Silicon phthalocyanines as photosensitizers for cancer treatment: an *in vitro* study

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ABSTRACT

Photodynamic therapy (PDT) is a quite new treatment for several types of cancers, particularly skin cancer, as well as for bacterial and fungi infections. It is based in the use of three elements: a photosensitizing compound, light and oxygen; the combination of them induces cell death. There is a wide range of photosensitizers that can be used for PDT, mostly porphyrins; however, new photosensitizers with better properties are under study. In this sense, silicon phthalocyanines (SiPCs) are well known synthetic dyes with promising photophysical properties (strong absorption in red region of the visible spectrum, strong singlet oxygen production, among others) to be used as photosensitizers in PDT. In this study we have tested the photochemical properties of two SiPCs (1 and 3) to destroy three cancer cell types in vitro: HeLa (cervical cancer), SCC-13 (skin cancer) and MDA-MB-231 (mammary cancer). By using the MTT assay we evaluate the cell death after PDT, demonstrating that both SiPCs were able to destroy the three cancer cell types, being SCC-13 cells the most sensitive and MDA-MB-231 the most resistant cell line to PDT with SiPCs. To identify SiPCs targets, we evaluated the intracellular localization of the compounds by fluorescence microscopy and by using known probes for specific organelles (Lysotracker® and Mitotracker® for labelling lysosomes and mitochondria, respectively). The results obtained indicated that both compounds were located in lysosomes. The induced cell death was caused by the production of ROS, which was determined with DHF-DA assay. Cytotoxicity and localization were also studied in spheroids (3D cultures as small rounded tumors) formed by SCC-13 cells and MDA-MB-231 cells, indicating that SiPC 1 was placed in lysosomes too. When treated with the compound, the diameter of spheroids was observed to decrease. In the case of MDA-MB-231, this size was smaller than the other cell line. Overall, the results obtained indicated that both SiPCs, but particularly SiPC 1, constitute excellent candidates to be applied for cancer treatment.

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ABBREVIATIONS

ABBREVIATION	MEANING
BSA	Bovine serum albumin
DHF-DA	2,7-dichloro-dihydrofluorescein diacetate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dymethyl sulfoxide
EGF	Epidermal growth factor
FBS	Fetal bovine serum
H_2O_2	Hydrogen peroxide
M-PCS	Metallic phthalocyanines
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
$O_2\bullet^-$	Superoxide anion radical
$^1O_2$	Singlet oxygen
PBS	Phosphate-buffered saline
PC	Phthalocyanine
PDT	Photodynamic therapy
POLY-HEMA	2-hydroxyethyl methacrylate
PS	Photosensitizer
ROS	Reactive oxygen species
SIPCS	Silicon phthalocyanines
SUBPCS	Subphthalocyanines
ZNPCS	Zinc phthalocyanines

INTRODUCTION

1.1. Photodynamic therapy: Basic concepts

Photodynamic therapy (PDT) has become a potential alternative to chemotherapy and radiotherapy for the treatment of cancer (Agostinis *et al*., 2011; Mohanty *et al*., 2017). PDT is a non-invasive and localized therapy with minimal or no damage to healthy tissues and a high healing process (Sivasubramanian *et al*., 2019). It has several advantages over conventional therapies including: (i) low systemic toxicity, due to the fact that PSs are only activated in the presence of light; (ii) ability to selectively destroy tumors accessible to light; (iii) high efficacy and (iv) that can be applied with other therapeutic modalities (chemotherapy, surgery, radiotherapy or immunotherapy). PDT causes the induction of cell death by the combined effect of a compound with photosensitizing properties, called photosensitizer (PS), visible light, and O_2. At the moment, it is being used as the treatment of accessible tumors, such us, lung, bladder, gynaecological neoplasms and particularly in dermatology for the treatment of non-melanoma skin cancers (Barata *et al*., 2015). In addition, PDT has been seen to be able to destroy microbial cells, including bacteria, fungi and viruses (Mohanty *et al*., 2017).

1.2. Components of PDT

As it was mentioned before, PDT is a multi-stage process in which three principal elements participate: a PS compound, visible light and O_2. None of these are individually toxic, but together initiate a photochemical reaction that culminates in the generation of ROS (reactive oxygen species), mainly a highly reactive product termed singlet oxygen ($^1O_2$), the main responsible to destroy cancer cells.

Photosensitizers are chromophores able to interact with photons with a determined wavelength leading to the formation of a high excited state able to form ROS (Wainwright et at., 1998). An ideal PS has a broad list of properties, including: (i) to be chemically pure, with known composition and good stability; (ii) selectivity to target tumor cells, fast accumulation inside them; (iii) minimal toxicity in absence of light; (iv) high absorption between 600 and 800nm (red to deep red), since light penetration into the tissues increases with the wavelength; and (v) capacity for forming a substantial yield of ROS upon irradiation (Banerjee *et al*., 2017).

An important number of synthetic or natural PSs have been studied, and some of them are nowadays being already applied for specific tumoral or pretumoral lesions in clinic. In general terms, PSs can be classified in two ways, attending to their generations (first, second and third generation) (Kwiatkowski *et al*., 2018) or their chemical structure (tetracyclic compounds or similar and compounds) (Pérez-Laguna *et al*., 2018). First generation PSs are the first ones demonstrated to be effective, but with some disadvantages such as low selectivity and high skin phototoxicity. Examples of them are the haematoporphyrin derivative (HPD), a mixture of mono-, di- and oligomers of porphyrins, the porfimer sodium, commercially known as Photofrin®. Second generation PSs present improved physical, chemical and therapeutic properties, able to absorb longer wavelengths and with fewer side effects. They are molecules derivate from a tetrapyrrole structure, similar to that of the protoporphyrin (PpIX) contained in hemoglobin with low toxicity (Wainwright *et al*., 2010) such as: synthetic porphyrins, benzoporphyrins, chlorins, bacteriochlorophylls, pheophorbides, naphthalocyanines, natural hydroxyquinone chromophores such as hypericins and, also, in this group we find the phthalocyanines (PCs). The third generation of PSs come from second generation conjugated to agents, such as nanoparticles, which improve selectivity of the compounds.

In the field of PSs, nanotechnology (characterised by the development of devices which size of 100 nm or lower) has become an important tool for the design of biocompatible particles covering by PSs to enhance its delivery to targeted cells, for example the design of dendrimers (Ideta *et al*., 2005; Obaid *et al*., 2016).

