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Subcellular localization and photodynamic activity of Photodithazine (glucosamine salt of chlorin e6) in murine melanoma B16-F10: an in vitro and in vivo study

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Subcellular localization and photodynamic activity of Photodithazine (glucosamine salt of chlorin e6) in murine melanoma B16-F10: an in vitro and in vivo study

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ABSTRACT

Photodynamic therapy (PDT) is already a good option for the clinical treatment of several lesions, including mainly non-melanoma skin cancers. However, cutaneous melanoma treatment remains a challenge when using PDT. One of the reasons for its reduced efficacy is the high pigmentation of melanoma cells. The object of our study is to evaluate the feasibility of the Photodithazine as a photosensitizer for melanoma. Photodithazine is already used in some malignant tumors with satisfactory results and has significant absorption band around 660 nm where the absorption of melanin is low. In this study, we measured the subcellular localization and photodynamic activity of Photodithazine (PDZ) in murine melanoma B16-F10 cell culture. Additionally, a PDT procedure was applied in an animal melanoma model. This first result demonstrates that Photodithazine is more localized at mitochondria in B16F10 cell culture and the cell viability is reduced to less than 90% using 1 μg/mL (PDZ) and 2 J/cm2. We also noticed a rapid PDZ (less than one hour) accumulation in a murine melanoma model. The treatment of melanoma resulted in 20 % more animal survival after one session of PDT compared with the control group. More studies are required to evaluate the cytotoxic effects of Photodithazine at human melanoma.

Keywords: subcellular localization; Photodithazine; B16F10; murine melanoma; confocal microscope; fluorescence-lifetime imaging microscopy

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1. INTRODUCTION

Photodynamic therapy (PDT) is an effective treatment for many diseases especially non-melanoma skin cancers [1–3]. Most of the treatments for these cancers and premalignant lesions may be overcome with the PDT established protocols, leading to better aesthetical results and costs reduction compared to surgical procedures. However, more efficiency is still necessary to treat other skin lesions such as melanoma, since the light penetration is limited in pigmented lesions and it is a very aggressive cancer [4].

Some strategies developed to increase treatment depth includes optical clearing [5] and application of photosensitizers (PSs) using microneedles [6]. Additionally, new chlorin- and porphyrin-based photosensitizers are tested in *in vitro* and *in vivo* studies using murine melanoma cell line (B16F10) [7–11]. However, most of these PSs are not yet approved for clinical applications.

One good candidate for melanoma treatment is the photosensitizer Photodithazine (PDZ), a glucosamine salt of chlorine (e6), which is already approved in some countries for clinical applicati*ons* [12–14]. It allows, compared to hematoporphyrin derivatives such as Photofrin and Photogem, an enhanced light transmittance through biological tissues due to a higher absorbance in a red-shifted wavelength, the absence of aggregation, fast clearance from an organism, and more appropriate photocytotoxicity [15,16].

PDZ is produced from *Spirulina platensis,* an N-methyl-D-glucosamine chlorine e6 derivative produced by VETA-GRAND Co. (Moscow, Russia). This modification on Ce6 is to stabilize and (make a hydrophilic compound) solubilize the compound in water [17]. The formula contains a mixture of chlorine e6 (Ce6) conjugated with di-N-metil-D-glucosamine (60%), Chlorine p6 e purpurins. [18]. Its maximum absorption is around 400 nm (Soret band) with low peaks at 505, 600 and 670 nm (Q-bands). This photosensitizer was used for clinical treatments of tumors in bronchi, esophagus, skin, and oral cavity. After the treatment, 53.7% of the patients presented a complete response, and 46.3% partial remission. [12]. Photodithazine is also used in many studies for microbial inactivation showing good results [19–22]. Besides the studies about PDZ, there is not much cellular information about its subcellular localization. [15]. Cells in a monolayer cell culture are more suitable to evaluate it, due to a more uniformly exposition to both photosensitizer and light when compared to PDT application on tissues, where light and photosensitizer distribution depend on their penetration in

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deeper layers. Because a higher density of these agents is needed to be used to reach these layers, the uniformity of monolayer cell culture allows studies of mechanisms that lead to more effectiveness of photodynamic therapy by observing the cell viability, biochemical processes based on fluorescence, and the localization of photosensitizer molecules on cellular organelles.

The photosensitizer molecules localization inside the cells can be detected by labeling their organelles so that fluorescence measurements can be used to monitor the exact position from both, and compare their locations. By determining the colocalization between the photosensitizer and particular cellular structures, it may be possible to foresee the death pathways induced by PDT. [23]. In this study, the cytoskeleton and mitochondria were labeled using fluorescence markers to investigate the photosensitizer distribution and quantify the *in vitro* and *in vivo* PDT effect in the murine melanoma models.

2. MATERIAL AND METHODS

2.1. Cell line and culture conditions

The B16F10 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). The cells were cultured in Dulbecco's Modified Eagle's Medium (Cutlab, Campinas, SP, Brazil) with 10% of fetal bovine serum (Cutlab, Campinas, SP, Brazil) and 0.1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, U.S.A.). They were maintained in a humidified atmosphere with 5% CO_2 and at 37ºC in an incubator (Sanyo, Tokyo, Japan). The cells were grown as monolayer overnight in flasks of 75 cm^2 with 20 mL of medium. When the culture confluence reached 70%, the medium was removed, and Trypsin with EDTA was added to detach the cells. The reaction of Trypsin was stopped by the addition of medium.

