result can be quantified by measuring the optical density in the range 550-690 nm [20]. The MTT assay is only a measure of cytotoxicity, but it was considered appropriate in this part of the study as the only aim was to show in principle that cells could be killed.

### 4.1. Response to conventional PDT

To optimize singlet oxygen production, the maximum dose of photosensitizer tolerated by the cells without causing toxicity itself (dark toxicity), was determined. Varying concentrations of hypericin and mTHPC were made up in serum free media from stock solutions and 0.1 mL added to the wells in a 96 well plate, each concentration in triplicate, for 3-4 or 24 h incubation times. Control cells were treated similarly but incubated with serum free media instead of drug. They were then washed twice with PBS, then complete media was added and after a further 24 h incubation, a MTT assay was conducted to assess cytotoxicity. The highest doses that did not cause a significant reduction in cell survival compared to the corresponding control ($p < 0.05$) for each set of conditions were termed the maximum sub-lethal doses. These were the doses used in all subsequent *in vitro* experiments for each set of conditions. Using these doses, the cell lines were then tested to assess the lowest light dose that could achieve a PDT effect, (blue light at 420 nm, 7 mW/cm$^2$, Lumisource Biotech, Oslo, Norway). Care was taken to ensure that external lighting was minimised during all steps involving photosensitizers: window blinds were drawn, lights were switched off, and plates were wrapped in aluminium foil, even in the incubator, to avoid inadvertent photoactivation.

After 3-4 h incubation, in the absence of light, the maximum sub-lethal dose for hypericin was 12.5 μM and for mTHPC was 18.4 μM. After 24 h, it was 10 μM for hypericin and 3.7 μM for mTHPC. Using these drug doses, there was no significant difference in the minimal light dose required for a PDT effect between short and long incubation times for either photosensitizer. The threshold light doses were lower for mTHPC (21 mJ/cm$^2$) than for hypericin (350 mJ/cm$^2$) due to the strong absorption of mTHPC at 420 nm (Fig. 1). These results confirmed that

