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Photodynamic Efficacy of Cercosporin in 3D Tumor Cell Cultures†

Mantas Grigalavicius1, Maria Mastrangelopoulou1, Delmon Arous1,2, Asta Juzeniene1, Mathilde Ménard3, Ellen Skarpen4, Kristian Berg1 and Theodossis A. Theodossiou1*†

1Department of Radiation Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway
2Department of Physics, University of Oslo, Oslo, Norway
3Institut Charles Gerhardt Montpellier, UMR-5253 CNRS-UM-ENSCM cc 1701, Montpellier cedex 05, France
4Department of Core Facilities, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway

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ABSTRACT

In the present work, we study the photodynamic action of cercosporin (cerco), a naturally occurring photosensitizer, on human cancer multicellular spheroids. U87 spheroids exhibit double the uptake of cerco than T47D and T98G spheroids as shown by flow cytometry on the single cell level. Moreover, cerco is efficiently internalized by cells throughout the spheroid as shown by confocal microscopy, for all three cell lines. Despite their higher cerco uptake, U87 spheroids show the least vulnerability to cerco-PDT, in contrast to the other two cell lines (T47D and T98G). While 300 μm diameter spheroids consistently shrink and become necrotic after cerco PDT, bigger spheroids (>500 μm) start to regrow following blue-light PDT and exhibit high viability. Cerco-PDT was found to be effective on bigger spheroids reaching 1mm in diameter especially under longer exposure to yellow light (~590 nm). In terms of metabolism, T47D and T98G undergo a complete bioenergetic collapse (respiration and glycolysis) as a result of cerco-PDT. U87 spheroids also experienced a respiratory collapse following cerco-PDT, but retained half their glycolytic activity.

INTRODUCTION

Photodynamic therapy of cancer (PDT) (1) is cancer treatment modality which utilizes a photosensitizing drug (photonsensitizer, PS), light of the appropriate wavelength ensuring PS activation, and interstitial molecular oxygen. The light-activated sensitizer reacts with the surrounding oxygen, generating toxic reactive oxygen species (ROS) and prominently singlet oxygen. In vivo, PDT can destroy cancer through a combination of (1) cell phototoxicity, (2) vascular shut down leading to tumor starvation and (3) immunogenicity triggered by the primary treatment-induced inflammation. In in vitro PDT studies, however, only the photocytotoxic action is evaluated, except if a specialized model has been designed. The main restraint of PDT is the limited depth of light penetration into tissue. Light in the red end of the visible spectrum can penetrate a few millimeters (~2 mm) into tissue while in the blue end the corresponding penetration depth is a couple of hundred micrometers (~300 μm at ~450 nm, (1)). In that context, an ideal clinical PDT photosensitizer would need to exhibit strong absorbance at or longer than 650 nm (deep red).

Cercosporin (cerco) is a naturally derived perylenequinone PS, like hypericin and the hypocrellins. It is produced by the Cercospora family of plant pathogens, however, its total synthesis in the lab has also been reported (2). Cerco has not been extensively studied as an anticancer photosensitising agent due to its short wavelength of activation (peak at ~470 nm). Nevertheless, it has been studied for its photodynamic activity within the remit of phytopathology (3) as it is a very powerful phototoxin for plants and crops. In that context, cerco has been reported to have a high singlet oxygen quantum yield, ranging from 0.84 in D2O to 0.97 in dioxane (4).

In our recent work on 2D cell cultures (5), we evaluated the capacity of cerco as a photosensitizer on three mammalian cancer cell lines, namely MCF7 (human breast adenocarcinoma), T98G and U87 (both human glioblastoma multiforme). We found that cerco is a very potent PS, efficiently killing all the cell lines investigated at different light doses applied. The cell killing essentially followed the PS loading patterns: U87 that presented half the cerco uptake of the other two cell lines, also demonstrated the lowest sensitivity to cerco-PDT, as compared to the other two cell lines in the study. In the same work, we also investigated the metabolic profiles of the three cell lines before and after treatment with cerco-PDT. We found that PDT treatment collapsed the metabolism in MCF7 and U87 cells while T98G cells, despite of their respiratory activity also dropping to background levels, retained about 1/3 of their glycolytic activity following cerco-PDT.