2.2. Subcellular localization

Two hundred thousand of B16F10 cells were seeded on a 35-diameter mm Petri dishes (IBIDI, Germany). Mitochondria were stained with 100 nM of MitoTrackerRed CM-H_2XROS (Molecular Probes, Thermo Fischer Scientific) for 45 min at 37 ºC. The cytoskeleton was first fixed with pre colled acetone -20 °C for 5 min and, then, stained with phalloidin conjugated with AlexaFluor (R) 532 (Molecular Probes, ThermoFischerScientific) for 20 min at room temperature. After that, the cells with each probe individually were incubated with 5 μg/mL Photodithazine for 1 hour. The samples were imaged by a confocal microscopy (LSM 780, Zeiss, Germany) at the excitation and emission wavelengths 405 (diode laser) 640-680 nm, 594 (HeNe Laser) 605-650 nm and 532(Ar Laser) 540-580 nm, respectively, for Photodithazine, AlexaFluor, and MitoTrackerRed. By using the JaCop plugin at ImageJ software, we calculated two parameters to evaluate the co-localization between Photodithazine and mitochondria or cytoskeleton: Pearson and Mander's coefficients.

2.3. Fluorescence-lifetime imaging microscopy

Fluorescence-lifetime imaging microscopy (FLIM) was performed using an inverted fluorescence confocal microscope from Zeiss (LSM 780, Zeiss, Germany) coupled with a FLIM system (Picoquant, Berlin, Germany). A 405 nm diode pulsed laser with 40 MHz of repetition rate was used for the PDZ excitation. The intensity and lifetime fluorescence signals were collected using a band-pass filter (650 ± 40) nm. The detected photons were counted by a time correlated single-photon counter, and the measurements were analyzed by using the Symphotime software (Picoquant, Berlin, Germany), which converts fluorescence lifetimes into a color scale and generates an image with false colors. Each fluorescence lifetime image was obtained by collecting 1000 counts at the peak value, and the image resolution was fixed at 512 × 512 pixels.

For the measurements on the confocal microscope, $2x10^5$ cells were plated on 35-diameter mm Petri dishes (IBIDI, Germany) containing DMEM medium with 10% of fetal bovine serum. After cellular adhesion, the cells were incubated with a 5 μg/mL Photodithazine solution for 1 hour in the dark at 37º C. We analyzed the fluorescence images before and after applying a light dose of 10 J/cm^2. PDT assay was performed with a home-made LED light source emitting at 660 nm. The FLIM images were reconstructed using an exponential fitting model (SymPhoTime software, PicoQuant GmbH), in which an average fluorescence lifetime of PDZ before and after PDT is displayed.

2.4. *In vitro* photodynamic therapy

Ten thousand B16F10 cells were seeded in 96-well plates (Corning, Corning, NY, U.S.A.) to attach overnight. Then they were washed twice with PBS and incubated for 1 hour in darkness with different concentrations of Photodithazine (0, 0.1, 0.5, 1, 5, 10, 20, 40, 60, 80 and 100 μg/mL) obtained from VETA-GRAND and diluted in DMEM medium (Cutlab, Campinas, SP, Brazil) without Fetal Serum Bovine. The cells were irradiated in DMEM medium without phenol with a

homogeneous system of LEDs of 20 mW/cm^2 at 660 nm. The exposure time was calculated to obtain 2, 4, 6, 8 and 10 J/cm^2 of light fluence. The cell viability was evaluated using MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, U.S.A.) diluted in DMEM (Cutlab, Campinas, SP, Brazil) without Fetal Bovine Serum. The B16F10 cells were incubated with MTT for 4 hours at final concentration per well of 0.5 mg/mL. The supernatant was removed and to dilute the formazan generated, DMSO was added. The absorbance at 570 nm and 690 nm was measured using a multi-well spectrophotometer (Multiskan™ GO Microplate, ThermoFisher, Waltham, MA, U.S.A.). The difference in MTT absorbance indicates the cell viability. Two controls were performed, one without photosensitizer and light and the other with photosensitizer only. The first control was considered with maximum cell viability.

2.5. Murine melanoma model

One billion B16F10 murine melanoma cells were intradermally injected in the flank of nude mice (University of São Paulo – Centro de Bioterismo). All animal procedures were approved by the Animal Committee of São Carlos Institute of Physics – Protocol: 03/2014. The treatment started when the tumors reached 5 mm in diameter.

2.6. *In vivo* photosensitizer kinetics

The photosensitizer in a concentration of 1.0 mg/kg was injected intravenously into the animals, and the kinetics in the tumor and the healthy skin was determined using laser-induced fluorescence spectroscopy (LIFS). It was used a diode laser coupled to a bifurcated fiber (Y-fiber) for excitation at 408 nm and the fluorescence collection at 660 nm. The optical fiber was placed on the tumor surface, and the PDZ fluorescence emission was collected by a USB 2000 Ocean Optics® spectrometer (Ocean Optics, U.S.A.).[24] To reduce any coupling issue between the fiber and the tissue, the fluorescence intensity of the photosensitizer (660 nm) was normalized by the skin fluorescence at 500 nm.

2.7. *In vivo* photodynamic therapy

For the PDT, the animals were anesthetized following the Animal Committee protocol, using isoflurane 5% for induction and 2% for maintenance. The photosensitizer was administered intravenously at a concentration of 1.0 mg/kg, and the light-drug interval was determined by the kinetics studies. The irradiation was performed using a diode laser emitting at 660 nm, light dose of 150 mW/cm^2 and fluence of 300 J/cm^2. After the treatment, the animals were kept in micro-isolator cages with access to food and water *ad libitum* and observed twice a day. The endpoints were weight loss greater than 15%, uncontrolled pain, ulcerated and/or infected tumor, and tumor size larger than 1.5 cm.