Light. There are a wide variety of light sources that can be used in PDT, between them light emitting diodes, which are devices based on activated semiconductors by electric current and no heat is generated (Woodburn *et al*., 2001). Another important factor is the penetration of the light into the tissues, which depends on two factors, the light wavelength and the type of tissue. The penetration of light into tissue increases with its wavelength as it has been indicated before. The penetration can vary with the type of tissue due to its optical spreading and the optic absorption due to the presence of chromophores such as haemoglobin or even water that can absorb specific wavelengths interfering with the treatment (Yoon *et al*., 2013, Anderson *et al*., 2017). Because of it, there is a frame between 600 and 1200nm called optic window of the

tissue, which is the reference for PDT. In any case, wavelengths upper than 850nm are not recommended to induce the photodynamic reaction, being the optimal therapeutic window situated between 650 and 850nm (red and near infrared). So that, the best PS must be able to absorb in this range, so the light spreading is the minimum and the penetration in the tissue is the maximum (Yoon *et al.*, 2013). PSs with strong absorbance in the deep red are for instance: chlorins, bacteriochlorins and PCs.

Oxygen. This element is present in the cells in its molecular form with two unpaired electrons whose spins are parallel located in two separated orbitals in the outer zone, so it makes the molecules around it highly susceptible to radical formation (Zhou *et al.*, 2016).

1.3. Photochemistry bases

PSs are compounds that in combination with certain radiations result in a photochemical or photophysical alteration. The PS is in a ground non-excited state (S_o) (with all the electrons with paired spins in the low energy orbitals) and modifies this electronic structure with the absorption of visible light (photons) shifting first to an electronically excited singlet state (S_1) (Vermeersch *et al.*, 1991; Kharkwal *et al.*, 2011). The excited singlet state is very unstable and can emit this excess energy as fluorescence and/or heat to reach the again the single state. Alternatively, an excited PS may undergo an intersystem crossing to form a more stable triplet (T_1) state with along lived excited state. The excited triplet state can transfer the energy to other molecules closely situated to the PS by using two pathways: type I and type II (Woodburn *et al.*, 2001). The following cascade of reactions causes cell death (Kwiatkowski *et al.*, 2018) (**Figure 1**).

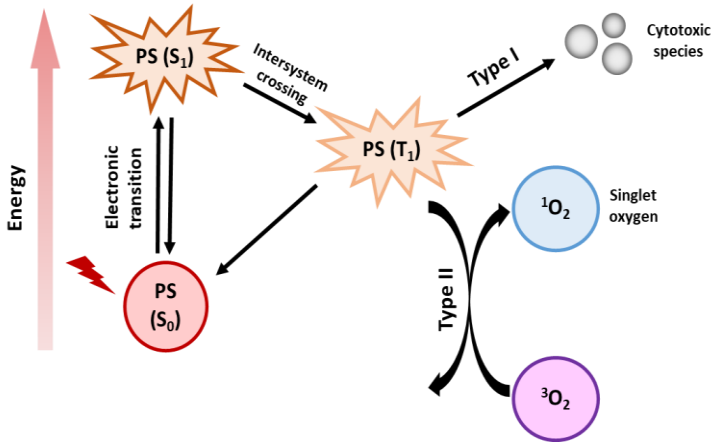

Figure 1. Modified Jablonski diagram. With the application of light, the photosensitizer (PS) in its ground state is excited by absorption of a photon, to a singlet state. By intersystem crossing the PS in its triplet stat, transfers its energy from this point following two pathways: type I produces cytotoxic species; and type II where this energy produces the excitation and transition of the O_2 in its ground state (which is $^3O_2$) to an excited singlet state ($^1O_2$).

The type I pathway involves electron transfer to a variety of organic molecule (lipids, proteins, nucleic acids, etc.) in the cellular microenvironment, forming different ROS, superoxide anion radical (O_2•-), the hydroxyl radical (OH•) or hydrogen peroxide (H_2O_2), among others (Agostinis *et al*., 2011; Kwiatkowski *et al*., 2018).

In the type II, the PS in the triplet excited state can transfer its energy to molecular oxygen (O_2) to form singlet oxygen ($^1O_2$), a potent oxidizing agent (Hamblin *et al*., 2015; Kwiatkowski *et al*., 2018). The highly reactive singlet oxygen has limited effects since it presents a very short life, being the place where the PS is localized the main cell target for damage (Josefsen *et al*., 2008; Abrahamse *et al*., 2016).

Most PSs are believed to operate via type II rather than type I mechanism (Kwiatkowski *et al*., 2018).

1.4. Phthalocyanines

Because of the positive results obtained in research related to this therapy, the interest in PDT is growing up, and so the development of new compounds and delivery systems for them. Porphyrins are some of the most studied PSs nowadays, but there are several other types that are also being studied, for example phthalocyanines (PCs). PCs are nanocompounds that in comparison with porphyrins, present higher absorption at longer wavelengths. PCs are some of the earliest synthetic compounds studied for cancer treatment since they are innocuous in the absence of light but able to induce high phototoxicity. In this sense, others and we have demonstrated the photosensitizing effects of several phthalocyanines both in vivo, for treatment of mice tumors, and in vitro, for the induction of cytotoxic effect on several cell lines. Moreover, ZnPCs are been applied in phase I/II clinical trials for the treatment of several types of cancer (Doustvandi *et al*., 2017; Kuzyniak *et al*., 2017).

- Chemical structure

A PC molecule is a macromolecule with an 18-π electron conjugated ring and a central cavity that allows the accommodation of different metal ions, which makes a huge influence in the properties of the compound. Due to their structure, these compounds are difficult to dissolve in most organic solvents (Arslan, 2016). When they

present a metallic atom, they are able to generate a high production of singlet oxygen (Pérez-Laguna *et al*., 2018). Metallic PCs have another characteristic, which is the modification of the axial groups bonded to the metal ion, which can contribute to many of the properties of these compounds (Van de Winckel *et al*., 2015).

- Absorption/emission spectrum

Two major bands compose their spectra: the Q band, which corresponds to the transition from the ground state to the first excited state (between 620-720nm); and the B or Soret band, which is the transition from ground state to second excited state (near 300 nm). The Q band is sharp and intense with a splitting, being the responsible of the blue colour of these molecules. The B band is instead broad with low intensity (Ueno *et al*., 2012; Brozek-Pluska *et al*., 2015). In the case of metallophthalocyanines, the band spectrum is different, having only one Q band.

Because the presence of the four peripheral benzenes, they present a strong absorption of the far red light (between the wavelengths of 600 nm and 850 nm) which is inside the optical therapeutic window. Because of this characteristic, PCs are excellent PSs for cancer treatment and the object of study in this work (Arslan, 2016).

1.5. Silicon phthalocyanines

PCs present a particular disadvantage; they tend to aggregate in aqueous solution due to the planarity of their aromatic system, making them inactive (Verhoeven *et al*., 1996). Their solubility can be improved when their peripheral and/or axial substituents of the macrocycle are modifying as well as the central metal (Woodburn *et al*., 2001). In the case of silicon phthalocyanines (SiPCs), the Si in the central cavity of the molecule allows to add two axial substituents that can prevent aggregation or even enhance its photochemical characteristics (Kishen *et al*., 2012). Depending of the substituents, SiPCs can be differentiated in SiPC 1 and SiPC 3, being both the object of study (Van de Winckel *et al*., 2015) (**Figure 2**).