In the present study, we evaluated the effect of cerco-PDT in 3D (spheroid) cultures of three mammalian cancer cell lines, namely T47D (human breast adenocarcinoma), U87 and T98G (both human glioblastoma multiforme). It is well known that multicellular spheroids provide a system of intermediate complexity between solid tumors in vivo and cell monolayers in...
culture (6), as they model the 3D–cell interactions and also the 3D structural barriers to cell nutrients, substrates and oxygen. In that context, the rationale and motivation for conducting the present study was to compare our recent 2D cell culture results with the corresponding cerco-PDT effects in 3D cultures.

MATERIALS AND METHODS

Chemicals and reagents. Agarose, cercosporin from Cercospora hawaii (cerco), antimony A, glycogenol (Oligo), carboxyl cyanide 4-(trifluoromethoxy)sphenyldihydrazone (FCCP), mycothiazol (Myxo), RPMI 1640 without phenol red, l-glutamine, penicillin/streptomycin, trypsin, poly-γ-l-lysine hydrobromide, fluorescein diacetate (FDA) and Dulbecco’s phosphate-buffered saline (PBS) with or without calcium chloride and magnesium chloride were purchased from Sigma-Aldrich Norway AS (Oslo, Norway). Corning™ Cell-Tak cell and tissue adhesive, Corning™ Matrigel® Matrix, and Corning™ 96-well clear ultra low attachment microplates were purchased from Fisher Scientific (Göteborg, Sweden). TrypLE™ Express Enzyme without phenol red, propidium iodide (PI), LIVE/DEAD™ cell stain kit and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Oslo, Norway). All consumables used for Seahorse XF96 metabolic analysis were purchased from Agilent Technologies (Santa Clara, CA).

Generation of spheroids. One human breast cancer (T47D) and two human brain cancer (T98G and U87) cell lines were employed to initiate the spheroid formation in 2D culture. The cells were maintained in RPMI 1640 without phenol red, supplemented with 10% FBS, 100 U mL^-1 penicillin/100 μg mL^-1 streptomycin and 2 mM l-glutamine at 37°C in a 5% CO2 humidified atmosphere. The spheroids were generated in agarose-coated flat bottom or ultra low attachment U-shaped 96-well microplates. The agarose was boiled in PBS for 10 min under continuous stirring prior dispensing into 96-well microplates and allowed to cool down for at least 1 h before setting the cells. The flat bottom microplates were coated with 1.5% (wt/vol) agarose and following the transfer of 100 μL cell suspension into each well they were centrifuged at 800 g for 20 min. The initial cell number required to create spheroids of similar size at day 4 after seeding varied from 500 to 5000.

PDT experiments. Cercosporin was dissolved in 100% DMSO to yield a 4 mM stock solution and then further diluted in RPMI complete medium as required for each experiment. Spheroids were incubated with cercosporin (6 μM) in complete medium for 4 h and washed prior to irradiation with phosphate buffer saline (PBS) containing calcium and magnesium. The blue light irradiations were performed in PBS from the top of 96-well plates using a Lumisource B3 yellow lamp peaked at approximately 450 and 590 nm, respectively (Norway) with four OSRAM L 18/67 M cercosporin lamps (ICL, Natick, MA). The L channel in the La*b* space of each RGB image was enhanced by performing contrast-limited adaptive histogram equalization (CLAHE) before being converted into grayscale images by eliminating the hue and saturation information while retaining the luminance. Utilizing the imbinizer Matlab function, grayscale images were then binarized to appropriately mask the whole spheroids by iteratively sweeping through different threshold sensitivities. Locally adaptive thresholding, based on mean intensity in the neighborhood of each pixel (Bradley’s method (7)), was necessary as nonuniform background illumination varied across the images due to spheroid spatial displacement over time. Of the spheroids that the Matlab program was inadequately able to automatically delineate, polygon structures were manually outlined and measured on the raw spheroid images using the Zeiss microscopy software Axiovision release 4.8.2.