3. RESULTS

3.1. Subcellular localization

The cytoskeleton in blue was observed close to cell membrane and nucleus (Figure 1A). It interacts through connection gap junction, focal adhesion, desmosomes, and hemidesmosomes among cell-cell and cell-matrix interaction. The fluorescence image (Figure 1C) merged between cytoskeleton (Figure 1A - blue) and Photodithazine (Figure 1B -red) do not present magenta (blue + red) spots that indicate an observed co-localization. The Pearson's and Mander's coefficients were calculated to know whether the generation of ROS species could alter this structure (Table 1).

We obtained a Pearson's coefficient of (0.58 ± 0.01), which does not present a high linearly relationship as the two Mander's coefficients. The first coefficient M1 for the pair (cytoskeleton/Photodithazine) was (0.58 ± 0.01), and the M2 for the pair (Photodithazine/cytoskeleton) was (0.27 ± 0.01). The first one indicates there were more fluorescent pixels of the cytoskeleton in the same pixel of Photodithazine fluorescence than the second one. Also, M2 indicates there was a little Photodithazine fluorescence with the cytoskeleton. These results suggest the absence of co-localization between cytoskeleton and Photodithazine. Therefore, the damage to this cell structure should be minimum or none.

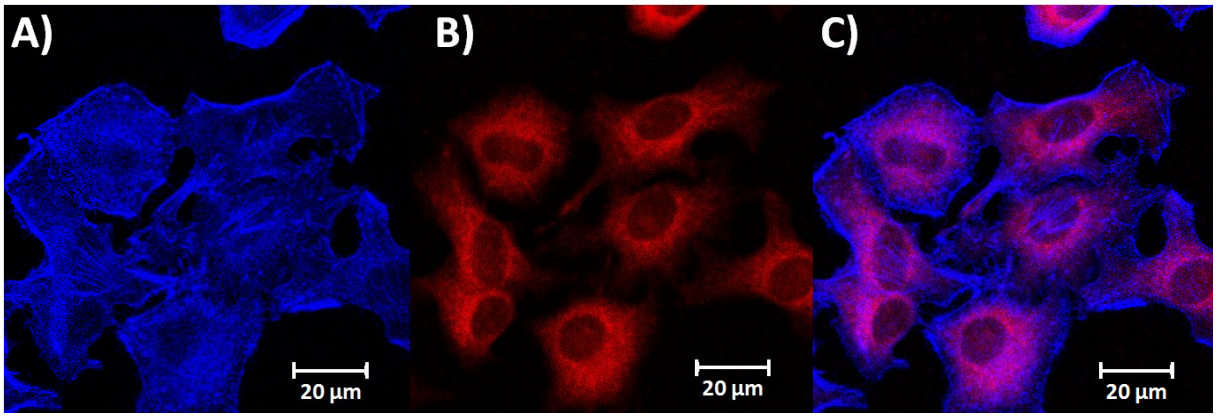

Figure 1 - Subcellular location of PDZ in Murine melanoma cells B16F10 at 5 µg/mL for 1 hour: A) cytoskeleton fluorescence; B) Photodithazine fluorescence; C) overlay of images of (A) and (B). Scaler bar: 20 µm.

The fluorescence of the mitochondrial probe (green) was observed throughout of cell's cytoplasm and in minor intensity inside the nucleus (Figure 2A), while photosensitizer's fluorescence was more intense in the nucleus surroundings (Figure 2B). The superposition of both fluorescence images (Figure 2C) is represented by yellow (green + red) spots indicating the existence of both mitochondrial and Photodithazine fluorophores, i.e., green and red pixels, respectively. To estimate the colocalization of these two fluorophores objectively, we used the ImageJ software with JaCop plugin to measure Mander's and Pearson coefficients.

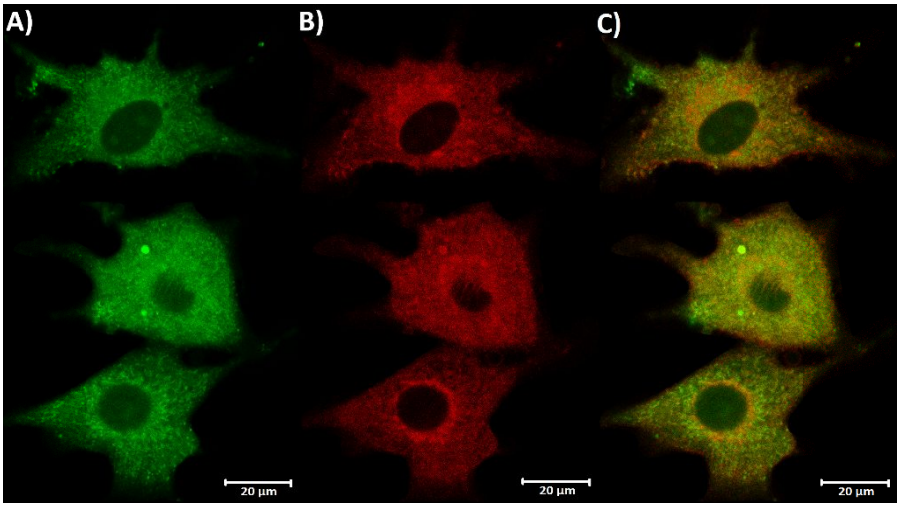

Figure 2 – Subcellular location of PDZ in Murine melanoma cells B16F10 at 5 µg/mL for 1 hour. A) mitochondria fluorescence; B) Photodithazine fluorescence; C) overlay of images of (A) and (B). Scaler bar: 20 µm.

