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Photochemical internalization of bleomycin and temozolomide – *in vitro* studies on glioma cell line F98

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Here we evaluate the photosensitizer meso-tetraphenyl chlorin disulphonate ($TPCS_{2a}$) in survival studies of rat glioma cancer cells in combination with the novel photochemical internalization (PCI) technique. The tested anticancer drugs were Bleomycin (BLM) and Temozolomide (TMZ). Glioma cells were incubated with $TPCS_{2a}$ ($0.2\,\mu g\,ml^{-1}$, 18 h, 37 °C) before BLM or TMZ stimulation (4 h) prior to red light illumination (652 nm, $50\,mW\,cm^{-2}$). The cell survival after BLM (0.5 μM)-PCI (40 s light) quantified using the MTT assay, was reduced about 25 % after 24 h relative to controls, and 31 % after TMZ-PCI. The supplementing quantification by clonogenic assays, using BLM (0.1 μM), indicated a long–term cytotoxic effect: the surviving fraction of clonogenic cells was reduced to 5 % after light exposure (80 s) with PCI, compared to 70 % in the case of PDT. In parallel, structural and morphological changes within the cells upon light treatment were examined using fluorescence microscopy techniques. The present study demonstrates that PCI of BLM is an effective method for killing of F98 glioma cells, but smaller effects were observed using TMZ following the "light after" strategy. The results are the basis for further *in vivo* studies on our rat glioma cancer model using PDT and PCI.

1 Introduction

Every year at least 18 000 patients are diagnosed in the United States with the malignant brain tumor glioblastoma multiforma (GBM), a cancer arising from glial cells who constitute the connective tissue of the nervous system[1]. Despite being a rare disease GMB is the most common form of primary brain tumor, and is also the most aggressive one. The diffuse and infiltrative growth of these cancer cells, as well of their high resistance to conventional cancer therapies, complicate their complete elimination[2]. The overall survival time after diagnosis is often less than a year[3,4] and there are needs for new therapeutics strategies that can prolong survival and improve life quality for this group of patients.

Different to conventional photo-dynamic therapy (PDT) with light sensitizers that are designed to directly kill cancer cells, the photochemical internalization (PCI) technology is based on accumulation of a sensitizer in endosomal and lysosomal membranes[5–8]. The activation of light is also here producing singlet oxygen ($^1O_2$)[9] however, it produces ruptures of endocytical membranes resulting in a release of loaded macromolecules, such as chemotherapeutics, into the cytosol. Such site-specific delivery techniques can be used for enhancing drug delivery using light and have earlier been tested in several human and animal cell lines (*in vitro*)[10–12] as well as for *in vivo* treatment studies parallel to clinical trials[13]. In addition to the development of various drug delivery enhancing methodology, there is also an ongoing development of new more traditional PDT agents. New systems based on polymers[14,15], nanoparticles[16,17] and systems specially designed to have multiphoton excitation capability[18–21], are being developed. We foresee that the combination of new light activating molecules along with methods for targeted delivery such as PCI will result in better clinical applications in the future.

The performance of the novel chlorin photosensitizer $TPCS_{2a}$ (tetraphenylchlorin disulphonate) with two adjacent sulphonated groups (also named Amphinex®: PCI Biotech, Oslo) is the focus of the present study on rat glioma cancer cells (F98). The main objective was to compare effects of $TPCS_{2a}$ in combination with the two chemotherapeutics bleomycin (BLM)[5–7,22] and temozolomide (TMZ)[23] by using the PCI- *in vitro* model system. This technique might be suitable for cancer therapy and TMZ is a chemotherapeutic al-

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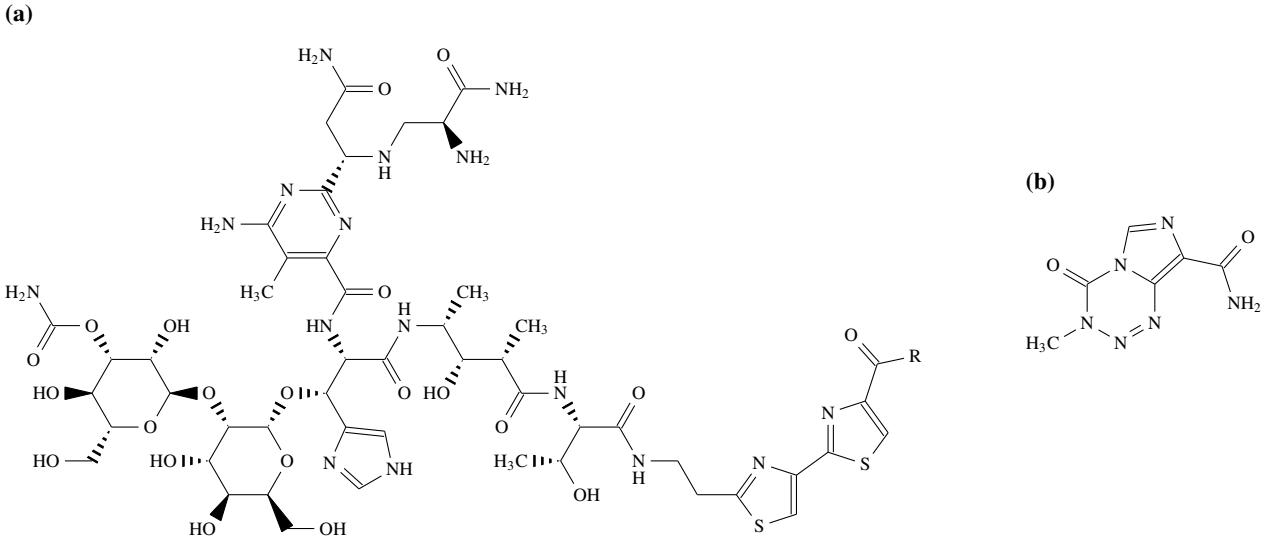

Fig. 1 Structural formula of the tested cytoctatica BLM in (a) and TMZ in (b) using the $TPCS_{2a}$-photochemical internalizartion (PCI) technology on the rat glioma cell line (F98).The terminal R consist of 4 four different substituents according to Baxter Oncology, GmbH.

ready approved and utilized for GBM treatment in Norway. In addition, the $TPCS_{2a}$-stability in glass and plastic tubes were spectroscopically measured after different intervals at 4 °C, documenting practical information using the novel and effective $TPCS_{2a}$-molecules for PCI-PDT. Our results on the cell model show that a combination of the anticancer drug BLM together with the PCI technology indicates possible treatment of gliomas, whereas the results obtained using TMZ under the identical conditions were not conclusive.

2 Materials and Methods

2.1 Chemicals

Thiazolyl Blue Tetrazolium Bromide powder, 3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide, was used for MTT assays (Sigma-Aldrich). A stock solution (30 mg ml^{-1}) of the amphiphilic chlorin $TPCS_{2a}$, formulated with polysorbate 80 (Tween), was kindly provided by PCI Biotech AS (Lysaker, Norway) and stored at 4 °C in a light protected box. The powder of lyophilized (freeze-dried) BLM (Baxter Oncology GmbH, Germany) was delivered in small, sealed bottles, each containing 15 000 international units (IE) according to the European Pharmacopeia (Ph. Eur.) (or 15 IE following the United States Pharmacopeial (USP) convention). TMZ powder (VetranalTM, analytical standard, SKU 76899) was purchased from Sigma-Aldrich in a package size of 10 mg and dissolved in 1 ml dimethyl sulfoxide (DMSO, Sigma-Aldrich) covered in aluminium foil and frozen. Other chemicals were of highest quality commercially available.

2.2 Cell culture

The undifferentiated malignant glioma cell line F98 (No. CRL-2397^{TM}), ordered from ATCC (American Type Culture Collection, LGC Standards, UK) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with glucose (4.5 g l^{-1}) (Cat. No. BE12-604F, Lonza), supplemented with extra L-glutamine (5 %), fetal bovine serum (FBS, 10 %), penicillin/streptomycin (1 %) and amphotericin B (0.1 %). The cell line were maintained in a humidified atmosphere of 5 % CO_2, 95 % air at 37 °C. The subculturing of cells was performed when confluence reached 80 to 100 %, i.e. about twice a week.