Confocal and epifluorescence microscopy. The spheroids were examined with a Zeiss LSM 880 Airyscan microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany), equipped with an Ar-Laser Multiline (458/488/514 nm), a DPSS-561 10 (561 nm), a Laser diode 405-30 CW (405 nm) and a HeNe laser (633 nm). The objectives used were the Zeiss plan-Apochromat 20× NA=0.8 air, the 25× NA=0.8 oil DICII or the 10× NA=1.4 air DICII for a larger field of view. Image acquisition, processing and visualization were performed with the ZEN 2.3 SPI basic software (Carl Zeiss). The spheroids were either fixed with 2% formaldehyde and subsequently processed for scanning between a thin glass plate and cover slip or transferred to a cloning ring mounted on a MatTek glass bottom dish without fixation. In both cases, spheroids were immobilized using a Matrigel® Matrix/media mixture (50:50, vol/vol).

In case of epifluorescence microscopy, green- and red-filtered images (live/dead or FDA/PI assay) were obtained by using an inverted microscope Axiosvert 200M (Zeiss, Germany) in reflection mode while the images without optical filtering were acquired in transmission mode (achroplan objective with magnification/numeric aperture 4×/0.1, resolution 2560 × 1920, spatial resolution 1.208 pixels μm^-1, bit depth 24, xenon lamp voltage 2.5–10.0 V). Camera exposure times were the same for PDT and control (CTRL) groups for the same spheroid (green FDA channel 50–120 ms, red PI channel 50–900 ms, nonfluorescent imaging channel 750–1000 μs). The spheroids were incubated with FDA (green, 8 μg mL^-1) and PI (red, 20 μg mL^-1) for 5 min and then washed with PBS containing Ca and Mg just before the imaging.

Spheroid image analysis. The spheroid areas were automatically delineated and quantified with in-house made software developed in Matlab (Mathworks, Natick, Massachusetts) and in-house developed Java software (OpenOdds, The Netherlands). In order to simulate the absorbance-fluorescence profile of cercosporin in spheroids, 40 000–60 000 cells were inoculated into 96-well plates 24 h prior to 4 μM cerco incubation for 4 h. Cells were subsequently washed twice with PBS and scanned for their 2D excitation-emission (20 nm bandwidth) in the Tecan spectrUM M10 plate reader. In order to determine the loading of spheroids with cerco, twenty to thirty spheroids were incubated with 6 μM cercosporin in complete RPMI media for 4 h before transferring them from agarose-coated microplates. Dissociation of these 3D cell structures into single cell suspensions was performed in 1 mL of TrypLE™ Express Enzyme after a single wash in PBS. The resulting cell suspensions were washed again with PBS and strained through a 35 μm mesh screen before the flow cytometry experiments. The flow cytometric analyses were performed using a LSRII (BD Biosciences, Franklin Lakes, NJ) and at least of 10 000 events for each sample. Cercosporin fluorescence was excited at 488 nm and registered after a bandpass filter at 670±14 nm. Flow cytometry data were analyzed using the FlowJo v.7.6.1i software (Treestar Inc., Ashland, OR).

RESULTS

Spectral response and relative quantity of loaded cercosporin. Initially, we incubated the cells with 4 μM cerco for 4 h. At the end of the incubation, the cells were scanned from the plate underside to produce a 2D excitation vs emission spectra. A representative pseudocolor contour map is shown in Fig. 1b for...
dose of 0.9 J cm$^{-2}$ uptake by T98G, T47D and U87 spheroids after incubating them with 6 micromolar cercosporin for 4 h. Then, the spheroids were disrupted and the absorbance of cell-internalized cercosporin was measured using flow cytometry. The control spheroids consisted in their vast majority of live cells (green for live) and the PDT-treated spheroids were incubated with 6 micromolar cercosporin for 4 h. Then, the spheroids were imaged in two ways as explained in materials and methods section. Representative micrographs are shown in Fig. 2. In order to evaluate the cercosporin uptake of the spheroids, we incubated them for 4 h with 6 micromolar cercosporin and then imaged the spheroids into single cell suspensions using TrypLE$^\text{TM}$ and disrupted the spheroids by squeezing between the slide and coverslip to reduce the spheroid z-dimensions so that the uniformity of cercosporin fluorescence could be evaluated without losing light due to spheroid size. In this case, as can be seen in the micrographs, the light could not penetrate through the whole spheroid height so the spheroid dimensions can be deduced by assuming symmetry in the orthogonal projections.