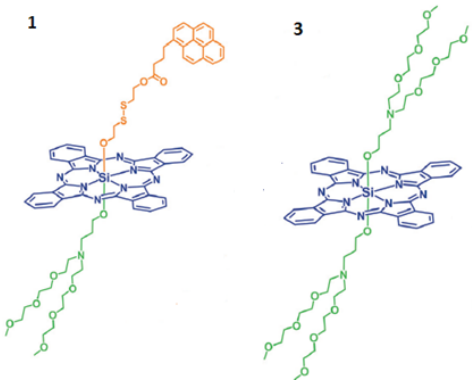

Figure 2. SiPC 1 and 3 chemical structure (obtained from Van de Winckel *et al*., 2015).

Previous to these studies, the reactive oxygen species generated by the irradiation of SiPC 1 and SiPC 3 were measured (Van de Winckel *et al.*, 2015) (**Table 1**).

Table 1. $^1O_2$ quantum yields of SiPC 1 and 3.

COMPOUND	Φ_Δ (%)
SIPC 1	4.7
SIPC 3	3.5

Because of the characteristics of silicon SiPCs, their potential use as PSs against cancer is being studied in *in vitro* cell cultures.

2. HYPOTHESIS AND OBJECTIVES

Our hypothesis is based on the affirmation that SiPCs are potential PSs to be applied in cancer treatment.

Therefore, our general objective is to determine the photosensitizing abilities of SiPCs to destroy cultured selected cancer cells *in vitro* as a first approach for the treatment of cervical adenocarcinoma (HeLa cells), squamous cell carcinoma (SCC-13 cells), and breast adenocarcinoma (MDA-MB-231 cells). The specific objectives are:

- To determine the ability of SiPCs to enter into the selected cancer cells grown as 2D and 3D cultures.
- To determine the dose-response to kill cancer cell lines.

3. MATERIALS AND METHODS

3.1. SiPCs preparation

SiPCs were synthesized as previously described in the Organic Chemistry Department, at the Universidad Autónoma of Madrid. Stock solutions were prepared in dimethyl sulfoxide (DMSO) (Van de Winckel *et al.* 2015). Incubation cell solutions were prepared in DMEM without FBS from the stock solutions, being the final concentration of DMSO always lower than 0.5% (v/v). To avoid the formation of precipitates and

ensure their entry in cells, stocks solutions in DMSO were subjected to agitation overnight the day before to their use, being the treatment solution prepared at the moment of the incubation. The absence of toxicity of DMSO concentration was also confirmed.

3.2. Cell cultures

For in vitro studies, we used three human cell lines: HeLa (cervical adenocarcinoma) (ATCC CCL-2), SCC-13 (squamous cell carcinoma) (Rheinwald and Beckett, 1981), and MDA-MB-231 (breast adenocarcinoma) (ATCC HTB-26). Bidimensional cultures (2D) of the cell lines were performed by using DMEM (Dulbecco's modified Eagle's medium high glucose 1X) supplemented with 10% (v/v) fetal bovine serum (FBS), 50units/ml penicillin and 50µg/ml streptomycin, all from Thermo Fisher Scientific Inc. Cell cultures were performed under standard conditions of 5% CO_2, 95% of relative humidity and 37ºC of temperature.

3.3. Spheroid cultures

Spheroids (3D cultures, as small rounded tumors) were prepared using MDA-MB-231 and SCC-13 cells in a specific medium made by DMEM (Dulbecco's modified Eagle's medium high glucose 1X) and F12 (F-12 Nutrient mixture, Ham, Gibco) in a (1:1) proportion, 2% B27 serum free supplement (Gibco), 20ng/mL EGF (Sigma), 0.4% bovine serum albumin (Sigma) and 4µg/mL insulin (Gibco). Before cells were seeded, it was necessary to precoat the multiwell plates with 1.2% poly-HEMA (2-hydroxyethyl methacrylate, Sigma) and left it overnight to create a film so cells could not be adhered to the plastic well. Then, cells were plated at a density of 40000 cells/mL, needing the spheroids 6 days to be formed.

3.4. Photodynamic treatments

Cells were seeded in 6, 12 or 24 well plates depending on the posterior evaluation. After reaching the 60-70% of confluence, cells were incubated with different concentrations of SiPCs (from 0.01µM to 1µM, prepared in DMEM without FBS) for 5h in darkness under standard conditions. Subsequently, cells were irradiated with red light emitting diode source, with a wavelength of 625nm, at doses between 0.61J/cm^2 and

12.24J/cm^2. After irradiation, cell medium was changed by DMEM with 10% FBS and left for 24h at 37ºC until evaluation.

3.5. Co-localization assays

When cells reached the 60-70% of confluence, were treated with 1µM of SiPC 1 and 2µM of SiPC 3 for 15h. After SiPC incubation, cells were also incubated with two different fluorescent probes for cellular organelles, Mitotracker® (for mitochondrias) and Lysotracker® (for lysosomes) (Invitrogen). In the case of spheroids, studies were carried out under the same conditions but only with SiPC 1 at a concentration of 1µM. Fluorescence of the compounds were observed under the fluorescence microscope (Olympus BX61 epifluorescence microscope, equipped with a HBO 100W mercury lamp and filter sets for fluorescence microscopy) by using blue light irradiation (450-490nm, BP 490 filter) for Mitotracker® and Lysotracker® probes and ultraviolet (360-370nm, UG-1 filter) and green light irradiation (570-590nm, DM 590 filter) for SiPCs.

3.6. Phototoxicity studies

The toxicity of SiPCs in cells was evaluated by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma) assay 24h after the phototreatment. The MTT assay is based in the activity of mitochondial enzymes that transform MTT in a insoluble compound called formazan. For that, a stock solution of MTT in PBS (1mg/mL) was prepared and diluted in DMEM (10% FBS) to obtain a final concentration of 50µg/mL to be applied in the cells and incubated for 3h at 37◦C. After removing the solution, DMSO was added to dissolve the formazan crystals formed giving a purple colour; its absorbance was measured at 542nm by spectrophotometry. The results obtained are represented as cell survival.

The toxicity of SiPC 1 on spheroids was determined 24h after irradiation by using two methods. The first one consisted in measuring the diameters of the treated spheroids with Image J and compared with those of controls. The second one was performed with two fluorescence dyes, acridine orange that stains in green both live and dead cells, and ethidium bromide which stains in red only cells that have lost membrane integrity.