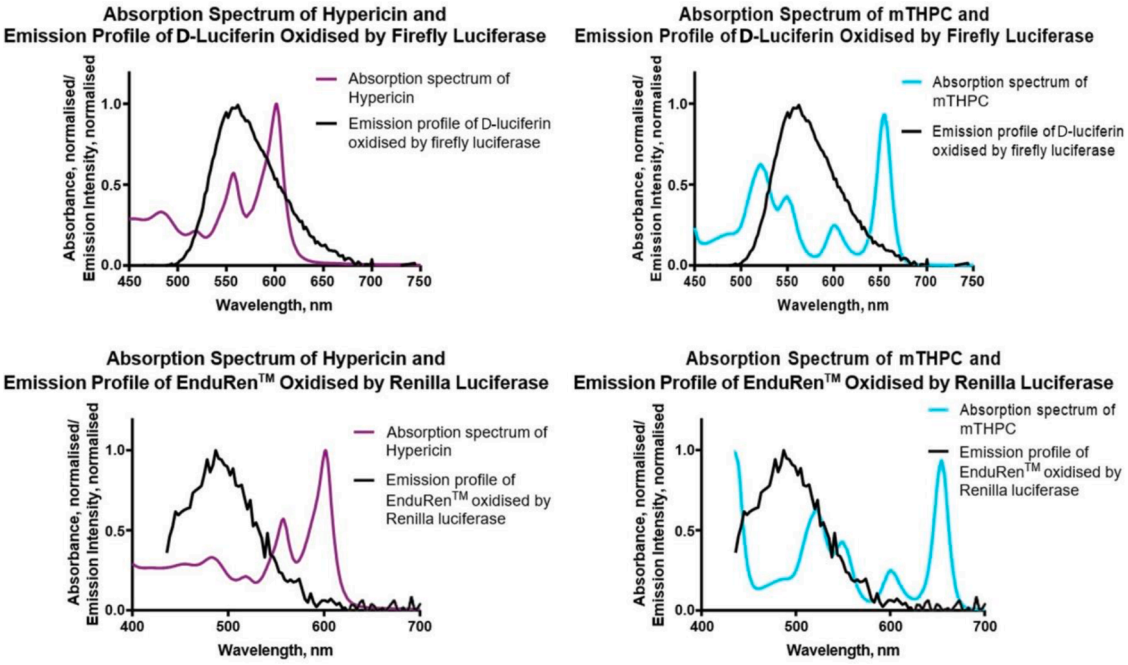


**Fig. 1.** Emission profiles of firefly luciferase from U87-luc cells, and Renilla luciferase from U87-hRluc cells compared with the Absorption Spectra of Hypericin and mTHPC. The peak bioluminescence of firefly luciferase lay between 546 – 574 nm, with the maximum at approximately 560 nm, which overlapped well with the absorption peaks of hypericin at 557 nm and mTHPC at 550 nm. The peak bioluminescence of Renilla luciferase lay between 451 – 505 nm, with the maximum at approximately 487 nm, which did not overlap so well with the absorption peaks of hypericin or mTHPC. The absorption spectrum for hypericin goes down to 400 nm, but that for mTHPC only goes down to about 420 nm.

U87 cells could be killed by conventional PDT. These drug doses were used in all subsequent experiments.

## 5. Generating bioluminescent cell lines

U87 cells were transfected with luciferases from 3 species – firefly, click beetle and renilla (a sea pansy). A plasmid containing firefly luciferase, CMV-luc, was kindly donated by Professor G Van der Pluijm. Plasmids containing click beetle and Renilla luciferase, CMV- CBG68luc and CMV-hRluc, respectively, were generated. The genes were purchased as vectors from Promega, along with a backbone vector (pcDNA3.1(+), Invitrogen), and multiplied by bacterial transformation. Once multiplied and extracted, the vectors underwent digestion by specific restriction endonucleases. The luciferase genes could then be ligated into the backbone vector, thereby forming the desired plasmids.

U87 cells were then transfected with CMV-luc, CMV-CBG68luc or CMV-hRluc using fugene-6 (Roche Biochemicals). Stable transfectants (U87-luc, U87-CBG68luc and U87-hRluc), were selected with 1mg/mL of neomycin. Neomycin-resistant clones were isolated, subcloned, and tested for luciferase activity [21]. One monoclonal and one polyclonal cell line for each of these lines was selected for further experimentation, based on the highest levels of bioluminescence measured on live cell assays.

## 6. Comparison of emission profiles of bioluminescence with the absorption spectra of the photosensitizers

The absorption spectra of hypericin (in DMSO) and mTHPC (in PBS) were recorded in a CARY 1E Varian spectrophotometer and corrected for the absorption profiles of DMSO and PBS. The emission spectra of U87-luc and U87-hRluc cells were measured by lysis of cultures of these cells, transferring the lysates into the wells of a 96 well plate (Corning). The natural substrate for U87-luc is D-luciferin. The natural substrate for U87-hRluc is coelenterazine, but as this is unstable in aqueous solution, 2 synthetic substrates have been developed, EnduRen™ and ViviRen™, which have different profiles in the peak amount of light generated and the duration of emission over time (Promega Corp.). For these spectral measurements, the substrates used were D-luciferin for U87-luc and EndoRen™, for U87-hRluc. After substrate administration the luminometer plates were put in a fluorometer (Thermo Electro Corp). The signal was captured between 400-800 nm. The results are shown in Fig. 1. The results for the photosensitizer absorption spectra and the emission spectra correlated well with published data for these compounds [22,23,24,25].

## 7. Characterising bioluminescence

The direct cytotoxicity of each substrate in the absence of a photosensitizer was measured after overnight incubation using a MTT assay. The maximum dose of D-luciferin that could be tolerated without direct cell kill was 2.5 mM. The maximum dose of EnduRen™ that could be tolerated by U87-hRluc cells without direct cytotoxicity was 60 μM.

The dose response curves for the peak light intensity as a function of the dose of substrate are shown in Fig. 2. In all subsequent experiments, the maximum sublethal doses were used, although for both D-luciferin and EndoRen™, the dose response curve plateaued, so the light produced by half the maximum sublethal dose was only slightly below that seen with the maximum sublethal dose.

The light intensity from D-luciferin peaked at around 3 min after its administration to U87-luc cells but dropped over a few minutes to a plateau at about 75% of the peak which persisted for at least an hour, as previously reported [12]. With U87-hRluc cells, the peak from EnduRen™ was much later at about 60 min, which faded over several hours, while the peak from ViviRen™ was much higher but fell to close to zero within an hour (results not shown).