In a second set of PDT experiments, cell spheroids were irradiated at three different sizes in case of T47D (0.27 to 0.81 mm$^2$) and one size in case of U87 (0.47 mm$^2$), while the PDT-treated spheroids almost exclusively consisted of dead cells (red), showing no sign of regrowth. The growth curves (Fig. 3a) confirm that the PDT-treated T47D spheroids exhibited sustained cell inhibition for all seeding densities (0.049 to 0.096 mm$^2$ for 500 cells, 0.074 to 0.096 mm$^2$ for 1000 cells and 0.104 to 0.096 mm$^2$ for 2000 cells), while the control spheroids exhibited continuous growth (0.050 ± 0.003 to 0.456 ± 0.024 mm$^2$ for 500 cells, 0.072 ± 0.003 to 0.534 ± 0.033 mm$^2$ for 1000 cells and 0.100 ± 0.005 to 0.642 ± 0.017 mm$^2$ for 2000 cells).

In a second set of PDT experiments, cell spheroids were irradiated at three different sizes in case of T47D (0.27 ± 0.02, 0.57 ± 0.02 and 0.81 ± 0.04 mm$^2$) and one size in case of U87 (0.47 ± 0.03 mm$^2$). The growth of both control (irradiated in the absence of cercosporin) and cercosporin-PDT treated spheroids was followed in time through measurement of their cross-sectional area. Representative images at selected time points are shown for T47D and U87 spheroids in Fig. 4a and b correspondingly while

**Study of spheroid uptake of cercosporin by confocal microscopy**

All spheroids were incubated with 4 micromolar cercosporin for 4 h. Then, the spheroids were imaged in two ways as explained in materials and methods section. Representative micrographs are shown in Fig. 2. In the top row, the spheroids were squeezed between the slide and coverslip to reduce the spheroid z-dimensions so that the uniformity of cercosporin fluorescence could be evaluated without losing light due to spheroid size. On top and right side of each micrograph, the representative orthogonal projections are shown. The confocal slice selected in the each main micrograph is represented as a blue line in the orthogonal projections. From these micrographs, it can be seen that in all spheroids (U87, T47D and T98G) the fluorescence is uniform throughout the x- or y-dimensions, even though the U87 spheroids were bigger.

The bottom row micrographs were obtained without any z squeezing of the spheroids and thus depict the actual z dimensions of the spheroids. In this case, as can be seen in the micrographs, the light could not penetrate through the whole spheroid height so the spheroid dimensions can be deduced by assuming symmetry in the orthogonal projections.

Once more as can be seen in the representative confocal slices, the fluorescence is uniform throughout the confocal slice area (even perhaps slightly more intense in the spheroid core), indicating that cercosporin was taken up well even ~150 mm deep in the spheroids.

**Spheroid responses to PDT treatment**

PDT-treated spheroids were incubated with 6 micromolar cercosporin for 4 h and then irradiated either with 1.3 J cm$^{-2}$ of blue light or 1.6 J cm$^{-2}$ of yellow light.