2.3 Survival studies using the MTT-assay for $TPCS_{2a}$, BLM and TMZ treatment

Culture dishes (Corning/Sarstedt, 60 mm × 15 mm) were provided with suspensions (3 ml) containing 0.4 − 0.5 million cells each at day 1, whereas the $TPCS_{2a}$ incubation (0.2 mg ml^{-1}, 18 h) started on day 2.[24] About 20 to 50 cell culture dishes were included in each experiment. The light treatments were performed at day 3 as described in subsections 2.4 and 2.5. On day 4 (or 5) the MTT working solution (0.5 mg ml^{-1}, 37 °C) was added after removal of old medium and washing twice (PBS). Further incubation (1 h, 37 °C) was obtained before the MTT solutions were discarded and replaced by isopropanol (2 ml). After shaking (Stuart orbital shaker, SSL1, 30 min, 80 RPM) the cell suspensions were centrifuged (5 min, RCF: 774 × g, Eppendorf centrifuge, 5810 R). Supernatants of pink color were diluted with appropriate

amounts of isopropanol before measuring the absorbance at 595 nm (Shimadzu UV-1700 spectrophotometer).

2.4 PDT experiments – Exposing cancer cells to laser light

At the end of $TPCS_{2a}$ incubation (0.2 μg ml^{-1}, 18 h) the cells were initially washed twice in PBS (2 to 3 ml, 37 °C) and the culture dishes provided with pure growth medium (3 ml, 37 °C), and incubated for about 5 h. Following incubation, and while contained in PBS, the dishes were illuminated (0 to 300 s, 652 nm, 50 mW cm^{-2}) with the Quanta System diode laser. Except during the actual laser treatment, dishes were covered in aluminium foil for as much of the time as possible. After exposure to laser light, the PBS was replaced with culture medium (3 ml, 37 °C), and all dishes were incubated for $\sim$ 24 hours.

2.5 PCI experiments – Incubation with BLM/TMZ, in addition to laser treatment

In PCI experiments (day 3) the chemotherapeutic solutions (0 to 1000) of BLM or TMZ were added, and the solutions were properly mixed prior to addition to the cells. As in the case of the PDT experiment, culture dishes were washed twice in PBS (2 to 3 ml, 37 °C) before incubation with pure or chemotherapeutics-containing culture medium (3 ml). After 3 to 4 h of incubation (37 °C), the F98 cells were exposed to laser light (0 to 180 s, 652 nm, 50 mW cm^{-2}) from the Quanta System diode laser (while containing chemotherapeutics solution/pure culture medium). Following another 1 to 2 h of incubation, dishes were washed once in PBS (2 to 3 ml), provided with fresh growth medium (3 ml) and incubated further overnight. At all times, unwanted light exposure was kept to a minimum by subdued lights and the use of aluminium foil.

2.6 Clonogenic assay

After the illumination procedure as earlier described for PDT or PCI treatment, the dishes were incubated for 1 to 2 h before washing (PBS) and cells scraping into 2 ml PBS. From each dish a homogeneous cell suspension (0.5 ml) was added to tubes containing PBS (10 ml), and further transfer of 0.5 ml solution to another PBS-containing (10 ml) tube. Again, the resulting solution (about 110 cells) was added (0.5 ml) to new culture dishes with culture medium (3 ml, 37 °C). All dishes were covered in aluminium foil and left in a CO_2 incubator (5 % CO_2, 37 °C) for 13-17 days, before the colonies were washed (H_2O), fixed with glutardialdehyde, 6 % in H_2O, stained with crystal violet (Sigma-Aldrich) and counted manually. For all measurements duplicate plates were used for each point and the experiments were carried out in duplicates or triplicates.

2.7 Light exposure using a Quanta System laser

Light exposure was carried out employing a Quanta System laser (TWI B-650, Solbiate Ololna, Italy). The laser emits red light (652 nm) with a variable power ranging from 0 to 500 mW. The laser beam was guided through an optical fiber (550 μm optical fibre: RBLF 550, Holmium), whose tip was mounted in a vertical distance of 12.3 cm above the bottom of culture dishes ($d = 6$ cm) placed directly underneath it. In this study only one power setting was used giving an approximately homogeneous irradiance (50 mW cm^{-2}) onto the sample.

2.8 Preparation and stability of $TPCS_{2a}$ stock solutions

The absorption spectra of $TPCS_{2a}$ solutions of different age, contained in standard polypropylene tube (15 ml, Cellstar®, Greiner Bio-One) and glass (11 ml, Pyrex®, Corning), were recorded. Each day from Monday till Friday a specific week, two $TPCS_{2a}$ solutions (10 ml, 150 μg ml^{-1}) were prepared from methanol (MeOH, HPLC grade) and $TPCS_{2a}$ stock solution (30 mg ml^{-1}). The mixtures were prepared in the dark, covered in aluminium foil and left in a fridge (4 °C) before the absorption spectra were recorded (Shimadzu UV-Visible (UV-1601PC spectrophotometer), using pure MeOH (HPLC grade) as reference solution. Absorption spectra recorded from four of the ten $TPCS_{2a}$ solutions (Supplementary information, Fig. 1) showed that no wavelength shifts however, there are minor differences in the absorbance value. These can be explained by slightly dissimilar dilution ratios during the initial making of the solutions or the dilution procedure. The absorbance spectra have typical maximum absorption band at 416 nm when dissolved in methanol. Moreover, the Soret band in the blue wavelength region is split into three distinct peaks, centered at 369 and 405 nm, besides 416 nm. However, in the context of PDT and PCI, the well-defined peak situated around 650 nm is the most as important as it is characteristic for $TPCS_{2a}$ and related PDT agents[25].

In order to confirm that a four days old $TPCS_{2a}$ solution displayed similar fluorescence characteristics as reported by Lilletvedt *et al.*[25,26], emission spectra were recorded (Supplementary information, Fig. 2) showing the distinct emission shown at 652 nm. The time-resolved fluorescence was also measured using an IBH time-correlated single photon counting spectrometer[27] (data not shown) giving a decay time of (8.58 ± 0.04) ns. This is as expected from porphyrin compounds.[28–30]

2.9 Fluorescence microscopy imaging

For the fluorescence microscopy 0.5×10^6 F98 cells were seeded out in Corning /Sarstedt dishes (30×10 mm) at day 1. After $TPCS_{2a}$ incubation (0.4 μg ml^{-1}, 18 h, day 2) the

cells were treated with light (180 s) after 3 h in PBS, using a Quanta system diode laser (652 nm, 50 mW cm^{-2}) at day 3, as described in subsection 2.4. The fluorescence microscopy was examined 1.5 h after illumination, by using an inverted microscope (Nikon Eclipse TE2000-S, Nikon Corp., Japan) equipped with a metal-halide light source (X-cite 120, Lumen Dynamics, Canada) and a filter cube with a 425DF45 exciter, 475DCLP dichroic mirror and 660DF50 emitter (all Omega Optical Inc., USA). Fluorescent images were captured with a Peltier cooled CCD camera (DS-5Mc with DSU2 Controller, Nikon Corp., Japan) operated from a PC running NiS Elements F ver. 2.33 (Nikon Corp., Japan) using a 60× ELWD Plan Fluor 0.70 NA objective (Nikon Corp., Japan).

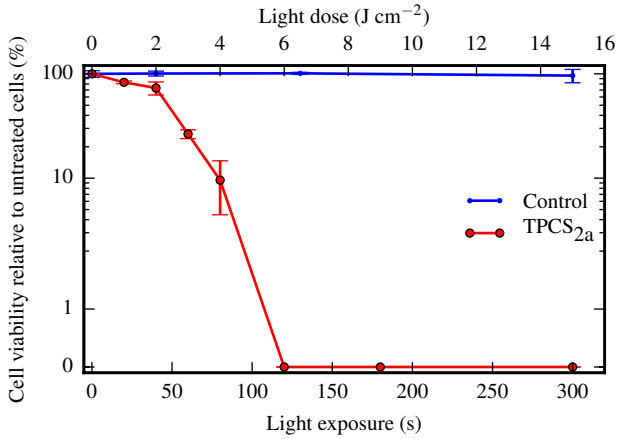

Fig. 2 Effects of photochemical treatment using TPCS$_{2a}$ on cell survival of F98 rat glioma cancer cells as a function of light dose. The cells were treated for 18 h with TPCS$_{2a}$ (0.2 µg ml^{-1}) and illuminated (in PBS) with different doses of red light (652 nm, 50 mW cm^{-2}) as described in section 2. Cell viability was measured by MTT 24 h post illumination. Data are presented as mean value and error bars ±SD of duplicates or triplicates, and this figure represent one of totally four independent experiments. The scale between zero and one on the y-axis is linear.