## 8. Bioluminescence Mediated Photodynamic Therapy (bPDT) in vitro

After these preliminary studies had established the best experimental

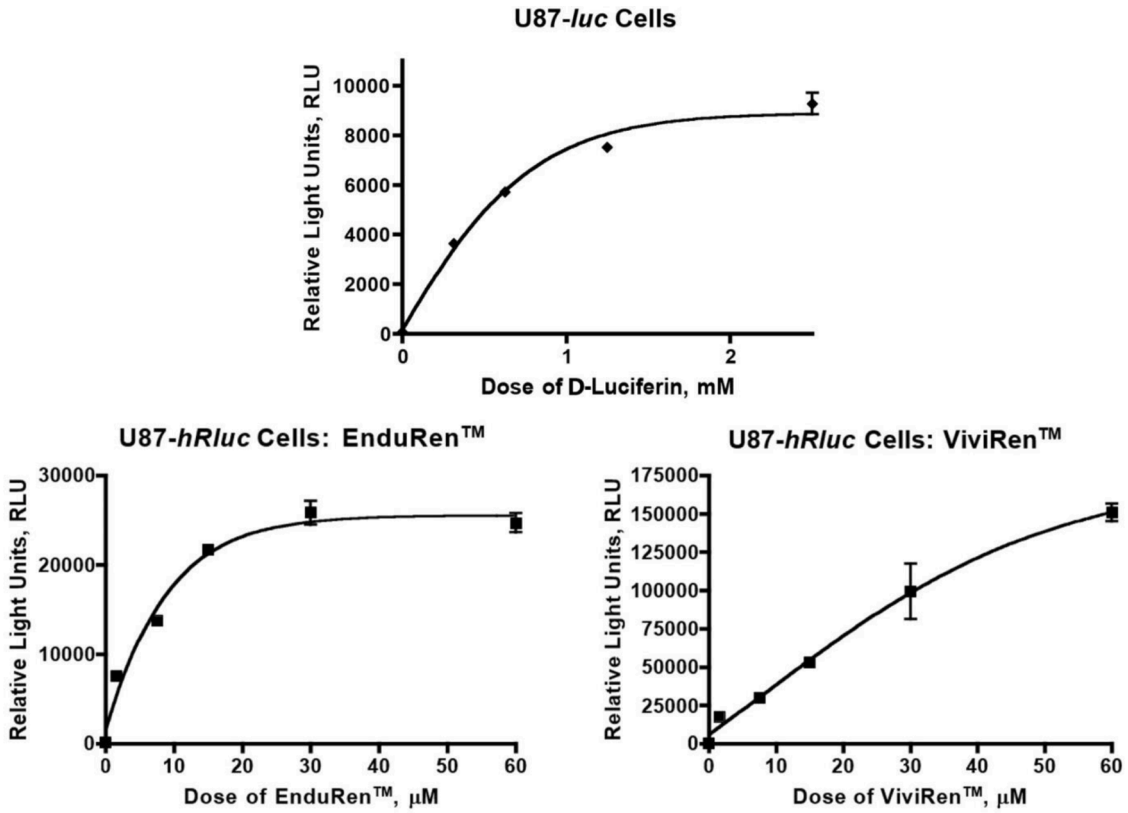


**Fig. 2.** Correlation of the peak intensity of light produced with the administered dose of substrate for d-luciferin on U87-luc cells and both EnduRenTM and ViviRenTM on U87-hRluc cells. The doses of each substrate were tested up to the maximum that could be given to each cell type without causing cell death in the absence of photosensitizer–2.5 mM for d-luciferin and 60 μM for EnduRen™. However for ViviRen™, although the maximum, non-toxic doses was 30 μM the maximum possible bioluminescence was achieved with 60 μM.

conditions, bPDT could be studied. Monoclonal and polyclonal luciferase transfected cell lines (U87-luc) were used together with control studies on non-transfected cells (U87). A limited number of experiments were also undertaken on U87-CBG68luc and U87-hRluc cells. All were kept under the same conditions and were maintained in complete media containing 1 mg/mL neomycin at all times. A live cell assay was conducted weekly on U87-luc cells to ensure that they continued to generate stable bioluminescence when treated with D-luciferin.

As before, following trypsinisation, sub-confluent cells were harvested, plated at a density of $1 \times 10^4$ cells per well in a 96 well plate and allowed to adhere over 24 h at 37C. Media was then aspirated and replaced with 0.1 mL of fresh complete media prior to experimentation.