In a first set of PDT experiments, T47D spheroids were grown from different cell inoculation numbers (500, 1000 and 2000) to different sizes (see below) and subjected to cercosporin-PDT. On day 17 from irradiation, fluorescence images were obtained with use of two dyes, PI (red for dead) and FDA (green for live). From those endpoint images, it can be seen that there is a profound difference in spheroid area between the two groups. Furthermore, the control spheroids consisted in their vast majority of live cells (green) while the PDT-treated spheroids almost exclusively consisted of dead cells (red), showing no sign of regrowth. The growth curves (Fig. 3a) confirm that the PDT-treated T47D spheroids exhibited sustained cell inhibition for all seeding densities (0.049 ± 0.003 to 0.043 ± 0.002 mm$^2$ for 500 cells, 0.074 ± 0.005 to 0.067 ± 0.005 mm$^2$ for 1000 cells and 0.104 ± 0.004 to 0.096 ± 0.009 mm$^2$ for 2000 cells), while the control spheroids exhibited continuous growth (0.050 ± 0.003 to 0.456 ± 0.024 mm$^2$ for 500 cells, 0.072 ± 0.003 to 0.534 ± 0.033 mm$^2$ for 1000 cells and 0.100 ± 0.005 to 0.642 ± 0.017 mm$^2$ for 2000 cells).

In a second set of PDT experiments, cell spheroids were irradiated at three different sizes in case of T47D (0.27 ± 0.02, 0.57 ± 0.02 and 0.81 ± 0.04 mm$^2$) and one size in case of U87 (0.47 ± 0.03 mm$^2$). The growth of both control (irradiated in the absence of cercosporin) and cercosporin-PDT treated spheroids was followed in time through measurement of their cross-sectional area. Representative images at selected time points are shown for T47D and U87 spheroids in Fig. 4a and b correspondingly while
From Fig. 4c, it can be seen that in the case of T47D there is a growth inhibition following PDT, where spheroid area dropped from $0.27 \pm 0.02$ to $0.21 \pm 0.01 \text{ mm}^2$ in eight days. Conversely, the nontreated T47D spheroids expanded in size from $0.22 \pm 0.02$ to $0.43 \pm 0.02 \text{ mm}^2$. The results were quite different for U87 cells: While cerco PDT conferred a temporary growth inhibition until day 3 after the PDT treatment from $0.47 \pm 0.03$ to $0.32 \pm 0.02 \text{ mm}^2$, the treated cells eventually caught up with the non treated cells which exhibited a slower (nonlinear) growth unlike the T47D ($0.42 \pm 0.05$ to $0.57 \pm 0.05 \text{ mm}^2$).

Having observed that PDT-treated, larger U87 spheroids caught up in area with their control counterparts, we also followed bigger T47D spheroids of $0.81 \pm 0.04$ and $0.57 \pm 0.02 \text{ mm}^2$ area after irradiating them with blue (Fig. 4d) and yellow (Fig. 4e) light correspondingly to see if they also exhibit a similar delayed trend. The growth curves from bigger spheroids in Fig. 4f show that the application of blue light on cerco-incubated spheroids resulted only in a weak PDT response when compared to their control group ($0.57 \pm 0.02$ to $0.73 \pm 0.06 \text{ mm}^2$ vs $0.50 \pm 0.02$ to $0.82 \pm 0.03 \text{ mm}^2$). The irradiation with yellow light, however, caused a significant growth inhibition from $0.81 \pm 0.04$ to $0.73 \pm 0.04 \text{ mm}^2$ in 9 days while the control spheroids exhibited continuous growth ($0.82 \pm 0.03$ to $1.25 \pm 0.04 \text{ mm}^2$).

We subsequently assessed the live-dead fractions of the T47D and U87 spheroids as the follow-up endpoint. The results can be seen in Fig. 5: In the first panel (a) the T47D spheroids with/without PDT treatment at day 8 are shown. It can be seen that apart from their profound difference in area, the control spheroids are in the vast majority consisted of live cells (green) while the PDT-treated spheroids are almost exclusively consisted of dead cells (red). The corresponding micrographs for U87 cells corroborate the observations in Fig. 4b. The end sizes of control and PDT-treated spheroids are comparable. Additionally, while the treated spheroids contain a significantly larger fraction of dead cells (red) they also contain a large fraction of viable cells. What is interesting in this case (PDT-treated spheroids) is that following PDT treatment, viable U87 cell groups detach from the main spheroid body and eventually start forming new spheroids (Fig. 4b, day 8, and Fig. 5b, white circles). Figure 5c represents yellow light-irradiated spheroids after 9 days of cerco-PDT treatment (initial size in diameter before treatment was $1010 \pm 26 \mu\text{m}$). Here, the treated spheroids experienced an observable shrinkage and only a small fraction of viable cells was observed in comparison with either the yellow-light control group or blue light-irradiated spheroids (Fig. 5d). Blue light irradiation had a marginal effect and induced only a delay in spheroid regrowth over the first week (Fig. 4f) that was followed by an increase in viable cells in the periphery of the spheroids in the next two weeks (Fig. 5d, irradiated at initial size of $802 \pm 12 \mu\text{m}$).
Metabolic studies in spheroids

