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ZINC(II) PHTHALOCYANINES AS PHOTOSENSITIZERS FOR ANTITUMOR PHOTODYNAMIC THERAPY

Running Title: Zinc(II) phthalocyanines as phototoxic agents

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To the memory of Prof. Dr. Josefina Awruch

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Highlights

- We present an updated summary on ZnPcs as antitumor photosensitizers
- ZnPcs localized in lysosomes, mitochondria or ER are efficient antitumor agents
- Apoptosis, necrosis and autophagy are the main cellular responses induced by ZnPc PDT
- Current efforts to improve *in vivo* PDT treatments with ZnPcs are discussed
- More clinical trials are required to evaluate ZnPcs as therapeutic agents

Abstract

Photodynamic therapy (PDT) is a highly specific and clinically approved method for cancer treatment in which a nontoxic drug known as photosensitizer (PS) is administered to a patient. After selective tumor irradiation, an almost complete eradication of the tumor can be reached as a consequence of reactive oxygen species (ROS) generation, which not only damage tumor cells, but also lead to tumor-associated vasculature occlusion and the induction of an immune response. Despite exhaustive investigation and encouraging results, zinc(II) phthalocyanines (ZnPcs) have not been approved as PSs for clinical use yet. This review presents an overview on the physicochemical properties of ZnPcs and biological results obtained both *in vitro* and in more complex models, such as 3D cell cultures, chicken chorioallantoic membranes and tumor-bearing mice. Cell death pathways induced after PDT treatment with ZnPcs are discussed in each case. Finally, combined therapeutic strategies including ZnPcs and the currently available clinical trials are mentioned.

Abbreviations: AIF, apoptosis-inducing factor; DCF-DA, 2´,7´-dichlorfluorescein diacetate; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; FAAD, Fas-associated death domian; GA, Golgi apparatus; LDH, Lactate dehydrogenase; NP, nanoparticles; PARP, poly(ADP-ribose) polymerase; PDT, photodynamic therapy; PEG, polyethylene glycol; PS, photosensitizer; Pcs, phthalocyanines; ROS, reactive oxygen species; UCNPs, upconverting nanoparticles; ZnPcs, zinc(II) phthalocyanines; ZnNcs, zinc(II)-naphthalocyanines.

***Keywords*:** Photodynamic therapy; Zinc phthalocyanines; Photophysical properties; *In vitro* antitumor assays; *In vivo* tumor models

1. Introduction

The first approaches to photodynamic therapy (PDT) are found in ancient texts such as the Egyptian medical treatise *Ebers Papyrus* and the *Atharvaveda* from the Hinduism. Despite this background, the observation of photochemical sensitization of tissues was first performed in detail by Raab (1900) in Germany and shortly afterwards by Von Tappeiner (1900), who coined the term "photodynamic action" to describe the treatment of skin tumors by using topical administration of eosin combined with sunlight (Kou et al., 2017). PDT is a therapeutic procedure based on the administration of a non-toxic photosensitizer (PS), which after activation by visible light in the presence of molecular oxygen, produces cytotoxic reactive oxygen species (ROS) that lead to cell damage (Henderson and Dougherty, 1992; Sharman et al., 1999; Detty et al., 2004; Plaetzer et al., 2009). Since the PS is preferentially localized into the cells to be treated and is activated only after irradiation of these cells, the PDT is a selective procedure frequently used for the treatment of a diversity of dermatological, ophthalmic and oncological diseases (Agostinis et al., 2011; van Straten et al., 2017). In clinical use, after topical or intravenous administration of a PS, the patient is irradiated with light of a suitable wavelength in the area to be treated and then maintained in the dark to minimize the probability of PS activation in other body regions. PS clearance

from the organism will allow the patient to resume usual activities with exposure to both solar and artificial light.