We obtained a Pearson coefficient higher than 0.7 showing a robust linear relationship [25] between the two fluorescence intensities. This relationship is independent of the background intensity and indicates that mitochondria and Photodithazine are highly present in the same regions. Furthermore, the Mander's coefficient M2 is represented by the pair Photodithazine/mitotracker, and the value was above 0.7 for the two images, which suggests that Photoditazine is highly concentrated on mitochondria, while M1 indicates that many mitochondria do not have Photodithazine. (Table 1) These two coefficients together provide a mutual evidence of the colocalization between the photosensitizer and many mitochondria. As a consequence, when the photosensitizer is activated, it may damage the mitochondria so that the caspase enzymes are released into the cell's cytoplasm, activating the caspase cascade [26]. This process may lead to apoptosis. One study confirming this effect was observed by the detection of pro-apoptotic genes when PDT was applied with Photodithazine in rats.[27]. During PDT irradiation, the harmed mitochondria may also lose part of their dehydrogenases, weakening the detected signal at MTT assay.

Table 1 – Colocalization Mander's and Pearson coefficients

Colocalization: Photodithazine and cytoskeleton		
Pearson	Mander's M1	Mander's M2
(0.58±0.01)	(0.58±0.01)	(0.27±0.01)
Colocalization: Photodithazine and mitochondria		
Pearson	Mander's M1	Mander's M2
(0.73±0.01)	(0.53±0.01)	(0.77±0.01)

3.2. Fluorescence-lifetime imaging microscopy

To analyze the PDT effects in fluorescence-lifetime, each decay curve generated by the sum of photons of three cells was used to calculate their respective fluorescence lifetimes. Analyses of images before and after PDT show an almost stable average fluorescence lifetime (Figure 3), suggesting the microenvironment of PDZ does not change during PDT, and the efficiency of PDT remains the same until 10 J/cm^2 of light dose. During the measurements, the PDZ presents an average fluorescence lifetime of (5.1 ± 0.2) ns. Additionally, we observed a decrease in the fluorescence intensity, especially in the surroundings of the cell membrane, which may indicate that PDZ is consumed first in this region. The fluorescence intensity decreases probably due to photobleaching of Photodithazine, but the average lifetime inside the cell remains almost constant.

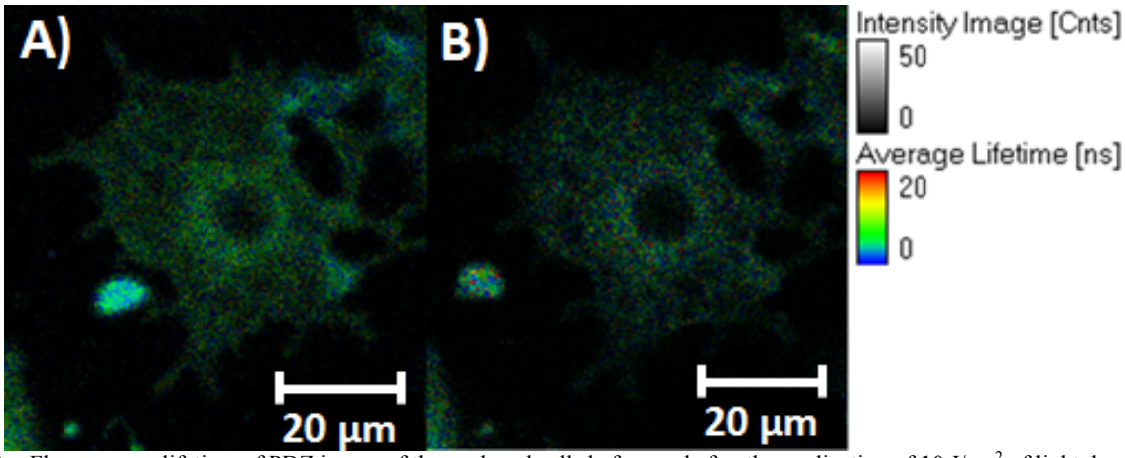

Figure 3 - Fluorescence lifetime of PDZ in one of the analyzed cells before and after the application of 10 J/cm^2 of light dose.

3.3. *In vitro* photodynamic therapy

The cell viability evaluated by the MTT colorimetric assay for the PS only control group did not show cytotoxicity for Photodithazine in concentrations between 0.1 and 100 mg/mL. (Figure 4). A significant reduction at around 90% of cell viability was obtained in the PDT group for concentrations above 1 µg/mL. For concentrations above 10 µg/mL, the PDT response reaches saturation in which a reduction of more than 90% of the cell viability was observed for each light dose. In this range, the control group without light presents a low toxicity as the concentration rise.