All spheroids were treated with 6 µM cerco and incubated for 4 h, before being subjected to PDT. One hour following the PDT treatment, control and treated spheroids were evaluated by the seahorse XFe96 analyzer for their oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) representing their respiration and glycolytic profiles correspondingly. The OCR trends in non-PDT-treated spheroids are presented in Fig. 6a. It can be seen that T47D spheroids exhibited higher basal OCR (~40 pmol min⁻¹/C0¹) than the other cell lines with U87 second (~30 pmol min⁻¹/C0¹) and T98G presenting much lower respiratory activity (~10 pmol min⁻¹/C0¹). Nevertheless, when the respective spheroid volumes were taken into account (T47D: U87:T98G ~ 3.6:2.9:1), the three cell lines exhibited comparable OCR (the normalized OCR profiles are shown in Fig. 6b).

It should be noted that the data in Fig. 6b reveal that T47D displayed the smallest reduction in oxygen consumption upon application of Oligo, while T98G spheroids exhibited the highest respiratory capacity among the three cell lines, when FCCP was added. In Fig. 6a, it can be seen that the respiration of all three spheroid types was profoundly subdued as early as one hour following PDT and was not responsive to any of the modulator stimuli (Oligo, FCCP, Myxo).

The glycolytic profiles of the three spheroid types can be seen in Fig. 6c and again in Fig. 6d following volume correction. It is evident from non-PDT-treated spheroids (Fig. 6d) that U87 exhibited the highest glycolytic activity (~23 mpH min⁻¹) with T98G a close second (~20 mpH min⁻¹) and T47D exhibiting the lowest basal glycolysis at ~13 mpH min⁻¹. All glycolytic profiles were responsive to the addition of Oligo, blocking ATP production from respiration. The application of PDT also abolished the glycolytic function in both T47D and T98G, while in U87, even though there was a profound reduction in the glycolytic rate, it was found to be at about 40% the glycolytic rate of control cells. The metabolic phenotypes of the spheroids (OCR vs ECAR) are shown in Fig. 6e and also in Fig. 6f following corrections for spheroid volumes, providing an overview of the findings in Fig. 6a–d.

DISCUSSION

The present study on the effects of cerco PDT in 3D spheroid cultures is an extension/follow-up of our previous study (5) where we studied the efficacy of cercosporin as a photosensitizer in 2D cultures.

Even though cercosporin is a very potent photosensitizer as concluded from both the studies, it is not used in conventional
deviation (SD).

PDT, due to its short wavelength of activation (~470 nm, Fig. 1b) which very much limits the depth of light penetration into tissue in contrast to clinically used photosensitizers absorbing in the red end of the visible spectrum (mTHPC, PpIX, chlorine e6, etc.). Nevertheless, cercosporin possesses a high singlet oxygen quantum yield (from 0.84 in D2O to 0.97 in dioxane, (4)) and could be very valuable in cases where external light administration is not required for the activation of the photosensitizer. One such case is our recently published work (10), where accelerated protons were shown to activate photosensitizers. More specifically cercosporin was activated by proton irradiation in M059K and T98G glioblastoma multiforma cells achieving significant differential cytotoxicity in comparison to cells irradiated in the absence of cercosporin. Another example of an application where cercosporin could also be very useful, is chemi- or bioluminescence PDT (11) where the photosensitizer is excited directly by a chemi/bioluminescent molecule in close proximity within the cell.