At molecular level, light irradiation at a specific wavelength delivers the necessary energy to produce an electronic transition of the PS from its ground state to an excited singlet state of short half-life. The loss of energy caused by the return of electrons from the excited level to the basal energy level (deactivation process) may involve fluorescence/heat emission or intersystem crossing to generate a lower energy excited triplet state. Deactivation of this triplet species can occur mainly by two mechanisms, known as type I or type II reactions, that generate reactive oxygen species (ROS) (Agostinis et al., 2011; Oliveira et al., 2011; Gomes et al., 2018). In type I reactions, the PS transfers an electron to biological substrates to form free radicals, which finally give rise to cytotoxic species such as superoxide anion, hydrogen peroxide and hydroxyl radicals. In type II reactions, the triplet oxygen ($^3O_2$) is converted into singlet oxygen ($^1O_2$), a highly cytotoxic species with a very short half-life, between 10 and 320 ns (Agostinis et al., 2011). As this species can diffuse 10-55 nm on average, a distance that represents one thousandth of the diameter of eukaryotic cells, the intracellular organelles containing the PS are considered to be the main sites where the phototoxic damage is triggered. The final consequence of the ROS formation is the induction of a cell death process, the destruction of the microvasculature and the activation of a local inflammatory reaction that may trigger an immune response (Engbreht et al., 1999; Castano et al., 2006; Buytaert et al, 2007; van Straten et al., 2017).

Many photo-activable molecules, including porphyrins, phthalocyanines, chlorines and bacteriochlorine derivatives, among others, have been found to be useful candidates for PDT applications (Gomes et al., 2018; Kou et al., 2017). In the 70´s, the use of PDT as a therapeutic option generated a renewed interest in the oncological field from the performance of systematized trials for tumors treatment (Diamond et al., 1972). Dougherty et al. (1975,

1978) carried out the first clinical trial in patients with Photofrin® as photosensitizer. This PS together with other porphyrin derivatives formed the first generation of PSs. This group of PSs showed some unfavourable characteristics, including low absorptions in the red light region (range between 600 to 800 nm, where tissue light penetration is high), poor solubility in polar solvents and skin phototoxicity (Allison and Sibata, 2010; Kou et al., 2017; Gomes et al., 2018). Due to this last feature, the patient has to take extreme precautions from environmental light exposure. The second generation of PSs emerged either from structural modifications made on the macrocycles of the first generation or from changes in the synthetic routes to generate new families of molecules. These new compounds, more similar to what is expected for an ideal PS, include chlorine or bacteriochlorin, benzoporphyrin (or new porphyrin derivatives), purpurins, texaphyrins and phthalocyanines (Kou et al., 2017; Gomes et al., 2018). Among these PSs, the phthalocyanines (Pcs) have drawn attention as promising antitumor phototoxic drugs. Besides various physicochemical characteristics that will be mentioned later, several Pcs coordinated to zinc, aluminum or silicon are very efficient generators of singlet oxygen (Paquette et al., 1991; Boyle et al., 1992; Margaron et al., 1996; Colussi et al., 1999). Afterwards, the third generation of PSs was developed to promote a higher and more specific accumulation of PS in target cells. The main strategies employed to achieve this objective involve PS binding to antibodies (Kudarha and Sawant, 2017; Pereira et al., 2014a) or incorporation to nanocarriers (Abrahamse et al., 2017; Yang et al., 2017).

In this review, among the extensive list of recognized PSs for cancer treatment, we focus on the characteristics of zinc(II) phthalocyanines (ZnPcs), as many variants of this type of photo-activable drugs with improved physicochemical and biological properties have been developed in the last years (Figure 1). The photophysical properties of ZnPcs, the *in vitro*

antitumor mechanism of action in 2D and 3D cell cultures, and the *in vivo* effectiveness as anticancer agents in animal models and clinical trials will be herein summarized.