For each set of laboratory conditions, wells were designated as one of 3 control groups – no treatment apart from PBS (C), photosensitizer only (D), D-luciferin only (L) or as treatment (D+L). Cells were incubated using the maximum sub-lethal photosensitizer doses established earlier from the conventional PDT studies. After incubating for the appropriate times (3-4 or 24 h, as before), all wells were washed twice with PBS and fresh complete media was replaced. D-luciferin was then added to the appropriate wells. An initial dose response study (Fig. 3a) showed that the most effective dose of D-luciferin was the maximum sub-lethal dose established earlier (2.5 mM) so this dose was used in all subsequent experiments. All cells were then incubated for a further 20-24 h prior to assay. For U87-CBG68luc and U87-hRluc cells, the substrates used were D-luciferin and EndoRen™, respectively.

The first experiments were done on U87 cells that had not been transfected. Subsequent studies used U87-luc luciferase-expressing cell lines, one monoclonal and one polyclonal, apart from a small number of experiments using U87-CBG68luc and U87-hRluc cells.

The variables studied included the choice of cell line (monoclonal or polyclonal) the photosensitizer (hypericin or mTHPC), and the incubation time after administration of the photosensitizer (4 or 24 h for hypericin, 3 or 24 h for mTHPC). As hypericin was reconstituted in DMSO, an extra control group of DMSO alone was added, but this did not affect the results (data not shown). To provide evidence that it is indeed bioluminescence activating the photosensitizer to cause a photodynamic effect that is mediating cytotoxicity, some experiments were undertaken with the addition of lycopene, which is an antioxidant. As the photodynamic effect is mediated by the generation of singlet oxygen, it was expected that the effect would be inhibited by an antioxidant.

To measure cytotoxicity after treatment, cells were plated in 2 parallel plates, treated similarly. The first was assessed by MTT assay as in the earlier work on conventional PDT and additionally by haemocytometry in which cells were stained with trypan blue and unstained cells, considered as viable, counted in a defined volume using a haemocytometer.

The second plate was for a growth assay; media was aspirated from the wells, which were then washed twice with 0.1 mL of PBS and 0.04 mL of trypsin added. After incubation for 1 min to allow the cells to detach, the cells in each well were resuspended in 0.1 mL of media, transferred to the wells of a 12 well plate and additional complete media added to each well to achieve a total volume of 1.5 mL. These were left to incubate until the control cells had reached subconfluence, typically 5 days, after which the surviving cells were counted by haemocytometry. The media was changed every other day. In both haemocytometry studies, the results were presented as a percentage of the number of cells surviving in the control (C) group. It is acknowledged that MTT is only useful for assaying a one log killing effect, but this was useful support for the better evidence from the haemocytometry and growth assay data in the bPDT studies.