3 Results

3.1 Cell survival after light treatment

When using PCI in the treatment of cancer, choosing appropriate light conditions is essential. Consequently, studies of the effect of photochemical treatment alone were performed with glioma F98 cells. The cytotoxicity of the photosensitizer TPCS$_{2a}$ together with red light illumination was found to be increasing with larger doses of light until completely cell death after about 120 s and light (Fig. 2). This was also documented by light microscopy using the example of lethal doses of LD$_{50}$ and LD$_{100}$ in Supplementary information, Fig. 3. The fluorescence microscopy of TPCS$_{2a}$ enriched F98 glioma cells verified the accumulation of photosensitizer in the cytoplasm after 180 s exposure to red light, as shown in the high magnification image in Fig. 3. More examples with light and fluorescence microscopy are shown in the supplementary materials section, Figs. 4 and 5.

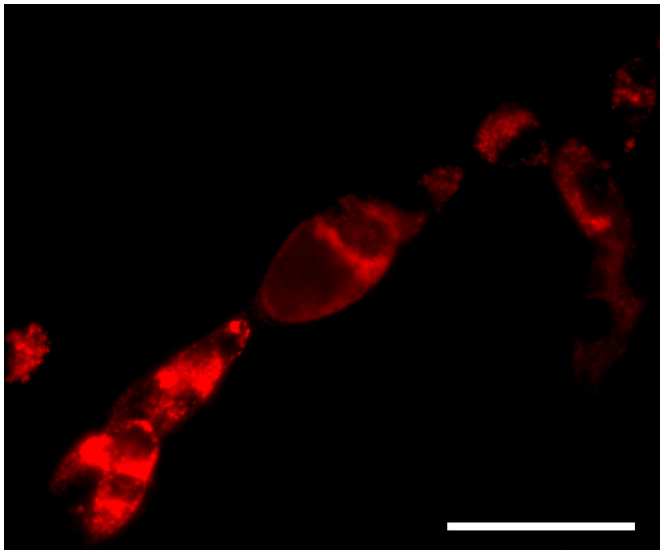

Fig. 3 Subcellular localization of TPCS$_{2a}$ in F98 glioma cells after incubation of TPCS$_{2a}$ (0.4 µg ml^{-1}, 18 h) and exposure (180 s) to red light. Scale bar is 50 µm. The cells were examined using an inverted microscope (Nikon Eclipse TE2000-S. Nikon Corp, Japan) 1.5 h post illumination as described in Material and Methods (section 2.9).

3.2 Cell survival after BLM and TMZ treatment

To be able to spot a potential increase in the biological activity of BLM due to photochemical internalization, the response of cells to either of the treatment strategies (*i.e.* the separate PDT and chemotherapeutic regimens), was determined. The viability of F98 cells following incubation with BLM and TMZ alone is displayed in Fig. 4. Each datapoint in this plot represents the average metabolic activity measured in 1-3 dishes from each of two similar independent experiments. According to the results, quite high doses of BLM are needed to cause a marked decrease in cellular viability. As much as 65 % of the cells were found to be functioning after a dose of 200 µM, whereas the fraction was reduced to about 25 % by a 1000 µM solution. The LD$_{50}$ dose appears to be close to 500 µM. For TMZ the effect was smaller. The cell viability decreased only by about 40 % by using a 1000 µM solution.

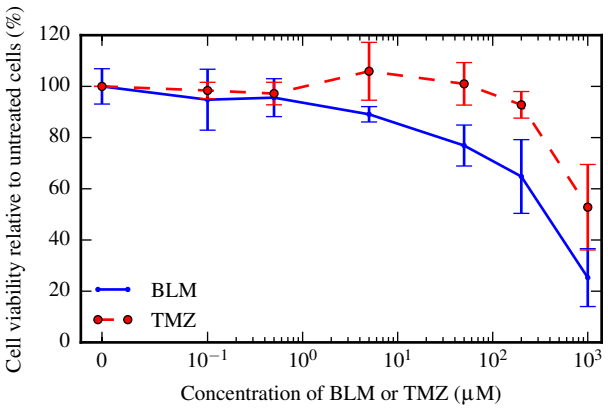

Fig. 4 Viability of rat glioma cells (F98), post 24 h illumination, as a function of bleomycin (BLM) and temozolomide (TMZ) concentration (0 to 1000 μM). The cells were incubated as described in Material and Methods using the MTT assay. The curves are normalized to 100 % viability for cells incubated in drug-free medium, and error bars are ± SD of the average viability measured in dishes included in two independent experiments.

3.3 BLM- and TMZ-based photochemical internalization

In order to investigate to what extent PCI can enhance the cytotoxic effects of BLM and TMZ, the cytotoxicity induced by different doses (0 to 1000 μM) of these compounds was investigated under various conditions of PDT. The cells were incubated with the PDT agent before adding BLM and TMZ, and the results are shown in Fig. 5a and 6a, respectively. In the (b)-panels the same data are normalized to the cell viability in the absence of BLM and TMZ, respectively, in order to see the contribution from the chemotherapeutic effect. The PCI effects alone can more clearly be seen for the longest illumination times in Fig. 5b, Fig. 6b and Fig. 8. The cytotoxic effects of BLM and TMZ have been normalized to 100 % for 0 μM BLM and 0 μM for TMZ, each of the illumination times. The histograms shown in Fig. 7 present BLM concentration required to reduce the cell viability by 50 % (LD_{50}) of normalized cell survival data from three BLM-PCI experiments. The presence of BLM seems to have minimal influence on the viability for doses up to around 50 μM. This was also indicated in data from the same experiment displayed as a function of light exposure instead of BLM concentration.

Conclusively, there are no or minor changes in cell viability for TMZ, but for BLM there are appreciable effects at concentration above approx. 100 μM. For this compound, the effect of illumination time is not critical up to approx. 80 s (see Fig. 8). The results indicate that the PCI technology can synergistically increase the cytotoxic effect of BLM in the F98 rat glioma cancer cells.

3.4 Clonogenic assay after PDT and BLM- and TMZ-based PCI

Long-term cytotoxic effects caused by an *in vitro* treatment strategy require a long-term sensitivity assay to be fully detected. Accordingly, clonogenic analyses replaced the MTT assay in some of the PCI experiments with BLM and TMZ (Fig. 8).

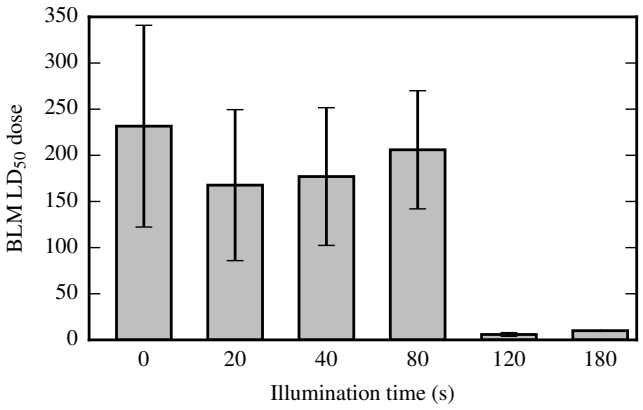

Fig. 7 Histogram presenting BLM concentration (μM) ±SEM at LD_{50} on normalized cell survival data from BLM-PCI experiments ($n = 3$) on F98 glioma cells. The cells were treated as explained in Fig. 5b and in Material and Methods, section 2 .

The PCI effects clearly displays lower relative clonal survival than the PDT effects for light doses exceeding 2.0 J cm^{-2} (40 s), using a BLM concentration as low as 0.1 μM. Whereas the surviving fraction of clonogenic cells was reduced to 5 % after 80 s of red light (652 nm) with PCI, nearly 70 % of clonogenic cells seemed to be viable after the same illumination time in the case of PDT. This difference in response is completely absent in the MTT-survival studies. However, treatment with BLM exclusively also appears to have larger impact on clonogenic cell survival than the level of energy metabolism in cells 24 h post treatment. As shown in a previous section, incubation with 0.1 μM BLM reduces viability with about 5 %, compared to the reduction of ∼ 30 % present in Fig. 4

The viability detected in F98 cells indicated that the sensitivity of F98 cells to TMZ alone (0 to 50 μM) is minimal. At higher TMZ concentration (1000 μM), the viable fraction of glioma cells remained close to 80 %, see Fig. 4. The cell clonal survival after illumination with red light 180 s (9 J cm^{-2}), results in only 9 % survival for $TPCS_{2a}$- and TMZ-PCI treatment.