2. Physicochemical properties of zinc(II) phthalocyanines

2.1. Zinc(II) phthalocyanines for PDT

Phthalocyanines are synthetic dyes that were first obtained by Braun and Tcherniac in 1907. Pcs are aromatic heterocycles that consist of four units of isoindoles bridged by nitrogen atoms. They have a characteristic UV-visible absorption spectrum with two main bands: the weak Soret band located in the UV zone of the spectrum at ~350 nm and the Q band, located in the red zone of the visible spectrum at around 680 nm, whose high intensity is one of the main characteristics of these dyes with a molar extinction coefficient of $1x10^5$ M^{-1} cm^{-1}, two orders of magnitude higher than most porphyrins (Leznoff and Lever, 1989). Their photochemical properties are strongly influenced by the nature of the coordinated central metal ion. Thus, the presence of diamagnetic elements in the central cavity, such as zinc (II), aluminum (III), gallium (III) or silicon, gives them high triplet quantum yields ($\Phi_T > 0.4$) with long triplet lifetimes ($\tau_T > 100$ μs) and adequate triplet energies ($E_{T1} = 1.2$ eV), making them efficient generators of singlet oxygen ($\Phi_\Delta > 0.4$) (Lagorio et al., 1993).

ZnPcs have advantageous characteristics including low dark toxicity, high chemical and photochemical stability, high therapeutic effect, minimal skin photosensitivity and excitation at wavelengths greater than 630 nm, which allows greater tissue penetration of radiation (Jori, 1992; Marino et al., 2010). Despite these attractive features, one disadvantage of Pcs is their poor solubility in organic solvents (Nyokong and Antunes, 2010). Their solubility can be increased by introducing peripheral (β position) and non-peripheral (α position) substituents in the Pc framework (Figure 1). The tetrasubstituted Pcs are usually more soluble than octasubstituted due to the formation of constitutional isomers and the high

dipole moment resulting from the asymmetric rearrangement of the substituents (Leznoff et al., 1985). However, the four possible structural isomers of a tetrasubstituted Pc are difficult to separate and purify (Hanack et al., 1993).

On the other hand, the formation of aggregates in solution decreases the capacity to produce singlet oxygen because the photochemical activity is exclusively related to monomer species. Thus, aggregates decrease not only the photoactivity of the PS but also limit the access to the neoplastic cells, affecting its bioavailability. The presence of bulky or electrically charged peripheral substituents bound to the macrocycle and/or axial ligands coordinated to the central metal minimizes the tendency to form aggregates (García Vior et al., 2009, Wang et al., 2012; Muli et al., 2015). Ke et al. (2009) demonstrated that tetra-α-substituted ZnPcs exhibit a reduced aggregating trend, higher photostability and enhanced photocytotoxicity toward human gastric carcinoma cells than the tetra-β-substituted counterparts. Our group also showed a similar behavior when the photodynamic effect of α and β cationic substitution of ZnPcs was studied in human nasopharynx KB carcinoma cells (Marino et al., 2010).

In order to improve water solubilization of ZnPcs, the synthesis of anionic derivatives containing sulfonic (Haywood-Small et al., 2006; Lan et al., 2016), phosphonic (Venkatramaiah et al., 2015) or carboxylic groups (Zhou et al., 2016) has been carried out. For instance, Zhou et al. (2016) demonstrated that a ZnPc substituted with 16 COOH groups existed in its monomeric form under physiological conditions and showed superior $^1O_2$ generation than other derivatives containing less COOH groups. To control the degree of aggregation in aqueous media, Ikeuchi et al. (2016) synthetized a water-soluble ZnPc bearing four 3-sulfone-linked propylsulfonyl at non-peripheral positions. Other authors have also demonstrated the importance of positive charges in ZnPcs (Wood et al. 1997, Li et al., 2008; Sekhejane et al., 2014; Wang A. et al. 2014). In this sense, we studied the synthesis and

photophysical properties of cationic bioisosteric ZnPcs containing peripheral chains bound to the macrocycle by oxygen, sulfur or selenium (García Vior et al., 2009; Marino et al. 2010; Gauna et al., 2011, Ezquerra Riega et al., 2018). A bathochromic shift of 8–10 nm for the Q-bands of ZnPcs was observed when sulfur or selenium were present, allowing the excitation of PSs in deeper regions of a tissue. In addition, although selenium ZnPcs reported the highest value of quantum yield of Φ_Δ, both selenium and sulfur substituted ZnPcs showed similar phototoxic activities in colon carcinoma cells (Ezquerra Riega et al., 2018).