3.7. ROS determination

ROS production was determined by using the fluorescent probe of 2,7-dichloro-dihydrofluorescein diacetate (DHF-DA, Abcam). A 75mM stock of DHF-DA diluted in ethanol was used. Cells grown on coverslips were incubated for 5h with SiPCs in the concentrations stablished in cytotoxicity assays and, during the last 50 minutes, DHF-DA was added directly to the well to obtain a final concentration of 6 x 10^{-6}M. Then, plates were irradiated without removing the medium in the presence of DHF-DA. Then, plates were immediately analysed by the fluorescence microscopy under blue excitation light (450-490nm). Immediately, photographs were taken of positive and negative cells of each condition and they were analysed by calculating the intensity of fluorescence of every cell with Image J.

3.8. Statistical analysis

Experiments were repeated at least three times. The statistical analysis was carried out with the version 6.05 of the program GraphPad Prism (GraphPad Software Inc, USA) used to make graphical representations and the statics. The statics differences were determined by applying the t Student test for independent samples (one-way ANOVA followed by Turkey's test) (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$).

4. RESULTS

4.1. Localization of SiPCs in monolayers of cell cultures

Before studying the potential photosensitizing effects of the SiPCs, it was necessary to determine if they were able to enter into the cells. For this, we performed a co-localization assay by the treatment of cells with 1µM of SiPC (1 and 3) for 15h followed by the incubation with the organelle fluorescent probes (for mitochondria and lysosomes). The subsequent evaluation was performed by using the fluorescence microscopy. The results obtained in SCC cells are shown in the **Figure 3**. As it can be seen, a red fluorescence inside cells could be observed after 15h of incubation with SiPC 1. SiPC 1 was mainly situated in a yuxtanuclear position. To determine the organelles in which the PC was located we performed co-localization experiments with well-known

biomarkers for mitochondria (Mitotracker®) or lysosomes (Lysotracker®). The superposition of the fluorescence images of SiPC 1 and of Lysotracker gives a yellowish fluorescence indicating that SiPC 1 was located in the lysosomes. Same results were obtained in the case of SiPC 3 in HeLa and MDA cells (**Annexed 1**).

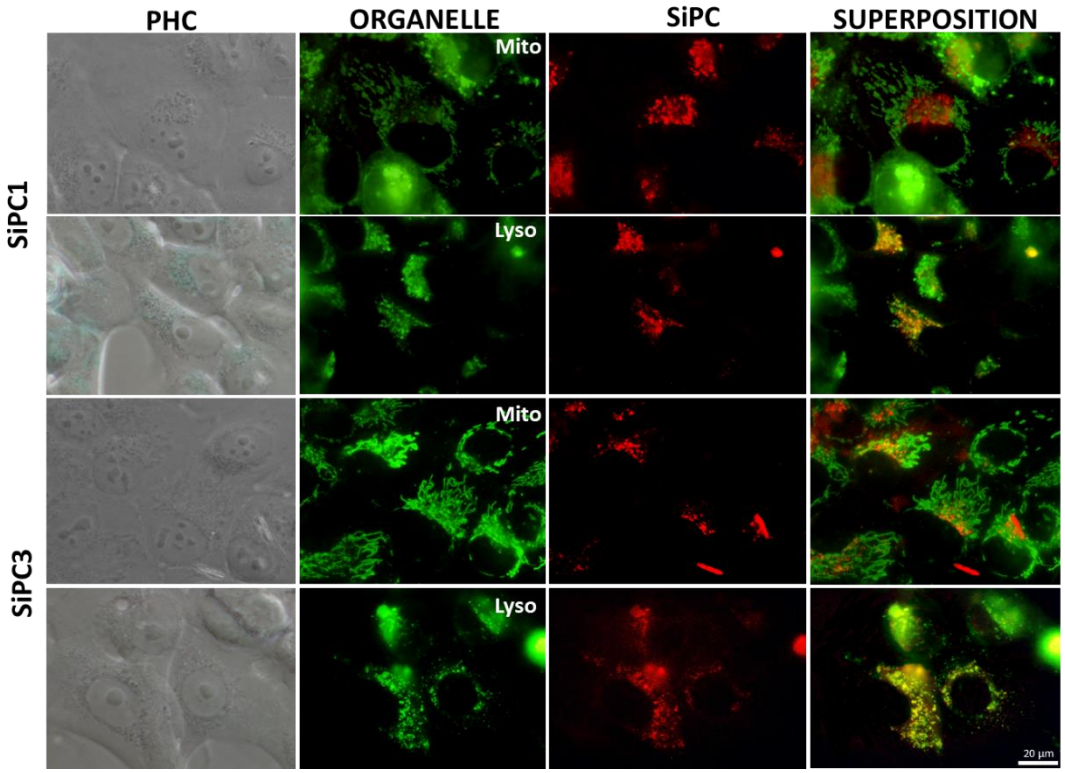

Figure 3. Localization of SiPCs in SCC cells. Cells were treated with 1µM of SiPC 1 and 2µM of SiPC 3 for 15h followed by the incubation with the fluorescent probes (Mitotracker® or Lysotracker®) according to the indications made by the manufactures. The first column corresponds to the cells observed under contrast phase microscopy, the second column to the organelle fluorescence under blue light irradiation (450-490nm); the third column to the SiPC fluorescence under green light irradiation (570-590nm) and the fourth column is the merge of the second and third columns. The yellowish colour in lysosomes indicates that the SiPC co-localize with these organelles.

4.2. Cytotoxicity in cells in monolayer

Once we know that both SiPCs were able to enter into the cells and were mainly located in lysosomes, we next evaluated their potential phototoxicity by using the MTT assay based in the activity of mitochondial enzymes. In a first approach, we analyzed the cellular toxicity induced in dark conditions by the SiPCs at different concentrations (from 0.01µM to 1µM in SiPC 1 and from 1µM to 2µM in SiPC 3) after 5h of incubation without irradiation. We also evaluated the potential toxicity induced by light irradiation alone. The results obtained indicated tha cell damage was not induced by SiPCs whem they

were applied at concentrations of 0.01µM of SiPC 1 or 1µM of SiPC 3, neither when cells were exposed to red light alone, without previous incubation with the PSs (**Table 3**).

However, when cells were first incubated with the PSs and then exposed to red light doses a variable phototoxicity was observed. The **Figure 4A** shows the decrease in cell survival of SCC cells after incubation with the SiPCs (1 and 3) followed by red light irradiation; the photokilling was dependent on the PSs concentrations as well as the light dose used. The figure also indicates that SiPC 1 was more effective than SiPC 3 since a higher cell toxicity was detected under the same treatment conditions. From these results, we selected the two lower concentrations: 0.01µM for SiPC 1 and 1µM for SiPC 3 for the rest of experiments.

Table 3. Cell survival under test conditions.