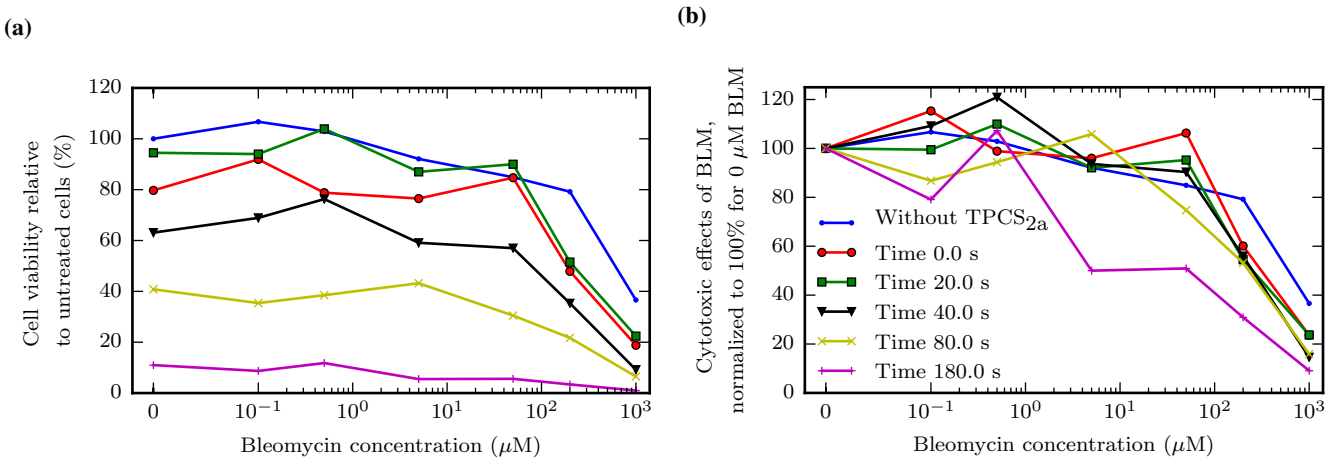

Fig. 5 $TPCS_{2a}$-PCI of bleomycin in F98 cells as function of red light exposure using different concentrations of bleomycin . Cells were incubated with $TPCS_{2a}$ (0.2 μg ml^{-1}, 18 h) before bleomycin incubation (0 to 1000 μM, 4 h) and illumination (652 nm, 50 mW cm^{-2}) from 0 to 180 s. Cell viability was assessed by the MTT assay 24 h post illumination, as described in Material and Methods. In (b) the values are normalized to 100 % for 0 μM bleomycin for each of the illumination times. Data for one of three representative experiments are shown and the statistics is summarized in Fig. 7.

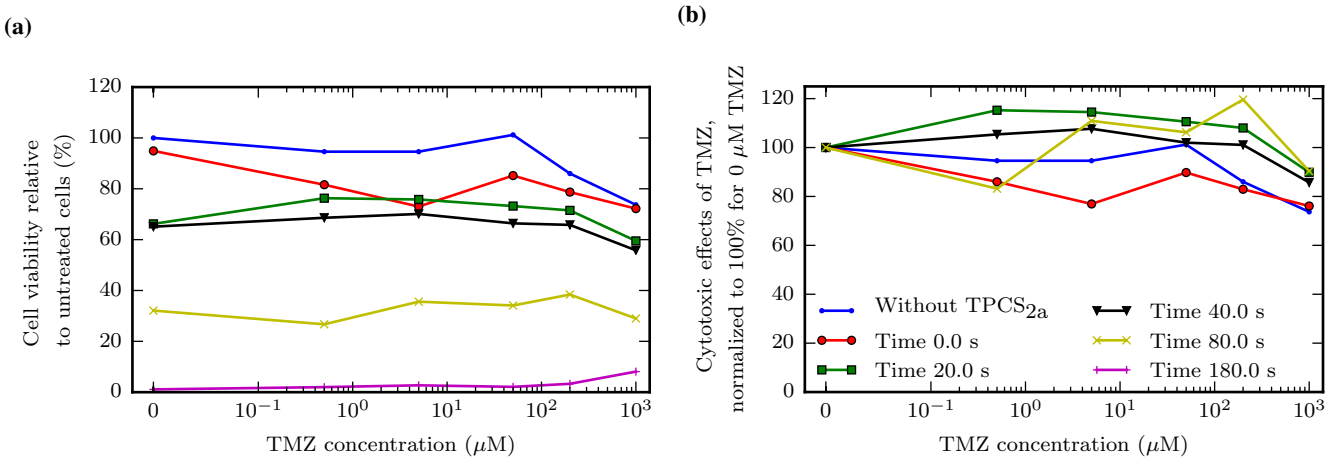

Fig. 6 $TPCS_{2a}$-PCI of temozolomide in F98 cells as function of red light exposure using different concentrations of temozolomide (a). Cells were incubated with $TPCS_{2a}$ (0.2 μg ml^{-1}, 18 h) before temozolomide incubation (0 to 1000 μM, 4 h) and illumination (652 nm, 50 mW cm^{-2}) from 0 to 180 s. Cell viability was assessed by the MTT assay 24 h post illumination, as described in Material and Methods. In (b) the values are normalized to 100 % for 0 μM temozolomide for each of the illumination times, except of 180 s since essentially complete cell death was achieved at the tested concentrations. Data for one of three representative experiments are shown.

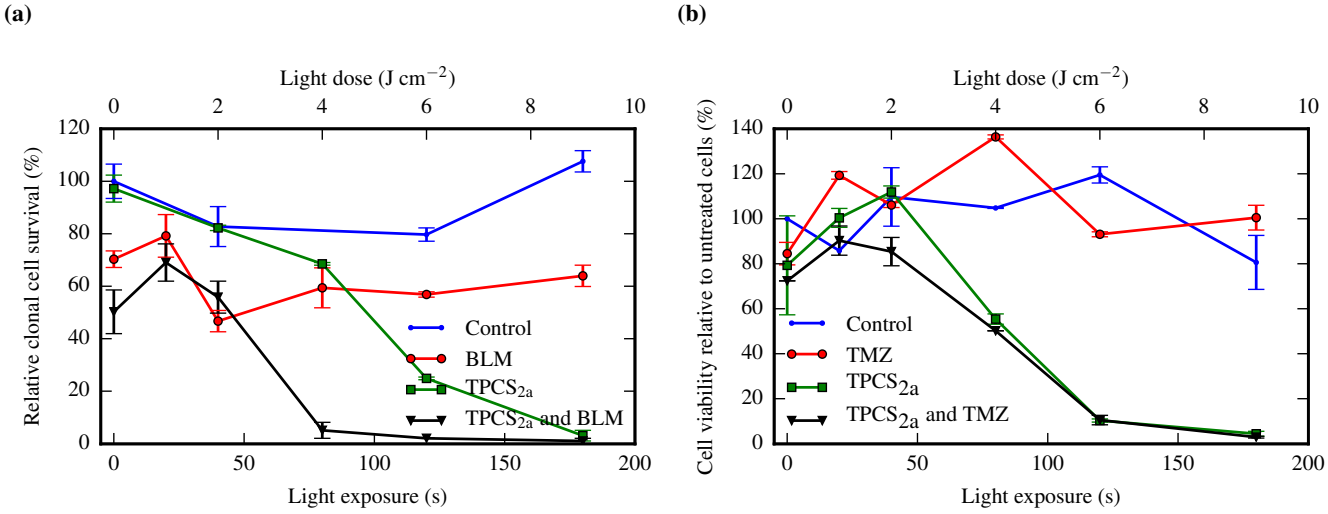

Fig. 8 Survival of clonogenic rat glioma cells (F98) as a function of light exposure (0 to 180 s at 652 nm, 50 mW cm^{-2}) after incubation with BLM (0.1 μM, 4 h) or TPCS$_{2a}$ (0.2 μg ml^{-1} alone or in combination, shown in (a). Effects of treatment with TMZ (0.5 μM, 4 h) or TPCS$_{2a}$(0.2 μg ml^{-1}, 18 h) alone or in combination were shown in (b).Control cells were treated with light alone. Data are presented as mean value ±SD of duplicates or triplicates, and this figure represent one of totally three independent experiments.