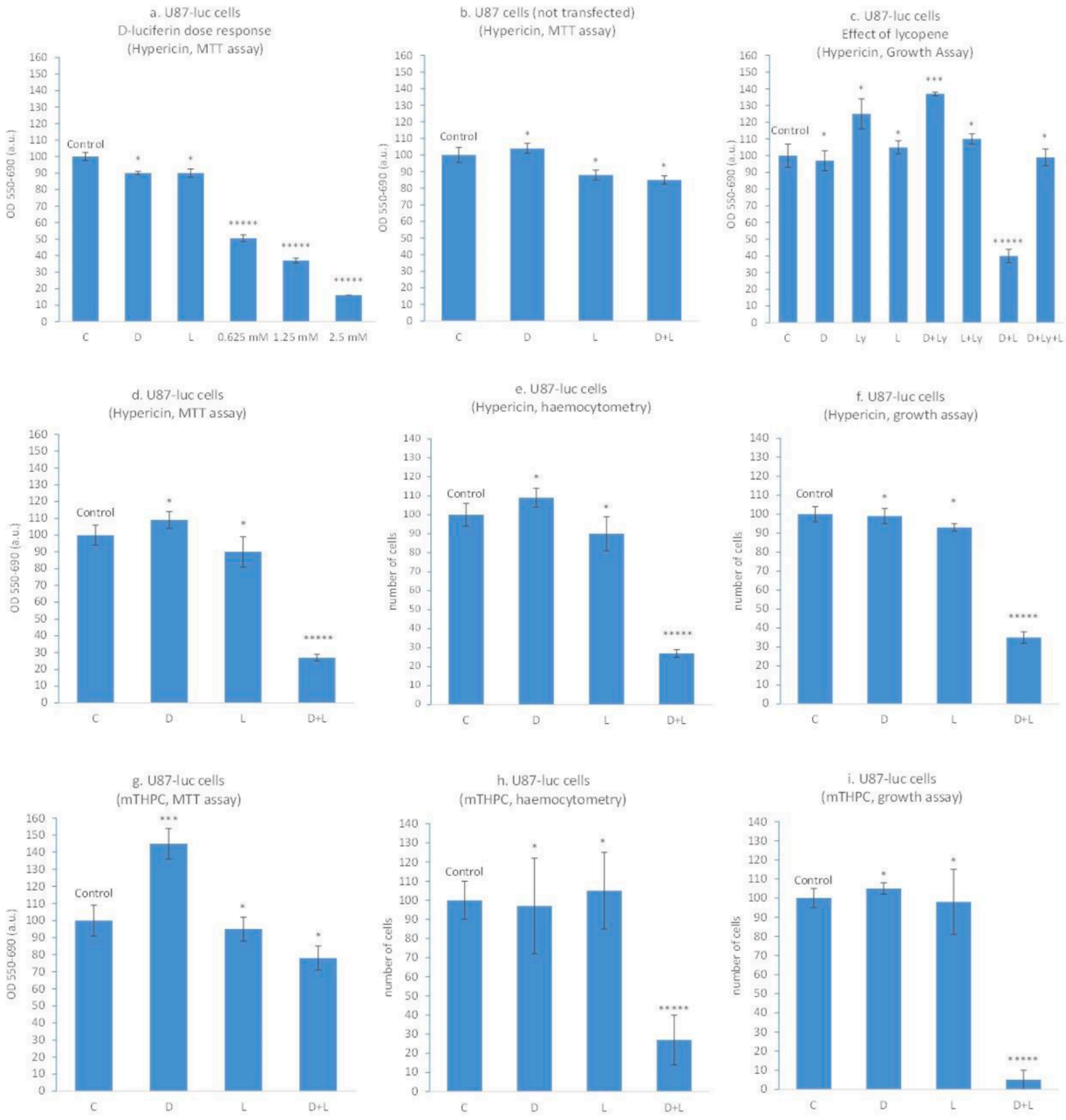


**Fig. 3.** Bioluminescence activated PDT (bPDT). Representative examples. Data are normalised so the result for the control cells without any additional reactants in each experiment is always 100. All other results can then be expressed as percentages. a) d-luciferin dose response. Monoclonal U87-luc cells incubated for 24 h with hypericin. MTT assay. In all subsequent experiments, the dose of d-luciferin used was 2.5 mM, the maximum dose that these cells could tolerate without causing cell death in the absence of a photosensitizer. b) Treatment of non-transfected U87 cells incubated with hypericin for 4 h. MTT assay . c) Effect of lycopene on bPDT effect. Polyclonal U87-luc cells incubated for 4hr with hypericin. Growth assay. d, e, f–Monoclonal U87-luc cells incubated with hypericin for 24 h. d) MTT assay, e) Haemocytometry f) Growth assay. g, h, i–Polyclonal U87-luc cells incubated with mTHPC for 3 h. g) MTT assay, h) Haemocytometry, i) Growth assay. C–Control cells treated only with PBS; D–Photosensitizer only (hypericin or mTHPC). Ly–Lycopene only; L–d-luciferin only; D+Ly–Photosensitizer and lycopene; L+Ly–d-luciferin and lycopene; D+L–Photosensitizer and d-luciferin; D+Ly+L–Photosensitizer, lycopene and d-luciferin. Asterisks indicate the degree of significance of the difference in cell numbers between each group and the control group–* $P>0.05$, ** $P\leq0.05$, *** $P<0.001$, **** $P<0.0001$.

## 9. Statistical analysis

Data was entered into GraphPad PRISM® Version 6 (GraphPad Software Inc.) and expressed as the mean of observations ± standard error (SE). The difference in values between groups was determined by analysis of variance (ANOVA) and Student-*t*-test using PRISM®. Results were considered significant when the value of $p \leq 0.05$.

## 10. Results for bioluminescence activated PDT (bPDT)

Representative examples of the key results are shown in Fig. 3 for U87-luc and non-transfected U87 cells. Not all combinations of treatment parameters were used but all those used were repeated 3 times.