		Cell survival (%) ± SD		
COMPOUND	Concentration	MDA-MB	HeLa	SCC-13
CONTROL	-	100 ± 3.1	100 ± 1.6	100 ± 2.3
RED LIGHT EXPOSURE	-	100 ± 1.4	99 ± 4.6	99 ± 1.1
SIPC 1	1µM	94 ± 2.7	92 ± 2.0	93 ± 3.7
SIPC 3	2µM	97 ± 1.6	95 ± 3.3	94 ± 3.0

The phototreatment also induced morphological changes. The **Figure 4B** shows the changes induced in the MDA cells as example; whereas control cells show the characteristic aspect of this cell line, the phototreatment induced cell retraction and the appareance of spherical morphologies. Similar results were observed in the other two cell lines (not shown).

We next determined the cell survival of the other two cell lines (MDA and HeLa) exposed to the selected concentrations of SiPCs at different red light doses (**Figure 4C**). The survival of the cell lines was dependent on the light dose employed, being lower with the increase of the irradiation. In addition, we observed that SiPC 3 induced the same effect in the three cell lines, whereas MDA cells were significantly more resistant to SiPC 1 than HeLa and SCC cells.

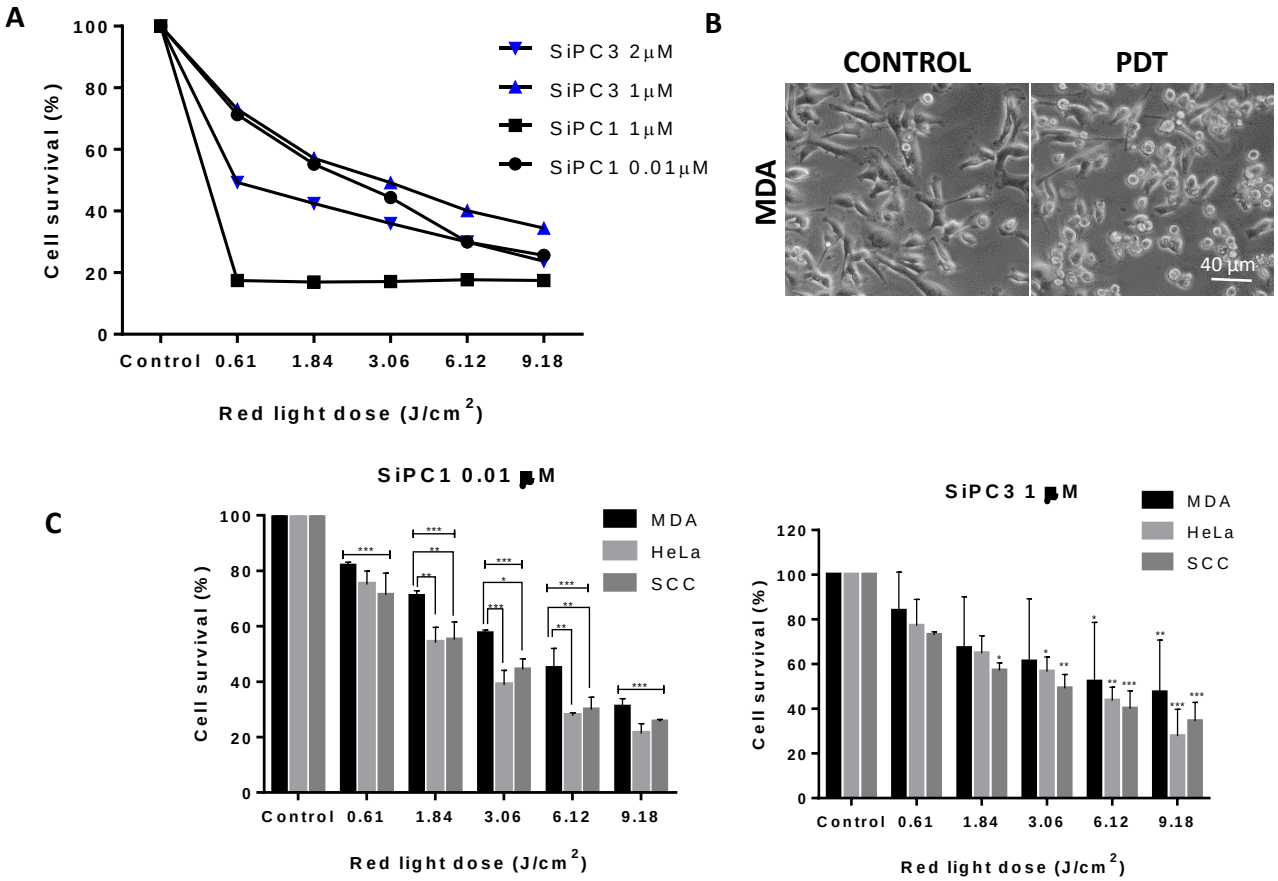

Figure 4. Dose-response of cells treated with SiPCs. (A) Relation of the concentration of both SiPCs (0.01µM and 1µM), the red light dose and cell survival in SCC cells. Higher concentrations were more effective, obtaining the 50% of cell death with lower light dose than with the lower concentrations. SiPC 1 induced higher death values than SiPC 3 for the same treatment conditions. **(B)** Morphological changes observed in MDA cells exposed to 0.01µM and 9.18J/cm^2. Treated cells acquired rounded morphology related to cell photodamage. **(C)** Cell survival in the three cell lines induced by the established concentrations of SiPCs (0.01µM for SiPC 1 and 1µM for SiPC 3) followed by different red light doses. Cell viability decreases significantly with higher doses of irradiation in both cases. MDA cells treated with SiPC 1 were more resistant. (*: p<0.05; **: p<0.01; ***: p<0.001).

4.3. ROS generation after PDT in cells in monolayer

PDT effects are mediated by ROS therefore, next we analysed the intracellular ROS production in the cells subjected to the phototreatments (**Figure 5**). For that, cells were incubated with the SiPCs and DHF-DA before being exposed to red light. After irradiation, ROS generation was evaluated by fluorescence microscopy differentiating between negative (no fluorescence) and positive cells (green fluorescence), which indicate that ROS was produced (**Figure 5A**). The **Figure 5B** shows the quantification of ROS production in relation with the red light dose in the three cell lines are obtained by

Image J from the fluorescence images. ROS production increases significantly with higher red light doses in the three cell lines treated with SiPC 1 and SiPC 3.