4 Discussion

The main objective of this study was to evaluate the response of glioma cells *in vitro* to the anticancer drugs BLM and TMZ in combination with light activation of the amphilic plasma membrane localizing photosensitizer TPCS$_{2a}$. A common feature among the majority of experiments performed *in vitro* is their ultimate goal of contributing to improved clinical practice in the future. In this respect, the F98 cell line appeared suitable, and it is appropriate for use in both *in vitro* and *in vivo* studies of a rat brain tumor and possesses biological characteristics closely resembling those of human glioblastoma[31].

The localized nature of PDT and PCI is partly a result of the selective accumulation of photosensitizers in cancerous tissue and the mechanisms involved in this high tumor-to-normal tissue distribution ratio are not fully understood[32]. Physiological properties of the tumor tissue itself seem to play important roles in accordance with *in vivo* studies on rat bladder cancer cells, presenting enriched fluorescence using an orthotopic rat bladder cancer model after intravesical instillation of the same photosensitizer[33].

Despite the fact that TMZ is a small and lipophilic molecule (Fig. 1), likely to penetrate the plasma membrane by passive diffusion[34], the viability detected in F98 cells, seems to be minimally attentuated even at high concentrations as shown in Fig. 4. The glioma F98 cells did not seem particularly sensitive to any of these drugs in separate, and in the absence of TPCS$_{2a}$, metabolic activity of drug-exposed cells was not influenced by increasing doses as revealed by the MTT assay. Light together with BLM have earlier been expected to give an additive toxic effect compared to each treatment alone[7], while the TPCS$_{2a}$-based PCI of TMZ would probably not give similar effects. This was as expected in the case of TMZ, since these alkylating agent molecules most likely do not associate with endosomal compartments, which is due to the fact that they are not taken into the cell via receptor-mediated endocytosis, but for PCI of BLM synergestic effects are indicated. But our results demonstrated a synergistic effect after PCI of BLM with TPCS$_{2a}$incubated glioma cells (Fig. 7). In addition, high activity of the DNA repair protein AGT (or MGMT) confers resistance of F98 cells towards alkylating agents like TMZ.[35] Nevertheless, expression in certain cancer cells of a repair protein named O-6-methylguanine-DNA methyltransferase (MGMT), or O-6-alkylguanine-DNA alkyltransferase (AGT), has been associated with poor therapeutic outcome of TMZ treatment and moreover, the MGMT/AGT gene is epigenetically silenced in a number of patients[36]. With a combination of radiotherapy and TMZ treatment, survival of patients with GBM can be significantly prolonged[37,38].

BLM is a glycopeptidic antibiotic utilized in chemotherapeutic regimens against a range of different cancers, including squamous cell carcinoma of e.g., the head and neck, testicular cancers and lymphomas[5]. The compound exerts its cytotoxicity by cleaving single- and double-stranded DNA at GC base pairs and one BLM molecule alone can produce 8-10 DNA breaks with a cytosolic presence as little as ∼ 400 molecules

per cell is lethal for about 50 % of cells *in vitro*[39,40]. In other words, BLM has a high intrinsic cytotoxicity. However, the large hydrophilic structure of BLM (molecular weight of $\sim$ 1.4 kDa, Fig. 1a) limits its penetration through the plasma membrane. Receptor-mediated endocytosis is typically required for the molecule to enter cells. Intracellularly, degradation by lysosomal hydrolases (prior to potential release from endolysosomal vesicles), or inactivation by the cytoplasmic enzyme BLM hydrolase, may prevent the compound from exerting its antitumor effect. Both *in vitro* and *in vivo* PCI has been shown to increase the therapeutic efficacy of BLM[5,7,22].

Results from the presented experiments shows the response of F98 cells to *in vitro* $TPCS_{2a}$-based PDT and PCI evaluated by means of a metabolic MTT assay using high cell numbers as explained in subsection 2.3 where the PDT and PCI experiments involved an utilization of a standard $TPCS_{2a}$concentration ($0.2\,\mu g\,ml^{-1}$)[41,42]. By lowering the amount of photosensitizer, less steep dose-response relationships may be obtained, causing varying inherent treatment sensitivity of different cell lines to be spotted more easily.

As shown in Fig. 2, the LD_{50} was determined to be approximately $2.5\,J\,cm^{-2}$ (50 s) for $TPCS_{2a}$-based PDT using $0.2\,\mu g\,ml^{-1}$. In contrast, rat bladder cancer cells (AY27) were shown to require no more than $1.9\,J\,cm^{-2}$ of the same laser light to die with a probability at 50 % (unpublished results, Gederaas *et al.*). In similar studies[22] it was reported an LD_{50} dose of $2.2\,J\,cm^{-2}$ for F98 cells exposed to PDT using the photosensitizer aluminium phthalocyanine disulfonate ($AlPcS_{2a}$) however, the usage of another illumination protocol (wavelength and irradiance) excludes direct comparison with their results.

By using the MTT assay the mitochondrial activity is documented, and consequently, the term "survival" assay should be used with caution. Reduced energy metabolism is also shown by cells temporarily resting, for instance due to cell cycle arrest. Obviously, the MTT assay does not provide information about the mode of potential cell death, as using fluorochrome tagging by e.g., AnnexinV Fluos or Propidium iodid for determing apoptotoc versus necrotic cells after PDT as demonstrated by Ekroll *et al.*[43]. The MTT assay is primarily a measure of acute toxicity, and cytotoxic mechanisms of BLM and other chemotherapeutics, as well as photodynamic cytotoxicity, need more than 24 hours to cause cancer cell impairment. However, together with clonogenic assays, the long-time cytotoxic effects (13-17 days post PDT) can be used to confirm the MTT assay. In this study an additional MTT-experiments was carried out both at 24 and 48 h after illumination, using a fixed concentration of both BLM (0.5 μM), of TPCS2a ($0.2\,\mu g\,ml^{-1}$) at an illumination of $2.0\,J\,cm^{-2}$ for 40 s. The results are summarized in Fig. 6 of supplementary information and as can be concluded, the PCI treatment causes largest reduction in viability, secondly after 'only PDT' with Amphinex, but the differences between 24 and 48 h waiting are small or insignificant. Results obtained by Selbo et al. suggested that more time is required for PCI effects to fully established.[44] According to Carmichael et al., the MTT assay is limited to detection of 90 % cell kill[45] and good experience from similar studies indicates that the absorbance was great enough for these actual PDT/PCI-protocols by using isopropanol as a solvent instead of DMSO or mineral oil.[43,46] Our results of postponing MTT evaluation from 24 to 48 h between light exposure and MTT analysis after PCI, suggest that a time duration of 24 h is acceptable for PCI effects to be revealed, but have to be performed in parallel with clonogen survival (Fig. 8). Our result (Figure S6) indicates that many PDT-exposed cells are capable to divide and proliferate despite showing low metabolic activity on the day after treatment. Moreover, BLM and PCI/PDT together indicated synergestic responses in the examination of clonal cell survival.

Chemosensitivity testing of a wide range of chemotherapeutic agents, on cell lines with vastly differing growth characteristics, has shown the MTT assay to give dose-response relationships comparable to those obtained with clonogenic and dye exclusion assays[45]. Nevertheless, some antineoplastics have been found to interfere with the MTT salt, causing incorrect absorbance values to be detected. According to Ulukaya *et al.*, chemical interactions between MTT and BLM are not an issue[47]. TMZ was not included in their study, but four out of five alkylating agents – including the TMZ analog dacarbazine – caused insignificant changes in absorbance. Anyway, in all experiments performed in the present report, extracellular chemotherapeutic molecules were removed from culture dishes the day before MTT evaluation, reducing possible interference between MTT and BLM or TMZ. In this respect, one should be aware that any washing procedure prior to MTT addition (or clonogenic analysis) could detach loosely attached (e.g. mitotic) cells from the culture dish bottoms and result in an artificial decrease in the number of dish substrates. Considered as the "gold standard" among sensitivity assays, the clonogenic assay is associated with more disadvantages than its long-term nature. A difficulty commonly experienced is clumping of colonies located close together, potentially causing too few colonies to be counted. Moreover, only cells that are actively dividing – and not reversibly non-dividing – are likely to be detected[48].