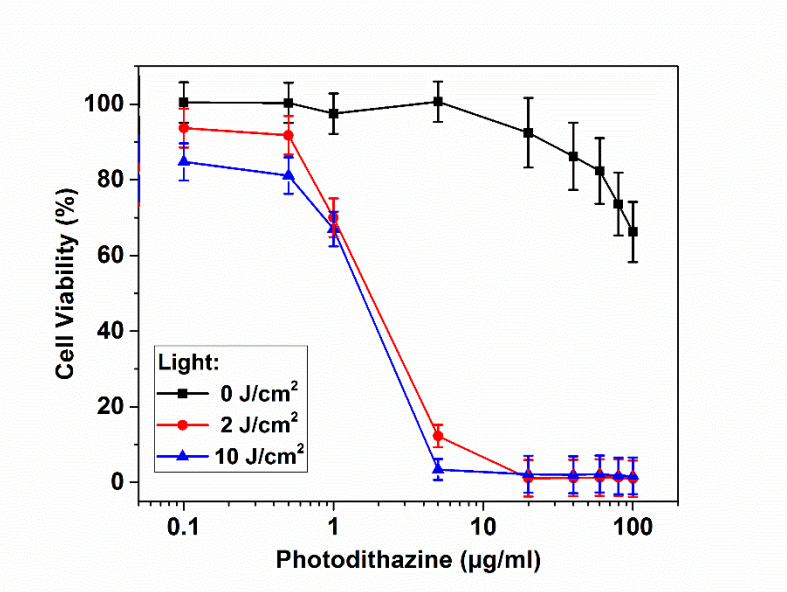

Figure 4 - Cell viability of B16F10 treated with PDT with different light fluences and Photodithazine concentrations analyzed through MTT assay.

3.4. *In vivo* photosensitizer kinetics

The photosensitizer kinetics measured with laser-inducer fluorescence spectroscopy can be seen in Figure 5. The LIFS assay showed that Photodithazine has a quick biodistribution and the peak for both, melanoma and normal skin was observed between 1 and 3 hours after the dye administration. Although there is a difference in the fluorescence intensity collected on the skin and the tumor, more studies are necessary to confirm the photosensitizer selectivity to melanoma. Photodithazine was eliminated around 24 hours after the injection. Based on these results, it was determined the light-drug interval of one hour for the PDT assays.

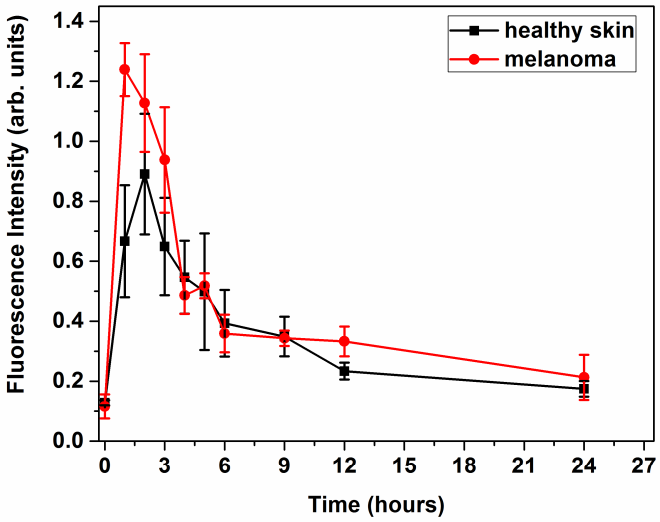

Figure 5 - Normalized fluorescence intensity of the photosensitizer at 660 nm in melanoma and healthy skin as a function of time. The highest peak is observed for both tissues between 1 and 3 hours after the photosensitizer administration.

3.5. *In vivo* photodynamic therapy

For the PDT assay, six animals were divided into two groups, control, and PDT group. After the treatment, the animals were observed twice a day, and the survival rate was determined (Figure 6). The day 0 in the survival assay is considered when tumors reached 5 mm in diameter, and the PDT group was treated. The animals from the control group survived between 10 and 14 days after that while the one-session PDT group survived between 10 and 20 days. The increase in the survival rate when compared to control and PDT groups was around 20%.

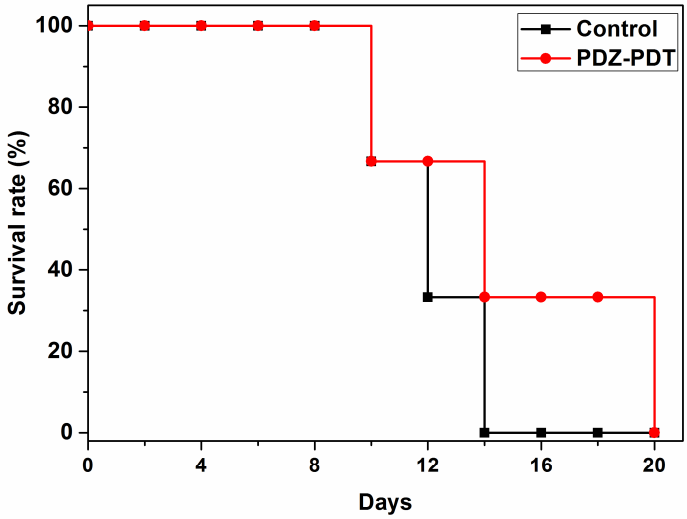

Figure 6 - Kaplan–Meier survival curves for B16F10 murine.

4. DISCUSSION

The photosensitizer uptake as its localization is related to the PDT biological effect. Most of the dyes are uptaken by the cells and located mainly in the cytoplasm or some organelles such as mitochondria, lysosomes and its membranes [28]. As the singlet oxygen produced during PDT has a short lifetime, the oxidation caused during PDT is limited to the areas where the photosensitizer is located, then the importance to investigate the dye localization in each cell line and to determine the biological effect of each drug [28].

In a previous study from Bachor et al. [29], the photosensitizer, Ce6, that has a similar structure of PDZ was observed bound to the mitochondria and, plasmatic and nuclear membranes in Human bladder carcinoma cells. No dye was observed inside nucleus or in the cytoplasm. However, Antonenko et al. observed the Ce6 in the cytoplasm in erythrocytes. [30].