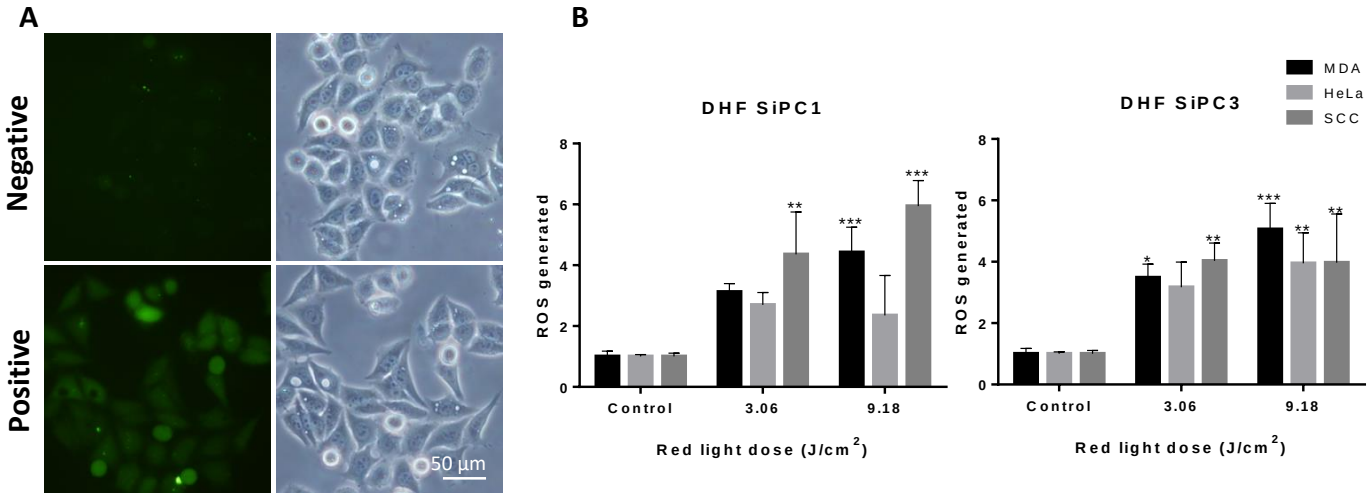

Figure 5. ROS generation in treated cells. (A) Control HeLa cells does not showed cells fluorescing in green, related with the production of ROS, whereas positive cells exhibited green fluorescence after phototreatment. **(B)** ROS production as a function of the red light dose applied after the phototreatment with SiPC 1 or SiPC 3. The levels of ROS were relatively similar with both SiPCs, being higher in SCC cells in comparison to the other two cell lines with SiPC 1 (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

4.4. Localization of SiPC 1 in spheroids

The results obtained indicated the photoactivity of the SiPC 1 and 3 in cancer cell lines cultured in vitro in 2D. Therefore, next we decided to test the activity of the PCs in 3D models (small rounded tumors *in vitro*), as an approximation to *in vivo* conditions. We performed the study with the MDA and SCC cells and with SiPC 1. SiPC 1 was selected due to its high photoactivity and since it was easier to be detected inside the cells in 2D cultures.

As can be seen in the **Figure 6**, SiPC 1 was able to enter in the spheroids being also located in lysosomes in both cell lines, MDA and SCC.

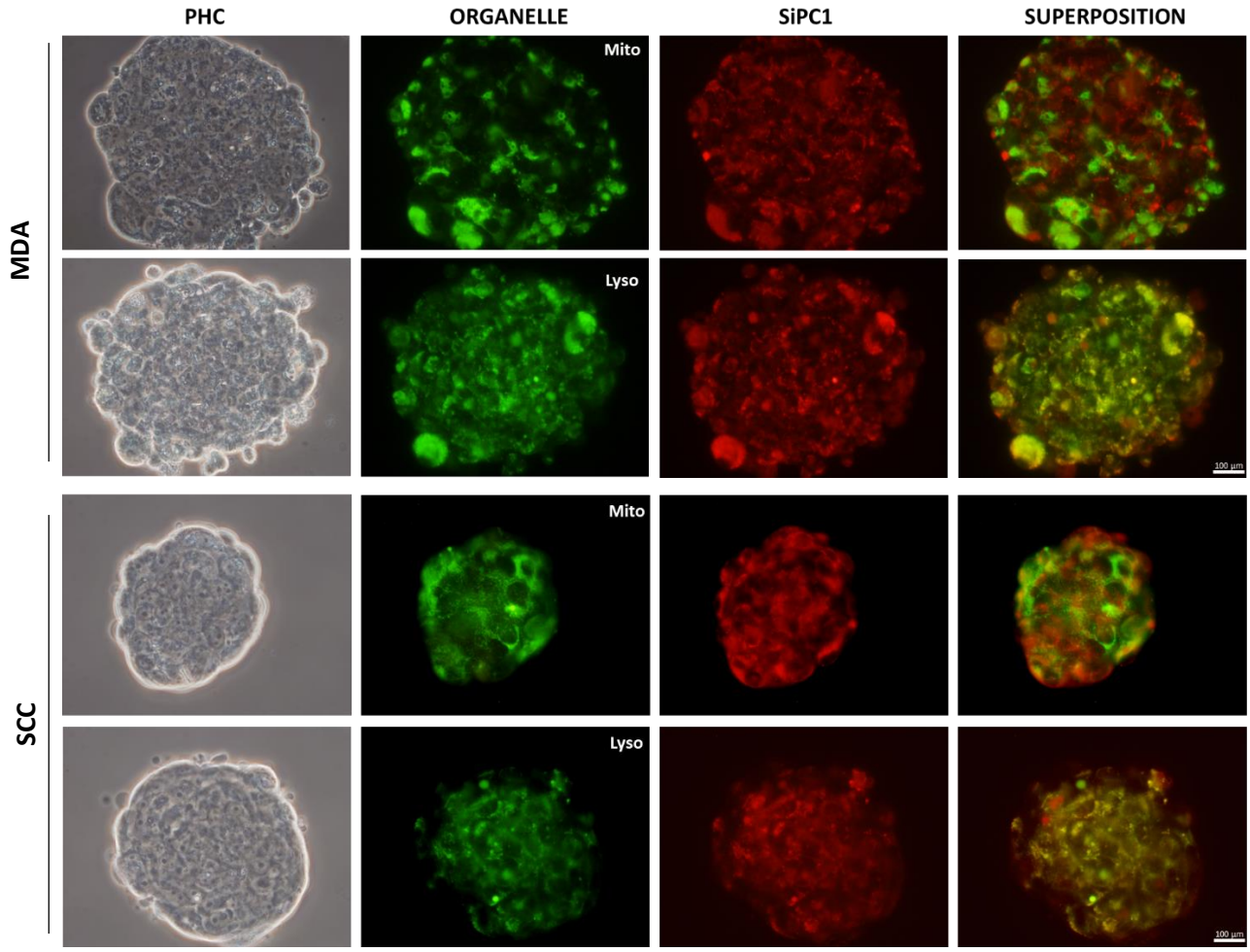

Figure 6. Localization of SiPC1 in MDA and SCC spheroids. Cells were treated with 1µM of SiPC 1 for 15h followed by the incubation with the fluorescent probes (Mitotracker® or Lysotracker®). The first column corresponds to the spheroids observed under contrast phase microscopy, the second column to the organelle fluorescence under blue light irradiation (450-490nm); the third column to the SiPC 1 fluorescence under green light irradiation (570-590nm) and the fourth column is the merge of the second and third columns. The yellowish colour in lysosomes indicates that the SiPC 1 co-localize with these organelles.