The mechanisms for producing cellular damage are different for TMZ and BLM, although DNA represents the target for both compounds. Following oral administration, TMZ is in physiological solution spontaneously converted to methyl-triazeno-imidazole-carboxamide (MTIC), which undergoes further conversion and ultimately causes a methyl group to be transferred to DNA[23]. Despite the fact that TMZ is a small and lipophilic molecule (Fig. 1), likely to penetrate the plasma membrane by passive diffusion[34], the viability de-

tected in F98 cells, seems to be minimal even at high concentrations (0 to 1000 μM) as shown in Fig. 4. One of the reasons for the low toxicity may be due to the ability of F98 glioma cells to alkylate/methylate DNA, because TMZ is a prodrug and an imidazotetrazine derivative of the alkylating agent dacarbazine, which has been available as an anti tumor agent in the US since 1999[49]. A combination of dacarbazine and BLM using the PCI technology, has previously been studied by Høgset *et al.*, but without any cytotoxic effects.

Finally, solutions of the amphiphilic photosensitizer $TPCS_{2a}$were visually and spectroscopically evaluated, as a small supplement to PDT and PCI experiments according to HPLC-analyses within a time window of five days, the absorbing and fluorescent properties of the solutions seemed not to be influenced by age or material of storage tube. The presented study has shown a clear dose-dependent transition from survival to complete cell death using different light doses on $TPCS_{2a}$-enriched F98 cells (Fig. 2). It should be pointed out that here it has not been focused on whether the cells died by an apoptotic or necrotic cell death process[50]. It remains to elucidate such details by comparing several cell lines known to respond to the a protocol for PDT- or PCI treatment similar to the one used here.

5 Conclusion

Photochemical internalization (PCI) is based on photo induced delivery of chemotherapeutics into the cytosol of tumor cells to reach their intracellular targets and exert their therapeutic activity, and the present results document that $TPCS_{2a}$-mediated PCI has the potential to enhance efficacy of BLM but not of TMZ in the tested rat glioma F98 cell line. The *in vitro* studies show that the cytotoxic effects are enhanced by $TPCS_{2a}$-mediated PCI using BLM in F98 cells and that cell death of TMZ was minimal even at high concentration (1000 μM). In general, the combination of PDT and BLM, but not TMZ, require further evaluation in glioma cells, but have so long given optimizing results for further improvements of the PCI technology and possible clinical applications.

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References

1 K. Hynynen, N. McDannold, N. A. Sheikov, F. A. Jolesz and N. Vykhodtseva, *Neuroimage*, 2005, **24**, 12–20.
2 M. Jamal, B. H. Rath, P. S. Tsang, K. Camphausen and P. J. Tofilon, *Neoplasia*, 2012, **14**, 150–158.
3 L. Paszat, N. Laperriere, P. Groome, K. Schulze, W. Mackillop and E. Holowaty, *Int. J. Radiat. Oncol.*, 2001, **51**, 100–107.
4 R. Helseth, E. Helseth, T. B. Johannesen, C. W. Langberg, K. Lote, P. Rønning, D. Scheie, A. Vik and T. R. Meling, *Acta Neurol. Scand.*, 2010, **122**, 159–167.
5 K. Berg, A. Dietze, O. Kaalhus and A. Høgset, *Clin. Cancer Res.*, 2005, **11**, 8476–8485.
6 O.-J. Norum, P. K. Selbo, A. Weyergang, K.-E. Giercksky and K. Berg, *J. Photochem. Photobiol. B Biol.*, 2009, **96**, 83–92.
7 O.-J. Norum, J.-V. Gaustad, E. Angell-Petersen, E. K. Rofstad, Q. Peng, K.-E. Giercksky and K. Berg, *Photochem. Photobiol.*, 2009, **85**, 740–749.
8 Y. Baglo, Q. Peng, L. Hagen, K. Berg, A. Høgset, F. Drabløs and O. A. Gederaas, *Photodiagnosis and Photodynamic Therapy*, 2015, **12**, 58–66.
9 P. Agostinis, K. Berg, K. A. Cengel, T. H. Foster, A. W. Girotti, S. O. Gollnick, S. M. Hahn, M. R. Hamblin, A. Juzeniene, D. Kessel, M. Korbelik, J. Moan, P. Mroz, D. Nowis, J. Piette, B. C. Wilson and J. Golab, *CA. Cancer J. Clin.*, 2011, **61**, 250–281.
10 K. Berg, M. Folini, L. Prasmickaite, P. Selbo, A. Bonsted, B. Engesaeter, N. Zaffaroni, A. Weyergang, A. Dietzea, G. Maelandsmo, E. Wagner, O.-J. Norum and A. Hogset, *Curr. Pharm. Biotechnol.*, 2007, **8**, 362–372.
11 H. Mojzisova, S. Bonneau, P. Maillard, K. Berg and D. Brault, *Photochem. Photobiol. Sci.*, 2009, **8**, 778–87.
12 K. Berg, D. Tran, P. K. Selbo and C. Rimington, *Compound PCT WO 03/020309*, 2001.
13 PCI Biotech AS, *Safety study of Amphinex based photochemical internalisation (PCI) of bleomycin in patients with cutaneous cancer*, `http://clinicaltrials.gov/show/NCT00993512`, NLM identifier NCT00993512, 2009.
14 Y.-E. K. Lee and R. Kopelman, *Methods Mol. Biol.*, 2011, **726**, 151–178.
15 C. Cepraga, T. Gallavardin, S. Marotte, P.-H. Lanoe, J.-C. Mulatier, F. Lerouge, S. Parola, M. Lindgren, P. L. Baldeck, J. Marvel, O. Maury, C. Monnereau, A. Favier, C. Andraud, Y. Leverrier and M.-T. Charreyre, *Polym. Chem.*, 2013, **4**, 61–67.
16 S.-H. Cheng and L.-W. Lo, *Curr. Drug Discov. Technol.*, 2011, **8**, 250–268.
17 C. Wang, L. Cheng and Z. Liu, *Theranostics*, 2013, **3**, 317–330.
18 K. Ogawa and Y. Kobuke, *Biomed Res. Int.*, 2013, **2013**, 125658.
19 D. Gao, R. R. Agayan, H. Xu, M. A. Philbert and R. Kopelman, *Nano Lett.*, 2006, **6**, 2383–2386.
20 T. Gallavardin, M. Maurin, S. Marotte, T. Simon, A.-M. Gabudean, Y. Bretonniere, M. Lindgren, F. Lerouge, P. L. Baldeck, O. Stephan, Y. Leverrier, J. Marvel, S. Parola, O. Maury and C. Andraud, *Photochem. Photobiol. Sci.*, 2011, **10**, 1216–1225.
21 T. Gallavardin, C. Armagnat, O. Maury, P. L. Baldeck, M. Lindgren, C. Monnereau and C. Andraud, *Chem. Commun.*, 2012, **48**, 1689–1691.
22 M. S. Mathews, J. W. Blickenstaff, E. Shih, G. Zamora, V. Vo, C. Sun, H. Hirschberg and S. J. Madsen, *J. Biomed. Opt.*, 2012, **17**, 1–8.
23 J. L. Villano, T. E. Seery and L. R. Bressler, *Cancer Chemother. Pharmacol.*, 2009, **64**, 647–655.
24 A. Weyergang, O. Kaalhus and K. Berg, *Mol. Cancer Ther.*, 2008, **7**, 1740–50.
25 M. Lilletvedt, H. H. Tønnesen, A. Høgset, L. Nardo and S. Kristensen, *Pharmazie*, 2010, **65**, 588–595.
26 M. Lilletvedt, S. Kristensen, H. H. Tønnesen, A. Høgset and L. Nardo, *J. Photochem. Photobiol. A Chem.*, 2010, **214**, 40–47.
27 E. Glimsdal, M. Carlsson, B. Eliasson, B. Minaev and M. Lindgren, *J. Phys. Chem. A*, 2007, **111**, 244–250.

28 B. Minaev and M. Lindgren, *Sensors (Basel).*, 2009, **9**, 1937–1966.

29 R. Vestberg, A. Nyström, M. Lindgren, E. Malmström and A. Hult, *Chem. Mater.*, 2004, **16**, 2794–2804.

30 B. Minaev and M. Lindgren, *Vibration and Fluorescence Spectra of Porphyrin-Cored 2,2-Bis(methylol)-propionic Acid Dendrimers.*, 2009, http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3345821&tool=pmcentrez&rendertype=abstract.