Despite the fact that both our 2D cell study (5) and our present work in 3D spheroid cultures are in agreement that cercosporin is a quite potent photosensitizer, there are several discrepancies in the effect of cerco-PDT on cell monolayers and 3D cultures. In 2D cell cultures, we found that U87 cells were the least efficient in uploading cercosporin as compared to MCF7 breast adenocarcinoma and T98G GBM cell lines following incubation with 4 μM cercosporin for 4 h. In the case of the 3D spheroids, however, incubation under the same conditions led to U87 cells showing the most efficient loading in the single cell level, double than both the T98G and T47D cells. One possible explanation for that is that the size (volume) of the spheroids was not the same for all cell lines but as delineated above it followed a ratio of T98G: T47D:U87 ~ 1:2.9:3.6. The substantially (three-fold) larger U87 spheroids may have the ability to hold more dye in the interstitium between cells, providing the actual cells with a bigger reservoir for uptake. It can also be seen from Fig. 2 that U87 spheroids are not that tightly packed with cells as T47D or T98G spheroids, probably making the diffusion of cercosporin easier.

In contrast to the findings of the 2D study, blue light irradiation was not very effective in the bigger spheroid models at the regime used (6 μs cercosporin for 4 h, with 1.3 J cm⁻² blue light at 450 nm), while the smaller T47D were found to be vulnerable (growth inhibited and necrotic) to PDT in the same conditions. The small T47D spheroids remain in a compact spheroid form and continuously shrink with time into a necrotic core, while the bigger T47D spheroids are more or less resistant to the blue light treatment, even more than similar size U87 spheroids. This is in agreement with our previous 2D cerco-PDT study (5), the extent of vulnerability tends to correlate with the uptake of cerco as U87 spheroids experienced a stronger initial insult of cerco-PDT compared to T47D spheroids in the same conditions. Another notable trend of the U87 spheroids is that following cerco-PDT with blue light which is more effective in the surface, even at a lower effective dose than blue light (blue:yellow ~ 0.9 J cm⁻²:0.44 J cm⁻²). This clearly demonstrates the limitations of shorter wavelength (blue) light in penetrating tissue; in smaller spheroids, blue light can more or less activate the PS in the entire volume of the spheroid, but not in larger spheroids. This is also evident from the spheroid microscopy experiments: When the spheroids are imaged in Matrigel (no suppression of their z dimension), the bottom end of the spheroids in the z stack image is dark, especially for U87 which are much larger. Yellow light conversely, although much less absorbed by cercosporin, has a far more detrimental effect on cercosporin treated spheroids, even at a lower effective dose than blue light.

Another notable trend of the U87 spheroids is that following cerco-PDT with blue light which is more effective in the surface, they tend to shed cell clusters which then form new viable spheroids (Fig. 4b, white circles). This is a sign of motility upon insult (of PDT in this case) and could be associated with the documented ability of GBM cells to readily metastasize mainly
Figure 5. Spheroid endpoint images of short (a–c) and long (d) follow-ups. Camera exposure times were the same for PDT and control (CTRL) groups for the same spheroid (green FDA channel 50–150 ms, red PI channel 50–900 ms). The spheroids were incubated with FDA (green, 8 µg mL\(^{-1}\)) and PI (red, 20 µg mL\(^{-1}\)) for 5 min and then washed with PBS containing Ca and Mg just before the imaging. Scale bars = 100 µm.
within the brain forming new islets (12). U87 cells form spheroids easier than both T98G and T47D, due to their natural tendency even in 2D cultures to form clusters. The U87 spheroids however are quite loose than the more “tightly packed” T47D spheroids.

In all spheroid cases, the outer layer of the spheroids remains more viable as they grow and that appears as a ring in the microscopy micrographs. As the spheroid size grows, the inner cell layers suffer from the deprivation of nutrients and poor oxygen diffusion and gradually become necrotic. This is closer to the reality in tumors (6), that is why 3D cultures are much better tumor models than 2D cell monolayers. Nevertheless the real tumors are much more heterogeneous in their constitution (various malignant cells, auxiliary normal cells and extracellular matrix) than the spheroids which purely consist of only the tumor cell line.