4.5. Cytotoxicity in spheroids

Next we evaluated the photoeffect of SiPC 1 in the 3D model by staining with acridine orange and ethidium bromide and by measuring the diameters of the tumors *in vitro* after the treatments. The staining with acridine orange and ethidium bromide revealed that spheroids treated with SiPC 1 and red light showed an orange fluoresce indicating that they were damaged (**Figure 7A and Annexed 2**). In addition, the size of the spheroids decreases significantly with the red light doses employed in both cell lines (**Figure 7B**).

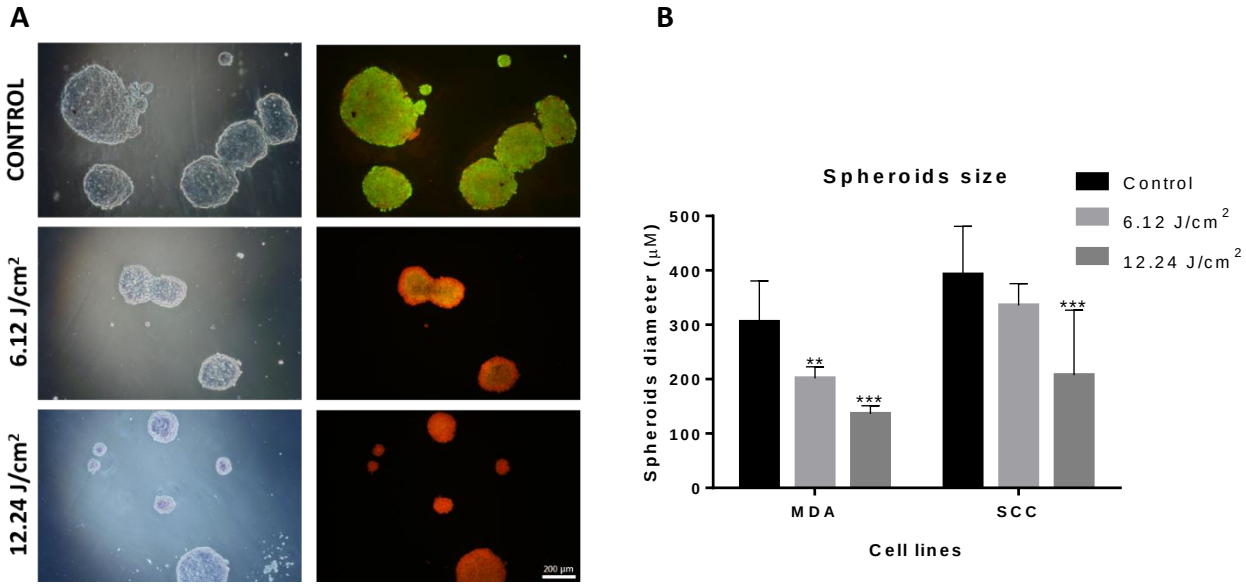

Figure 7. **Photodamage effects in spheroids. (A)** SCC spheroids stained with acridine orange and ethidium bromide; green fluorescence indicates alive spheroids whereas orange orange fluorescence is related with dead spheroids. **(B)** Changes in the diameter of spheroids after phototreatment. In both cell lines the size of spheroids decreases significantly with the red light doses employed (**: p<0.01; ***: p<0.001).

5. DISCUSSION

The development of new photosensitizers with better properties than the porphyrin, Photofrin, the main compound applied in clinic for PDT of cancer, constitute an important area of research. In this sense, different PSs haven synthetized and their photodynamic effects evaluated by using a wide range of cancer cell types in *in vitro* and i*n vivo* systems. In this sense, previous studies by using subphthalocyanines (SubPCs) synthesized in the same Organic Chemistry Department, at the Universidad Autónoma of Madrid where the compounds used in this work (SiPCs) have been made (Van de Winckel *et al*., 2015) have excellent photosensitizing properties for destroying SCC-13 and HeLa cancer cells. SiPCs belong to the group of phthalocyanines, macrocyclic compounds, similar to porphyrins but with two advantages over porphyrins as potential PDT agents including higher ROS generation and better spectroscopic properties (Arslan, 2016).

The subcellular localization of a PS is related to the cell death mechanism induced by PDT, as a consequence of the short lifetime of $^1O_2$, the main reactive species

produced after PDT, for which the primary localization of the PS determines the initial damage upon its activation (Xu *et al*., 2014). Our results indicated that both SiPCs were located in the cellular organelles lysosomes in the three cell lines with independence on the type of growth, 2D and 3D cultures. Some other PSs have been described to be located in lysosomes including SubPCs (Van de Winckel *et al*., 2018) with great efficiency in tumor cell photokilling. Also other studies have described localization of the compounds in lysosomes and in the perinuclear region of the cells, like ZnPCs (Hodgkinson *et al*., 2017). This last study indicated that PSs located in lysosomes produced lower effects after illumination than those situated in mitochondria (Hodgkinson *et al*., 2017). However, our results clearly have shown the high photosensity induced by both SiPCs in the studied tumor cells, even the PCs were situated in lysosomes.

One of the main characteristics of a good PS is the absence of cell toxicity in the darkness. Our results have clearly demonstrated that bboth SiPCs, even when they were used at high concentrations were non-toxic in absence of light. Other PSs also present this characteristic such as SubPCs or ZnPCs (Doustvandi *et al*., 2017; Van de Winckel *et al*., 2018).

A dose-dependent toxicity of PDT was observed in the three cell lines, with the selected concentrations of each SiPCs and the different doses of red light used. The phototoxicity was related with ROS production. ROS are the main effectors of cell death after PDT (Kwiatkowski *et al*., 2018). ROS generation was higher in SCC cells when treated with SiPC 1 but not with SiPC 3.

Other studies with ZnPC showed that maintaining the red light dose and changing the concentration of the PS resulted in an increase of cell death with the increase of the concentration (Doustvandi *et al*., 2017). These results are in agreement with these obtained in our work; cells were treated with two different concentrations of SiPC 1 and 3. We demonstrated that SiPC 1 is better than SiPC 3 because it requires llower concentration to induce the same or even more photoeffect. Studies carried out in breast, cervix and skin cancer cells with a BODIPY derivative followed light irradiation, indicated a phototoxicity related with the concentration of the compound for the same dose light (Gorbe *et al*., 2015). The authors demonstrated that HeLa and SCC cells resulted to be more sensitive to the treatment, while MCF-7 breast cancer cells were

the most resistant. Similar results have been also obtained with our three cell lines treated with SiPCs. Despite breast cancer cell lines are different in this case, results are the same indicating that both compounds have similar effect.