31 A. (LGC Standards), *Product information: F98 (CRL-2397)*, https://www.lgcstandards-atcc.org/Products/Cells_and_Microorganisms/By_Tissue/Brain/CRL-2397.

32 M. R. Hamblin and E. L. Newman, *J. Photochem. Photobiol. B Biol.*, 1994, **23**, 3–8.

33 Y. Baglo, L. Hagen, A. Høgset, F. Drabløs, M. Otterlei and O. A. Gederaas, *Biomed Res. Int.*, 2014, **2014**, 921296.

34 V. L. Bull and M. J. Tisdale, *Biochem. Pharmacol.*, 1987, **36**, 3215–3220.

35 L. D. Rhines, P. Sampath, M. E. Dolan, B. M. Tyler, H. Brem and J. Weingart, *Cancer Research*, 2000, **60**, 6307–6310.

36 M. E. Hegi, A.-C. Diserens, T. Gorlia, M.-F. Hamou, N. de Tribolet, M. Weller, J. M. Kros, J. A. Hainfellner, W. Mason, L. Mariani, J. E. C. Bromberg, P. Hau, R. O. Mirimanoff, J. G. Cairncross, R. C. Janzer and R. Stupp, *N. Engl. J. Med.*, 2005, **352**, 997–1003.

37 R. Stupp, W. P. Mason, M. J. van den Bent, M. Weller, B. Fisher, M. J. B. Taphoorn, K. Belanger, A. A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R. C. Janzer, S. K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J. G. Cairncross, E. Eisenhauer and R. O. Mirimanoff, *N. Engl. J. Med.*, 2005, **352**, 987–996.

38 P. A. Rønning, E. Helseth, T. R. Meling and T. B. Johannesen, *Neuro. Oncol.*, 2012, **14**, 1178–1184.

39 M. S. Mathews, *Enhancing the efficacy of phototherapy for malignant gliomas*, Doctoral thesis, UiO, 2013.

40 B. Poddevin, S. Orlowski, J. Belehradek Jr and L. M. Mir, *Biochem. Pharmacol.*, 1991, **42**, S67 – S75.

41 K. Berg, S. Nordstrand, P. K. Selbo, D. T. T. Tran, E. Angell-Petersen and A. Høgset, *Photochem. & Photobiol. Sci.*, 2011, **10**, 1637–1651.

42 M. E. B. Berstad, *MSc thesis*, UiB, Radiumhospitalet, Oslo, 2009.

43 I. K. Ekroll, *MSc thesis*, NTNU, 2009.

44 P. K. Selbo, M. G. Rosenblum, L. H. Cheung, W. Zhang and K. Berg, *PLoS One*, 2009, **4**, e6691.

45 J. Carmichael, W. G. DeGraff, A. F. Gazdar, J. D. Minna and J. B. Mitchell, *Cancer Res.*, 1987, **47**, 936–942.

46 O. A. Gederaas, S. A. Schønberg, S. l. Ramstad, K. Berg, A. Johnsson and H. E. Krokan, *Photochem. Photobiol. Sci.*, 2005, **4**, 383–9.

47 E. Ulukaya, M. Colakogullari and E. J. Wood, *Chemother.*, 2004, **50**, 43–50.

48 R. M. Hoffman, *J. Clin. Lab. Anal.*, 1991, **5**, 133–143.

49 M. Stevens, J. Hickman and S. Langdon, *Cancer Res.*, 1987, **47**, 5846–5852.

50 J. Berlanda, T. Kiesslich, C. B. Oberdanner, F. J. Obermair, B. Krammer and K. Plaetzer, *J. Environ. Pathol. Toxicol. Oncol.*, 2006, **25**, 173–188.

Supplementary

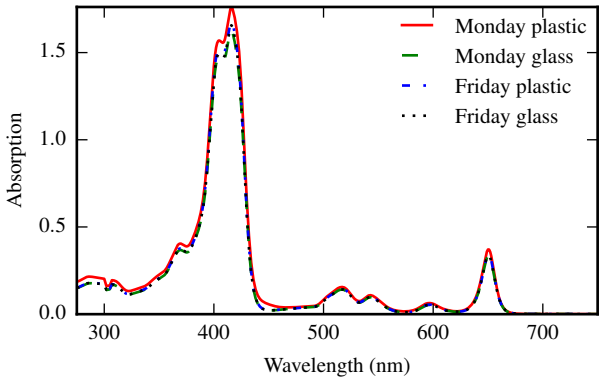

Fig. 1 Absorption spectra of $TPCS_{2a}$ in methanol (150 µg ml^{-1}) recorded immediately after preparation and after 4 days. The solutions were contained in plastic or glass tubes.

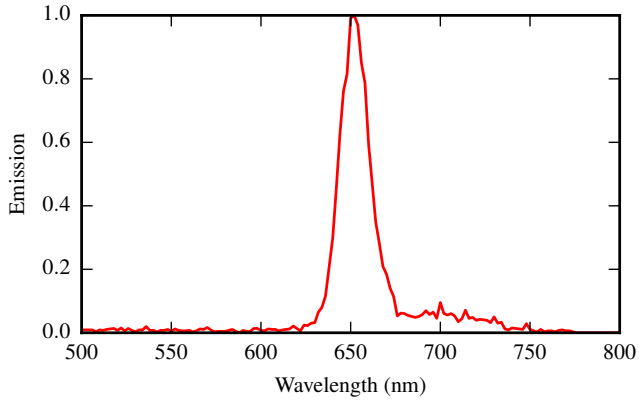

Fig. 2 Emission spectrum of $TPCS_{2a}$ in methanol for excitation at 403 nm.

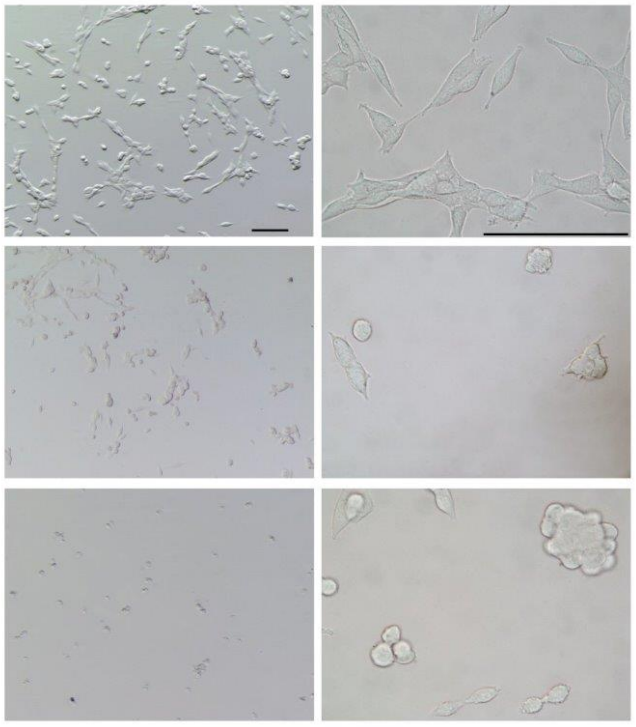

Fig. 3 Light microscopy images of glioma F98 cells after $TPCS_{2a}$ incubation (0.2 µg ml^{-1}, 18 h) and different light exposure (Quanta System laser (652 nm, 50 mW cm^{-2}). Enlargement 40 times (left column) and 60 times (right column). Upper panels: Control cells (no treatment). Middle panels: cells at LD_{50} dose (50 s of illumination). Lower panels: LD_{100} dose (120 s of illumination). The viability was quantified using the MTT assay as described in Material and Methods (Fig. 2). Scale bars in top panels are 0.1 mm.