The metabolic profile of the spheroids is also very different to that in 2D cell cultures (5). In cell monolayers, U87 cells exhibited much lower respiratory activity than T98G cells. When assayed in 3D formation, however, after volume corrections all three spheroids exhibited similar OCR. It must be noted that in the 2D studies, the cells were not corrected for size. Indeed, when adhered on the plates and stretched out T98G look much bigger than U87, however when their sizes were measured by a
Beckman-Coulter counter (Beckman-Coulter, Miami), these differences were not so profound (median volume: U87 ~ 3.3 × 10^3 µm^3, T47D ~ 3.3 × 10^3 µm^3 and T98G ~ 5.2 × 10^3 µm^3) and normalization would only reduce the U87/T98G OCR ratio from 1/3 to 1/2. This indicates that the spheroid respiration is regulated mainly by spheroid volume and not cell type. Similarly there were also discrepancies in the glycolytic rates between the 2D and 3D cultures. In cell monolayers, again T98G had a much higher glycolytic rate than U87 (~ three-fold) while in the 3D case the rates are comparable with U87 spheroids being marginally more glycolytic than T98G. Cerco-PDT collapsed the metabolism of both T98G and T47D, and also the respiration of U87, but not the glycolytic rate of the latter which continued at almost half capacity. This again is in disagreement with the 2D scenario where the cell lines were crashed metabolically (including U87) except for T98G which maintained their glycolytic rate at ~1/3. One more factor that contributes to the above observations is the much looser adhesion of U87 spheroids (vide infra), which may apparently allow both glycolysis substrates and oxygen easier access to the spheroid inner layers. As it has been shown in our previous work (5), cerco is subcellularly located in the endoplasmic reticulum/mitochondria. As a result, the PDT activation of cerco may confer considerable damage to the cellular respiration by damaging the electron transport chain (ETC) constituent complexes. In our previous work (13) using a similar naturally occurring perylenequinone PS, hypericin (HYP), we found that HYP-PDT damages irreversibly complex III of the ETC in DU145, human prostate carcinoma cells. In a more recent publication (14), we further saw that HYP-PDT caused a profound decrease of both the respiration and the glycolytic rate of MCF7 human breast adenocarcinoma cells, 1 h following irradiation. This is in good agreement with our 2D cerco findings (5), where cerco-PDT collapsed the respiration and profoundly diminished the glycolytic action in U87, T98G human glioblastoma multiforme and MCF7 human breast carcinoma cells.

From the above, it is obvious that the metabolic collapse of T47D cells 1 h following cerco-PDT is in good agreement with the concomitant growth inhibition/shrinkage of the T47D spheroids which gradually become necrotic. In contrast the U87 spheroids, although their respiration breaks down following cerco-PDT, apparently their quasi-functional glycolytic ability, could be one key factor that helped the spheroids retain their viability and their capacity to grow up to the size of the nontreated controls by day 8, despite their initial decrease in size up to day 3. This is also supported from the results of our previous work on cerco PDT (5) where U87 present with the lowest respiration of the 3 cell lines investigated (T98G, MCF7 and U87) yet their glycolytic activity is comparable to that of MCF7 indicating that they rely more on glycolysis for ATP production (Warburg effect) (15) and hence survival since ATP deficiency leads to bioenergetic collapse and cell death. (16,17).

Once again, in the present work, cerosporin was found to be a very potent photosensitizer. In the PDT field in general, the vast majority of the in vitro studies have been conducted in cell monolayers where cell killing, and cell-cell interactions where 3D structural considerations do not apply. It is therefore very important, especially for PDT applications where the light penetration into tissue is limited, to use 3D cultures which are structurally more similar to solid tumors. Even though the short activation wavelengths of cerco are limiting for classical PDT application in the clinic, it may prove very useful for emerging applications as for example proton-dynamic therapy (10).

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