Studies performed with SubPCs showed that maintaining the concentration of the compound and increasing red light doses resulted also in a decreased of cell survival (Van de Winckel *et al*., 2015). We have obtained similar results with both SiPCs.

Finally, spheroids are considered an excellent model to mimic the situation *in vivo,* better than monolayers of tumor cells, we evaluate the effect of SiPC-PDT on them. The results obtained indicated that SiPC 1 was able to destroy also spheroids since a reduction in their diameter after irradiation was observed. These results agree with those observed in the SubPCs, in which a decrease in the size of the spheroids indicated a good efficacy of the PDT (Van de Winckel *et al*., 2018).

Overall, our results have indicated that the compounds tested, SiPC 1 and 3, but particularly SiPC 1, showed high abilities to induce tumor cell photokilling, being therefore excellent candidates for further *in vivo* studies to be used for PDT in cancer.

6. CONCLUSIONS

From the results obtained in our work we can conclude that:

1. Both SiPCs show excellent photosensitizing properties to destroy breast, cervix and skin cancer cells in culture.
2. SiPC 1 and 3 can enter into the cancer cell lines in 2D and 3D (spheroids) models, being located both of them in lysosomes.
3. ROS generation produced after the phototreatment is the main factor for cell damage and thus for tumoral cell death.

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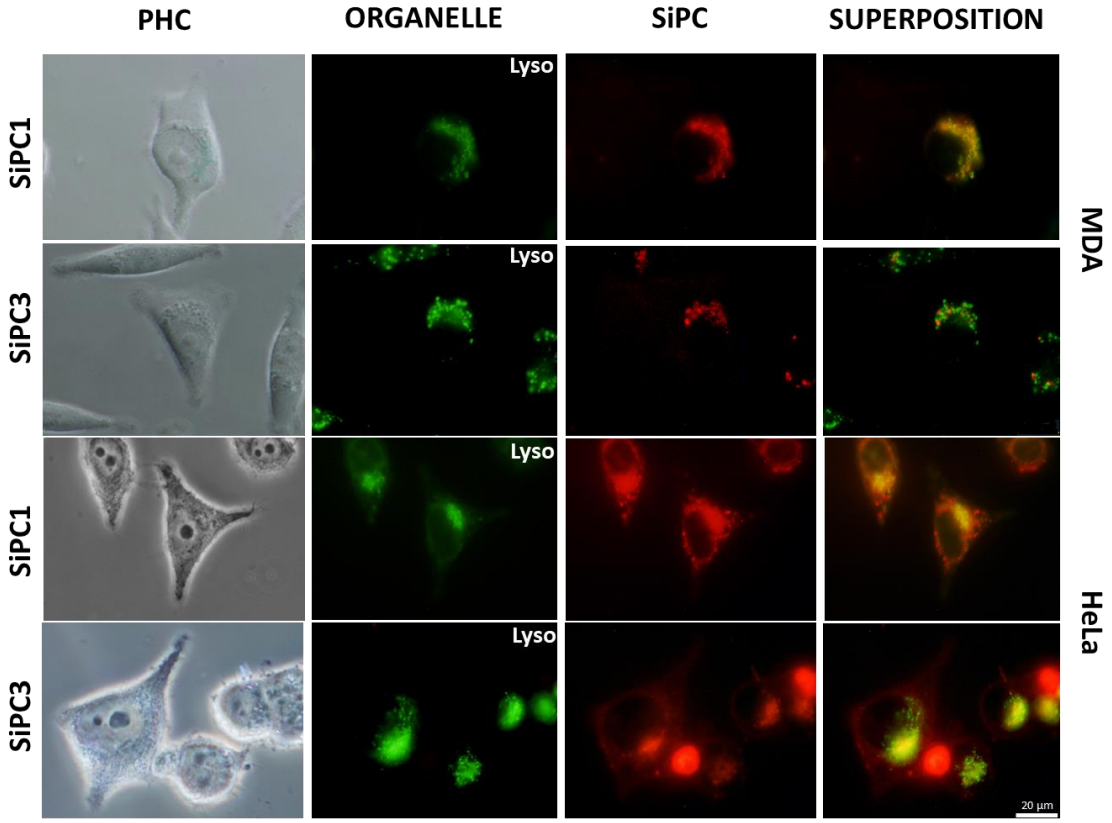
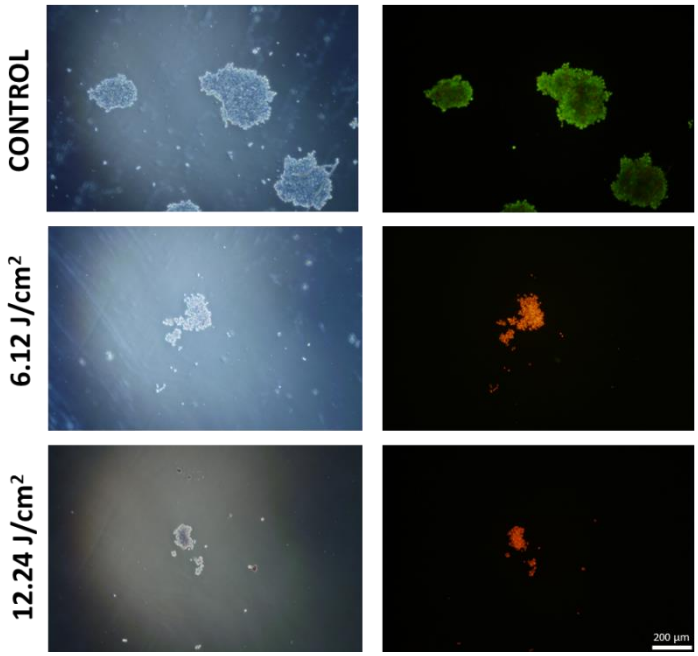
ANNEXED



Annexed 1. Localization of SiPC1 in MDA and SCC spheroids. Cells were treated with 1µM of SiPC 1 for 15h followed by the incubation with the fluorescent probes (Mitotracker® or Lysotracker®). The first column corresponds to the cells observed under contrast phase microscopy, the second column to the organelle fluorescence under blue light irradiation (450-490 nm); the third column to the SiPC fluorescence under green light irradiation (570-590 nm) and the fourth column is the merge of the second and third columns. The yellowish colour in lysosomes indicates that the SiPC co-localize with these organelles.



Annexed 2. **Photodamage effects in spheroids.** Stain of MDA spheroids with acridine orange and ethidium bromide, where green spheroids are alive and orange ones are dead. The diameter of the spheroids decreases when higher red light doses are applied.