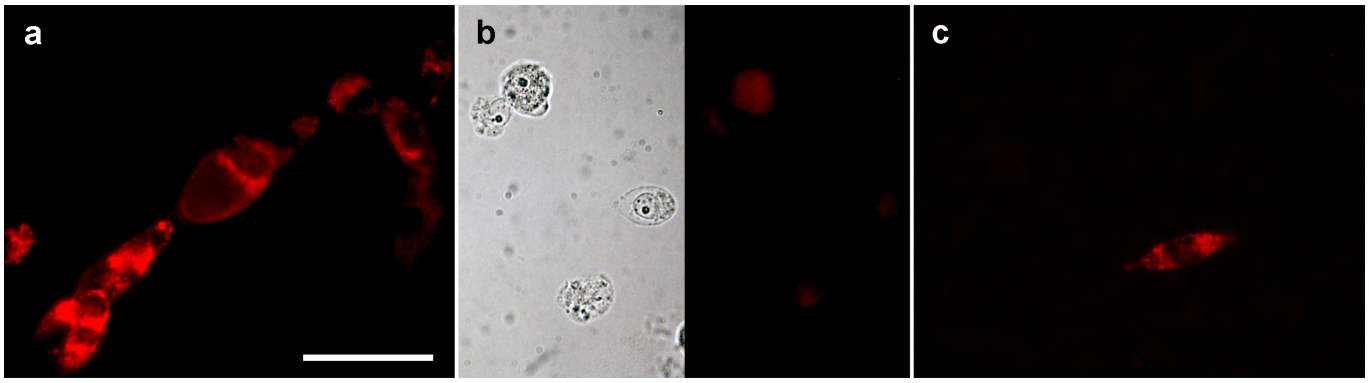

Fig. 4 Subcellular localization of $TPCS_{2a}$ in F98 glioma cells after $TPCS_{2a}$ incubation (0.4 μg ml^{-1}, 18 h) in a) and after PDT (180 s, 652 nm, 50 mW cm^{-2}) in b) and after BLM-PCI (160 μM BLM, 4 h) in c). The cells were examined on an inverted microscope (Nikon Eclipse TE2000-S) 1.5 h after illumination as described in Material and Method, section 2, in the main manuscript. No auto fluorescence was detected neither in control, nor treated cells, as shown in Supplementary Fig. 5a). Scale bar is 50 μm

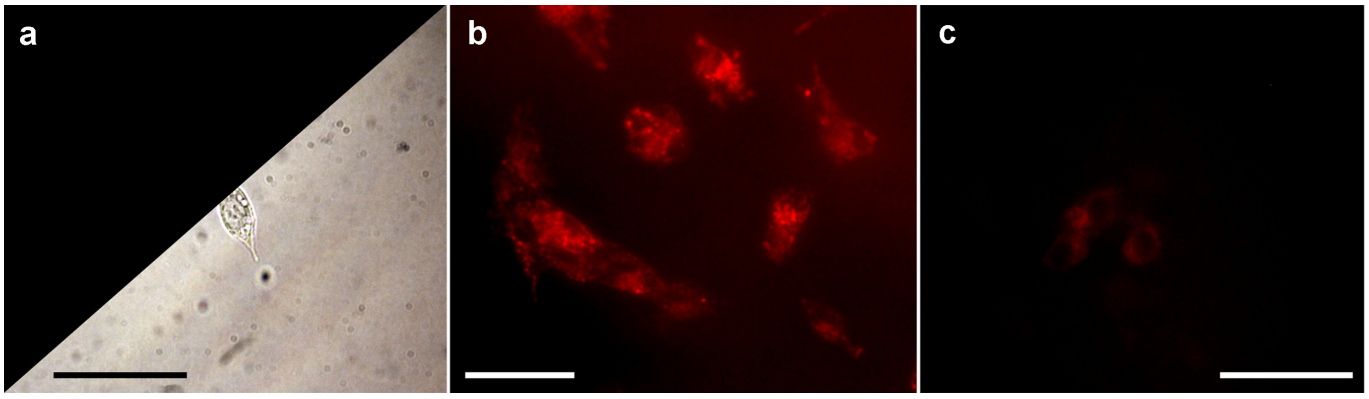

Fig. 5 F98 glioma cells presented without any treatment shown by fluorescence and light microscopy detecting no auto fluorescence. $TPCS_{2a}$ treated cells (0.4 μg ml^{-1}, 18 h) prior to TMZ incubation (500 μM, 4 h) and without light were presented in b) and in combination with red light treatment (180 s, 652 nm, 50 mW cm^{-2}) in c). The cells were examined on an inverted microscope (Nikon Eclipse TE2000-S) 1.5 h after illumination as described in Material and Method, section 2, in the main manuscript. Scale bars are 50 μm.

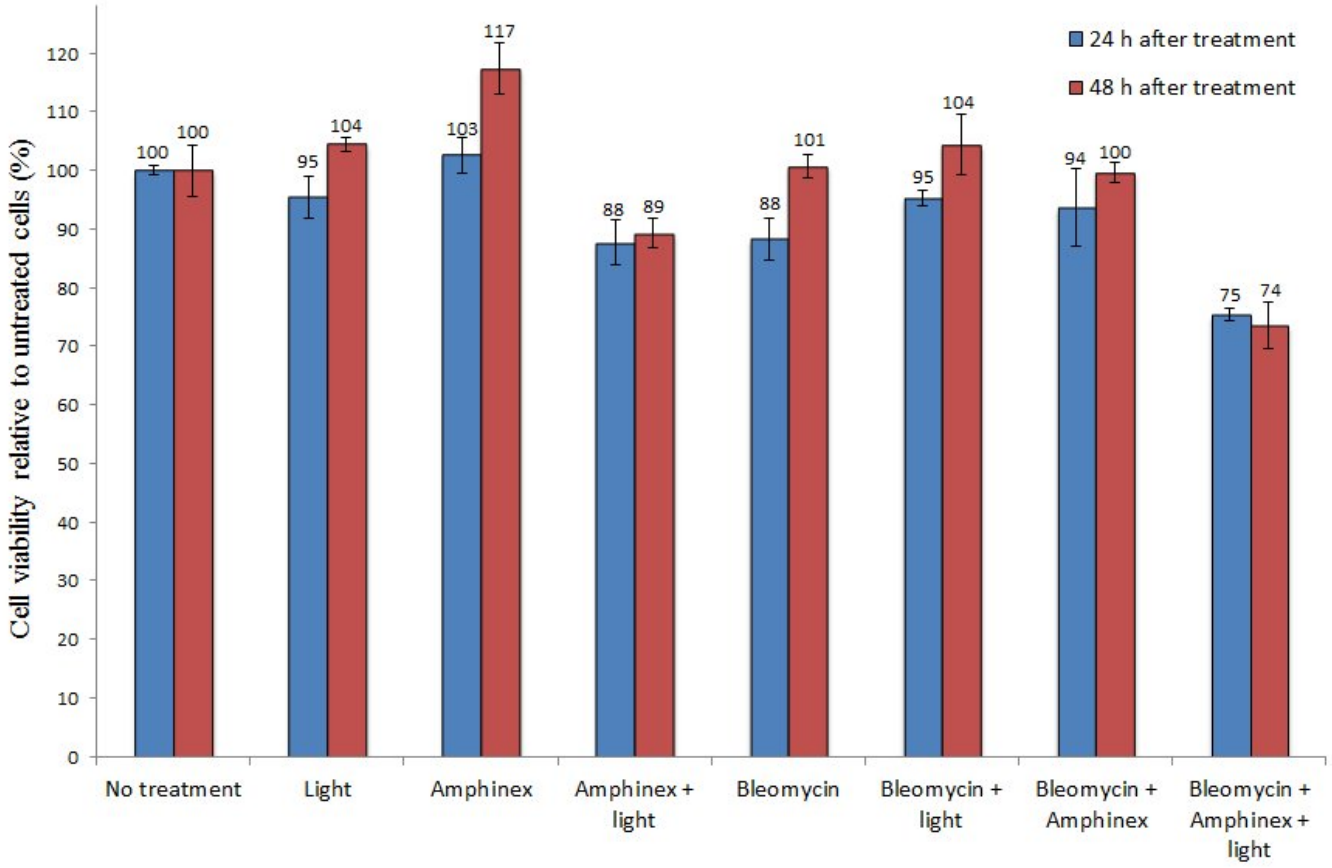

Fig. 6 Viability of rat glioma cells (F98) exposed to different combinations of light from a Quanta System laser diode (40 s, 652 nm, 50 mW cm^{-2}), Amphinex ($TPCS_{2a}$) 0.2 μg ml^{-1}, 18 h and BLM (0.5 μM,). Cell viability was measured by using MTT 24 h (blue bars) or 48 h (red bars) post treatment. Below each pair of bars, the relevant treatment combination is displayed. Error bars are based on two parallel runs.

Graphical abstract

Phot-Chemical Internalization and Illumination together with chemotherapeutics gives synergetic cell death in F98 glioma cell model.