A higher or lower presence of yellow spots in figure 2C may be subjective, since the visual analysis may lead to different points of view and the natural intensity variation also alter the results. Experiments performed by Luo et al. showed a high co-localization between chlorin e6 and mitochondria. On the other hand, when using the same pair of photosensitizer and organelle, other researchers visualized little or no co-localization at all. Dunn et al. [31] demonstrated that visual analysis might cause misinterpretations because a little variation in the color intensity of fluorophores may change the status of entirely co-localized to none co-localization at all.

Although many studies report co-localization between labeled molecules and cellular structures, most of them do not formally determine the appropriate coefficients. In a previous study, using Photodithazine as the photosensitizer, the researchers investigated the co-localization with mitochondria, lysosome, endoplasmatic reticulum and Golgi apparatus, and, by overlapping lysosomes and Photodithazine molecules in fluorescence images, the co-localization was considered more evident between Photodithazine and mitochondria. Also, they observed that fluorescence from Golgi apparatus, endoplasmatic reticulum, and Photodithazine was not co-localized [22]. However, this analysis may lead to mistakes concerning this co-localization once they were determined by observation. To optimize all parameters for PDT treatments using different cell lines, photosensitizer incubation times and different drugs, it is crucial to determine a standard method to quantify co-localization.

In this study, the co-localization was calculated by Mander's and Pearson coefficient, and it showed that Photodithazine is located mainly in the mitochondria of the melanoma cells.

The results indicate the mitochondria is one of the targets of this PDT application, and, consequently, a decrease of cell viability is already expected, since the MTT is converted inside these organelles. A reduction of over 90% in the cell viability was also a great result compared to other studies reported in the literature.

Wen et al. observed a cell viability decrease around 50% and 70% in the TC-1 cell induced with HPV-1 for the Photodithazine concentrations of 0.5 μg/mL and 0.75 μg/mL respectively, with 24 hours of light-drug interval. [22]. They also evaluated a rat leukemic cell line and reached the same decrease in the viability of 30% and 90% for the concentrations of 0.5 μg/mL and 1.0 μg/mL, respectively [32]. For the fluence of 6.25 J/cm^2 and Photodithazine concentration of 1 mg/mL, Wen et al. also, observed 90.6 ± 1.5% of the reduction in the A20 cellular viability [32]. When compared to B16F10 cells, the melanoma cells were more resistant to PDT, since 70 ± 8% of cell viability was presented after PDT under similar fluence and photosensitizer concentration (6 J/cm^2 of light dose and 1 mg/mL of Photodithazine concentration). The results above agree with melanotic melanoma cell line characteristics, as their proliferation reprogramming ability and apoptosis pathways. [33]

Diverse studies show that chlorin e6 (Ce6), a PS chemically similar to PDZ, also demonstrate a decrease in the cell viability up to 70% with concentrations above 2 μg/mL of Ce6 in cellular monolayers [34–37]. This result resembles our results to Photodithazine, which demonstrate a potential decrease in cell viability above 1 μg/mL concentration. Also, a decreased cell viability with concentration was observed when the PDT was applied on the uterus cell line SW480 with chlorin e6, which was similar to results from Li et al. of Photodithazine when using concentrations between 0.125 and 8 mg/mL and a light dose of 6 J/cm^2.

Since this effectiveness of PDT depends on many factors such as the photosensitizer concentration and fluence, several studies reported the effects when varying them. These factors could alter the cell death pathway as it was showed by Acedo et al. [38], who observed the dependence of apoptosis and necrosis for several light doses.

Chen and collaborators investigated the effect of PDT with methylene blue in murine melanoma model and noted that the treatment prolonged the animal's survival, but it was not able to treat the whole tumor and tumor regrowth was observed [39]. Photodithazine showed a fast accumulation in the tumor as a quick elimination from the organism. This characteristic decreases the phototoxicity and photosensitivity damages due to environment illumination after the treatment.

In our study, a single session of PDT with Photodithazine was evaluated, and an average of 40% on the animal survival rate was reached. These results show that despite the vast localization of the photosensitizer inside the cell, melanoma treatment with one PDT session is still not effective. Multiples PDT session may be an alternative to improve the PDT response in pigmented tumors.

5. CONCLUSION

In this study, murine melanoma cells were investigated in monolayer model using Photodithazine, a photosensitizer. Cellular uptake and intracellular co-localization of the photosensitizer was observed by cellular labeling components that were analyzed by confocal microscopy. Cell viability assays were performed to obtain the cytotoxic concentrations in the dark, using the MTT test. Using the less cytotoxic concentrations, photodynamic therapy trials were carried out to obtain the cytotoxic fluences. Finally, the average lifetime fluorescence of photosensitizer was measured to characterize the photodynamic therapy in FLIM (fluorescence lifetime imaging microscopy). The results indicated that longer dark incubation cause greater cytotoxicity at the concentrations above 0.1 mg/mL of Photodithazine. Thereby the results of photodynamic therapy experiments observed the decreasing from over 90% cell viability at low levels of Photodithazine using fluences above 2 J/cm^2. By calculating correlation coefficients, the high colocalization was observed in mitochondria. In the *in vivo* assays, Photodithazine showed accumulation in the tumor in a short interval (between 1 and 3 hours) and a quick elimination from the organism. The single PDT session increased the animal's survival in around 20% showing the potential of the technique.

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REFERENCES

[1] Szeimies, R.-M., Morton, C. A., Sidoroff, A.., Braathen, L. R., "Photodynamic therapy for non-melanoma skin cancer.," Acta Derm. Venereol. **85**(6), 483–490 (2005).