The dose response curve for D-luciferin showed an increasing cytotoxic effect with increasing dose, as the dose reached 2.5mM, the maximum dose that did not cause direct cytotoxicity in the absence of a photosensitizer, Fig. 3a. This dose was used in all subsequent studies.

There was no detectable bPDT effect on cells that had not been

transfected, Fig. 3b. This confirmed that it is the interaction between hypericin and the bioluminescence generated by the addition of D-luciferin that is the cause of cell death and not any direct chemical interaction between photosensitizer and D-luciferin. The addition of the antioxidant lycopene to U87-luc cells after their incubation with hypericin at the same time as the addition of D-luciferin was seen to abolish the bPDT effect. Fig. 3c. Results after incubation with mTHPC were similar and these are not shown. This further attests that the mechanism of cell death is attributable to a photodynamic effect that is mediated by singlet oxygen.

Figs. 3d, e and f show the results for the 3 assay techniques for U87-luc monoclonal cells treated with 24 h of incubation with hypericin followed by the addition of D-luciferin. 3d shows results of a MTT assay; 3e shows the results from haemocytometry; and 3f shows the results from a growth assay.

Figs. 3g, h and i show the results for the 3 assay techniques for U87-luc polyclonal cells treated with 3 h of incubation with mTHPC prior to addition of D-luciferin. 3g shows results of a MTT assay; 3h shows the results from haemocytometry; and 3i shows the results from a growth assay.

There were no more than very minor differences in the bPDT responses between the different incubation times for each photosensitizer (4 and 24 h for hypericin, 3 and 24 h for mTHPC), and between experiments with the monoclonal and polyclonal cell lines for each photosensitizer, none of which reached statistical significance (data not shown).

The key response in all cases was taken as the comparison between the no treatment group (C) and the treatment group (D+L). There were no significant differences between the untreated (C) and light only (L) controls in any experiment. In several cases, there was increased cell survival in the drug only (D) control group, which in a few instances reached significance, particularly with mTHPC. This suggested that the photosensitizer alone was stimulating cell growth. An example of this is shown in the MTT assay of Fig. 3g, although this was not seen on the haemocytometry, Fig. 3h, and growth assays, Fig. 3i, in the same experiment.

With hypericin, the bPDT effects were comparable with all 3 assay techniques with each combination of treatment parameters. With mTHPC, significant bPDT effects could be detected between the control (C) and treatment (D+L) groups with haemocytometry and growth assays, comparable to those seen with hypericin. For the MTT assay, the difference was only significant between the drug only (D) and the treatment groups (D+L). In some cases with both photosensitizers the reduction in cell survival was more profound with the cell growth assay than with the earlier haemocytometry analysis.

Over 16 experiments using hypericin, each repeated 3 times, the mean survival of treated cells compared to controls was 36%, (median 34%, range 13-62%, only 2 over 50%, $P<0.001$ or better in all but one measurement). In 8 comparable experiments with mTHPC, each repeated 3 times, the mean survival of treated cells was 35%, (median 34%, range 4-60%, only one over 50%. $P<0.001$ in all but one measurement). These mTHPC figures were based just on haemocytometry and growth assay.

Data for U87-CBG68luc and U87-hRluc cells are limited and are not shown. Using hypericin and the substrate D-luciferin, the results for U87-CBG68luc cells were very similar to those for U87-luc (20-23% cell survival compared with the no treatment control group, $P<0.001$). With mTHPC, there was less effect, but when assessed by haemocytometry and growth assay, this did just reach significance ($P<0.001$ on growth assay). For U87-hRluc cells, no effect could be detected using hypericin, most likely related to the poor correlation between the absorption peaks of the hypericin and the renilla luciferase emission spectrum (Fig. 1), but with mTHPC, where the spectral match was better, there was significant cell kill (cell survival 25% of controls on growth assay, $P<0.001$), but only with the longer, 24 h, incubation period.

## 11. Discussion

There is considerable interest in the potential of PDT for cancer treatment as it is relatively straightforward, usually minimally invasive with few significant side effects, repeatable without the cumulative toxicity associated with ionizing radiation, and has been shown to be effective against radioresistant and chemoresistant cells [3]. However, it is difficult to deliver light from an external source if the target cells are diffuse and their location not well known. Hence the interest in endogenous sources of light. Most work on endogenous light sources for PDT has focused on bioluminescence, although chemiluminescence (using luminol) and Cerenkov radiation (which requires radioactive isotopes) have also been shown to be effective .