[2] Grecco, C., Buzzá, H. H.., Stringasci, M. D., Andrade, C. T.., Vollet-Filho, J. D., Pratavieira, S., Zanchin, A. L., Tuboy, A. M., Bagnato, V. S., et al., "Single LED-based device to perform widefield fluorescence imaging and photodynamic therapy," Prog. Biomed. Opt. Imaging - Proc. SPIE **9531**, C. Kurachi, K. Svanberg, B. J. Tromberg, and V. S. Bagnato, Eds., 953121 (2015).

[3] Romano, R. A., Pratavieira, S., Silva, A. P. d., Kurachi, C.., Guimarães, F. E. G., "Light-driven photosensitizer uptake increases Candida albicans photodynamic inactivation," J. Biophotonics **9**, 1–10 (2017).

[4] Huang, Y., Vecchio, D., Avci, P., Yin, R., Garcia-diaz, M.., Hamblin, M. R., "Melanoma resistance to photodynamic therapy : new insights," 239–250 (2014).

[5] Pires, L., Demidov, V., Vitkin, I. A., Bagnato, V., Kurachi, C.., Wilson, B. C., "Optical clearing of melanoma in vivo : characterization by diffuse reflectance spectroscopy and optical coherence tomography," J. Biomed. Opt. **21**(8), 81210 (2016).

[6] Rodrigues, P. G. S., Campos de Menezes, P. F., Fujita, A. K. L., Escobar, A., Barboza de Nardi, A., Kurachi, C., Bagnato, V. S., "Assessment of ALA-induced PpIX production in porcine skin pretreated with microneedles," J. Biophotonics **8**(9), 723–729 (2015).

[7] Lim, D.-S. S., Ko, S.-H. H.., Lee, W.-Y. Y., "Silkworm-pheophorbide a mediated photodynamic therapy against B16F10 pigmented melanoma," J. Photochem. Photobiol. B Biol. **74**(1), 1–6 (2004).

[8] Meng, Z., Yu, B., Han, G., Liu, M., Shan, B., Dong, G., Miao, Z., Jia, N., Tan, Z., et al., "Chlorin p 6 -Based Water-Soluble Amino Acid Derivatives as Potent Photosensitizers for Photodynamic Therapy," J. Med. Chem. **59**(10), 4999–5010 (2016).

[9] RADZI, R., OSAKI, T., TSUKA, T., Imagawa, T., Minami, S.., OKAMOTO, Y., "Morphological Study in B16F10 Murine Melanoma Cells after Photodynamic Hyperthermal Therapy with Indocyanine Green (ICG)," J. Vet. Med. Sci. **74**(4), 465–472 (2012).

[10] Woodbum, K. W., Fan, Q., Kessel, D., Luo, Y.., Young, S. W., "Photodynamic therapy of B16f10 murine melanoma with lutetium texaphyrin," J. Invest. Dermatol. **110**(5), 746–751, Elsevier Masson SAS (1998).

[11] Chen, Z.-A., Kuthati, Y., Kankala, R. K., Chang, Y.-C., Liu, C.-L., Weng, C.-F., Mou, C.-Y.., Lee, C.-H., "Encapsulation of palladium porphyrin photosensitizer in layered metal oxide nanoparticles for photodynamic therapy against skin melanoma," Sci. Technol. Adv. Mater. **16**(5), 54205, IOP Publishing (2015).

[12] Stranadko, E. P., Ponomarev, G. V., Mechkov, V. M., RiaboV, M. V., Ivanov, A. V., Reshetnikov, A. V.., M-Koraboyev, U., "Publications - The first experience of Photodithazine clinical application for photodynamic therapy of malignant tumors."

[13] Ivanov, A., "One more PDT application of chlorin e6," BiOS 2000 …, 1–7 (2000).

[14] Romanko, Y. S., Tsyb, A. F., Kaplan, M. A.., Popuchiev, V. V., "Effect of photodynamic therapy with photodithazine on morphofunctional parameters of M-1 sarcoma," Bull. Exp. Biol. Med. **138**(6), 584–589 (2004).

[15] Correa, J. C., Bagnato, V. S., Imasato, H.., Perussi, J. R., "Previous illumination of a water soluble chlorine photosensitizer increases its cytotoxicity," Laser Phys. **22**(9), 1387–1394 (2012).

[16] Ricci Donato, H. A., Pratavieira, S., Grecco, C., Brugnera-Júnior, A., Bagnato, V. S.., Kurachi, C., "Clinical Comparison of Two Photosensitizers for Oral Cavity Decontamination," Photomed. Laser Surg., pho.2016.4114 (2016).

[17] Calixto, G., Bernegossi, J., de Freitas, L., Fontana, C.., Chorilli, M., "Nanotechnology-Based Drug Delivery Systems for Photodynamic Therapy of Cancer: A Review," Molecules **21**(3), 342, Multidisciplinary Digital Publishing Institute (2016).

[18] Uzdensky, a B., Dergacheva, O. Y., Zhavoronkova, a a., Reshetnikov, a V., Ponomarev, G. V., "Photodynamic effect of novel chlorin e6 derivatives on a single nerve cell.," Life Sci. **74**(17), 2185–2197 (2004).

[19] Carmello, J. C., Alves, F., Basso, F. G., Costa, C. A. D., Bagnato, V. S., Mima, E. G. D.., Pavarina, A. C., "Treatment of Oral Candidiasis Using Photodithazine (R)-Mediated Photodynamic Therapy In Vivo," PLoS One **11**(6), 1–18 (2016).

[20] Quishida, C. C. C., de Oliveira Mima, E. G., Dovigo, L. N., Jorge, J. H., Bagnato, V. S.., Pavarina, A. C., "Photodynamic inactivation of a multispecies biofilm using Photodithazine® and LED light after one and three successive applications," Lasers Med. Sci. **30**(9), 2303–2312, Lasers in Medical Science (2015).