Bioluminescence imaging (BLI) is widely used experimentally as a relatively inexpensive, fast and simple method of imaging tumor progression and response to treatment. Using bioluminescence to activate a photosensitizer has been studied much less but there is recent increasing interest in its potential.

This study has shown that U87 glioma cells transfected with firefly luciferase (U87-luc) can be killed by bioluminescence activated PDT (bPDT) using the photosensitizers mTHPC and hypericin and the substrate D-luciferin. Results using cells transfected with click beetle luciferase (U87-CBG68luc) and the substrate D-luciferin were limited, but supportive of the results using U87-luc. Using Renilla luciferase (U87-hRluc) and the substrate EndoRen with hypericin, no effect could be detected, most likely due to the poor match of the emission spectrum from U87-hRluc to the absorption spectrum of hypericin. Some effect could be seen using mTHPC, although the spectral match was not as good as with U87-luc.

The most promising in vivo bPDT results reported so far have been on subcutaneous CT26 (rat colon cancer) tumors in mice. The photosensitizer Ce6 (Chlorin e6) and substrate, (coelenterazine) were administered systemically, but the luciferase, in the form Luc-QD (a conjugate of Renilla luciferase and quantum dots) was injected directly into the target tumor. Near complete inhibition of tumor growth was achieved [14]. However, in this situation, bPDT has no advantage over laser activated, image guided interstitial PDT or any other local treatments and would not work to eliminate cells in unknown locations as with astrocytomas.

An ideal solution would be in vivo transfection of astrocytoma cells with luciferase. This would be very difficult although a possible mechanism has been demonstrated, in which advanced human prostate cancer lesions were visualised in living mice by a targeted gene transfer vector and optical imaging [26]. Mammalian cells have no natural luciferase, so this would ensure that systemic administration of a photosensitizer and an appropriate substrate would only generate light in the tumor cells, regardless of where the photosensitizer was taken up.

In the absence of transfection, another approach would be to look for ways to enhance the bPDT effect in tumor cells and reduce it in normal tissues such as the liver. To treat diffusely infiltrating cells at unknown locations, all components of treatment must be given systemically and not locally. The first requirement would be a deeper understanding of systemic bPDT. The initial step would be to treat normal mice systemically with, for example, a conjugate of photosensitizer and luciferase [15], followed by substrate to see what effects are produced in normal organs like the liver. There will certainly be effects in a range of normal tissues, but it is well established that many such effects heal safely [3]. The aim will be to determine the maximum doses of each component that can be tolerated with no unacceptable effects on structure or function during healing, by varying factors such as drug doses, drug light intervals, regimen of administration (single doses, repeated doses, continuous treatment, duration of treatment etc). The same studies must then be repeated on transplanted, non-transfected astrocytomas (located subcutaneously for convenience) to establish if there are any conditions under which astrocytoma cells can be treated without unacceptable effects in other organs. Some selectivity of uptake of mTHPC has been

shown in astrocytomas compared to adjacent normal brain [5] and if antibodies with any degree of selectivity should become available, this could help if conjugated to the photosensitizer/luciferase conjugate. These could, of course, also be used for simpler approaches such as conjugation to chemotherapy agents.

It is appreciated that this is a very early project in the development of bPDT and that there are many hurdles to be addressed to understand what ultimate potential it might have. It is proposed to continue this work by growing tumors in mice with the cells in the present study transfected with luciferase that generate the most light, and for which the spectrum of emitted light best matches the absorption spectra of hypericin and mTHPC, namely U87-luc. This will ensure that the luciferase is already in the cancer cells, wherever they are, and not anywhere else, and treatment will only involve administration of the photosensitizer and D-luciferin, both of which can be given systemically. This will show if bPDT can kill astrocytomas grown from transfected cells, without damage to adjacent normal brain, but it will still be a major challenge to take the concept further.

## Author contributions

Jane Ng: Conceptualisation, funding acquisition, methodology, investigation, data curation and analysis, visualisation, writing draft and review

Nico Henriquez: methodology, resources, supervision, investigation, review and editing,

Neil Kitchen: Conceptualisation, funding acquisition, resources, supervision, project administration and review

Alexander MacRobert: data analysis, writing and review

Norman Williams: data analysis, presentation and review

Stephen Bown: Conceptualisation, supervision, data curation and analysis, project administration, visualisation, writing and review

## Declaration of Competing Interests

None.

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