[21] Dovigo, L. N., Carmello, J. C., Carvalho, M. T., Mima, E. G., Vergani, C. E., Bagnato, V. S.., Pavarina, A. C., "Photodynamic inactivation of clinical isolates of *Candida* using Photodithazine ®," Biofouling **29**(9), 1057–1067 (2013).

[22] Wen, L. Y., Bae, S.-M., Do, J. H., Park, K.-S.., Ahn, W. S., "The effects of photodynamic therapy with Photodithazine on HPV 16 E6/E7 associated cervical cancer model," J. Porphyr. Phthalocyanines **15**(3), 174–180, World Scientific Publishing Company (2011).

[23] Mroz, P., Yaroslavsky, A., Kharkwal, G. B.., Hamblin, M. R., "Cell Death Pathways in Photodynamic Therapy of Cancer," Cancers (Basel). **3**(4), 2516–2539 (2011).

[24] de Paula Campos, C., de Paula D'Almeida, C., Nogueira, M. S., Moriyama, L. T., Pratavieira, S.., Kurachi, C., "Fluorescence spectroscopy in the visible range for the assessment of UVB radiation effects in hairless mice skin," Photodiagnosis Photodyn. Ther. **20** (2017).

[25] Malgady, R. G.., Krebs, D. E., "Understanding Correlation Coefficients and Regression," Phys. Ther. **66**(1) (1986).

[26] Kessel, D.., Oleinick, N. L., "Photodynamic therapy and cell death pathways.," Methods Mol. Biol. **635**, 35–46, NIH Public Access (2010).

[27] Silva, J. C., Ferreira-Strixino, J., Fontana, L. C., Paula, L. M., Raniero, L., Martin, A. A.., Canevari, R. A., "Apoptosis-associated genes related to photodynamic therapy in breast carcinomas," Lasers Med. Sci. **29**(4), 1429–1436, Springer London (2014).

[28] Bacellar, I. O. L., Tsubone, T. M., Pavani, C.., Baptista, M. S., "Photodynamic efficiency: From molecular photochemistry to cell death," Int. J. Mol. Sci. **16**(9), 20523–20559 (2015).
[29] Bachor, R., Shea, C. R., Gillies, R.., Hasan, T., "Photosensitized destruction of human bladder carcinoma cells treated with chlorin e6-conjugated microspheres.," Proc. Natl. Acad. Sci. U. S. A. **88**(4), 1580–1584, National Academy of Sciences (1991).
[30] Antonenko, Y. N., Kotova, E. A., Omarova, E. O., Rokitskaya, T. I., Ol'shevskaya, V. A., Kalinin, V. N., Nikitina, R. G., Osipchuk, J. S., Kaplan, M. A., et al., "Photodynamic activity of the boronated chlorin e6 amide in artificial and cellular membranes," Biochim. Biophys. Acta - Biomembr. **1838**(3), 793–801 (2014).
[31] Dunn, K. W., Kamocka, M. M.., McDonald, J. H., "A practical guide to evaluating colocalization in biological microscopy," AJP Cell Physiol. **300**(4), C723–C742 (2011).
[32] Wen, L. Y., Bae, S.-M., Chun, H.-J., Park, K.-S.., Ahn, W. S., "Therapeutic effects of systemic photodynamic therapy in a leukemia animal model using A20 cells.," Lasers Med. Sci. **27**(2), 445–452 (2012).
[33] Soengas, M. S.., Lowe, S. W., "Apoptosis and melanoma chemoresistance," Oncogene **22**(20), 3138–3151, Nature Publishing Group (2003).
[34] Luo, W., Liu, R.-S., Zhu, J.-G., Li, Y.-C.., Liu, H.-C., "Subcellular location and photodynamic therapeutic effect of chlorin e6 in the human tongue squamous cell cancer Tca8113 cell line.," Oncol. Lett. **9**(2), 551–556 (2015).
[35] Zhao, L., Yang, H., Amano, T., Qin, H., Zheng, L., Takahashi, A., Zhao, S., Tooyama, I., Murakami, T., et al., "Efficient delivery of chlorin e6 into ovarian cancer cells with octalysine conjugated superparamagnetic iron oxide nanoparticles for effective photodynamic therapy," J. Mater. Chem. B **4**(47), 7741–7748 (2016).
[36] Wawrzyńska, M., Kałas, W., Biały, D., Zioło, E., Arkowski, J., Mazurek, W.., Strządała, L., "In Vitro Photodynamic Therapy with Chlorin e6 Leads to Apoptosis of Human Vascular Smooth Muscle Cells," Arch. Immunol. Ther. Exp. (Warsz). **58**(1), 67–75 (2010).
[37] Li, Y., Yu, Y., Kang, L.., Lu, Y., "Effects of chlorin e6-mediated photodynamic therapy on human colon cancer SW480 cells.," Int. J. Clin. Exp. Med. **7**(12), 4867–4876 (2014).
[38] Acedo, P., Stockert, J. C., Cañete, M.., Villanueva, A., "Two combined photosensitizers: a goal for more effective photodynamic therapy of cancer.," Cell Death Dis. **5**, e1122 (2014).
[39] Chen, Y., Zheng, W., Li, Y., Zhong, J., Ji, J.., Shen, P., "Apoptosis induced by methylene-blue-mediated photodynamic therapy in melanomas and the involvement of mitochondrial dysfunction revealed by proteomics," Cancer Sci. (2008).