2.2. Third generation zinc(II) phthalocyanines

Even though lipophilic PSs show an increased tumor to normal tissue ratio (Stockert et al., 2004), the intravenous administration is difficult. To overcome this issue and avoid post-PDT skin photosensitivity, the so-called third generation PSs have been developed. They comprise second generation PSs associated to a carrier able to promote PS accumulation in tumor cells, to reduce aggregation tendency and minimize localization in normal cells. On these bases, formulation of ZnPcs in different nanosized delivery systems has been attempted. In the last years the most studied carriers include liposomes (de Oliveira et al., 2010; Garcia et al., 2011; López Zeballos et al., 2013; Kim et al., 2014), polymeric micelles (García Vior et al., 2013; Pucelik et al., 2016; Lamch et al., 2016; Debele et al., 2017) and different types of nanoparticles (NPs) (Camerin et al., 2010; Ping et al., 2016; Oluwole et al., 2016; Yurt et al, 2017), all of which showed an increase in the bioavailability, stability and transport of ZnPcs to the target tissue. Our group, after studying the incorporation of a lipophilic tetrasubstituted ZnPc into polymeric micelles and different liposomes, demonstrated a similar increase in solubility for both carriers, but a higher photodynamic activity against tumor cells in micellar formulations (López Zeballos et al., 2013; García Vior et al., 2013; Chiarante et al., 2017). ZnPc molecules have been also encapsulated into NPs of different nature, such as polymeric NPs (Conte et al., 2013; Ping et

al., 2016; Huang et al., 2016), TiO_2 NPs (Yurt et al, 2017; Lopez et al., 2010), gold NPs (Camerin et al., 2010; Manoto et al., 2017a; Mfouo-Tynga et al., 2018a, 2018b; Dube et al., 2018; Garcia Vior et al., 2019) and silica based NPs (Tu et al., 2012; Oluwole et al., 2016). Ricci-Junior et al. (2018), after evaluating the photobiological activity of three nanosystems (nanoparticles, nanoemulsions and mesoporous silica) containing ZnPc, demonstrated that all of these formulations could be used for clinical purposes in PDT. Furthermore, upconverting nanoparticles (UCNPs), based on the absorption and conversion of near infrared light to visible photons that can further activate the PS, have been efficiently employed as PDT carriers for ZnPcs (Guo et al., 2010; Tian et al., 2013; Wang H. et al., 2014a; Hou et al., 2015).

Targeting moieties that recognize specific sites expressed in tumor cells have been coupled to nanocarriers to improve ZnPcs accumulation. For instance, ZnPcs surrounded by a carbohydrate shell of galactose (Pereira et al., 2014b) or lactose (García Calavia et al., 2018) units have shown to increase tumor selectivity, since cancer cells are known to overexpress carbohydrate-binding proteins, such as galectin-1 and the glucose GLUT-1 transporter. Folic acid incorporated into micelles (Liang et al., 2014a) or UCNPs (Cui et al., 2013) has been employed for targeting cancer cells overexpressing folate receptor. Wang H. et al. (2014b) demonstrated the efficacy of ZnPc and UCNP encapsulated into lipid micelles carrying cell-penetrating peptides. Likewise, ZnPcs conjugated with peptides or antibodies directed to EGFR have been properly incorporated by cells overexpressing EGFR (Ongarora et al., 2012a; Broekgaarden et al., 2016a). UCNPs conjugated with ZnPc and the monoclonal antibody Trastuzumab were designed by Ramirez Garcia et al. (2018) for specific HER2-positive breast cancer detection and PDT.

3. *In vitro* photodynamic assays of zinc(II) phthalocyanines

3.1. ROS formation, cellular uptake and intracellular localization

As already mentioned, an appropriate PS should be capable of inducing an efficient formation of ROS after being irradiated. Among these phototoxic species, the main reacting species *in vivo* seems to be singlet oxygen (Agostinis et al, 2011; Gomes et al., 2018). Although this species has short life-time and short radius of action, cell damage is initiated in the intracellular sites where the PS is localized. Consequently, the oxidative damage begins the cascade of molecular events that finally lead to cell death (Oleinick et al., 2002; Chiu et al., 2010; Kessel, 2004). Based on the complexity imposed by cellular models, the ability of PSs to generate ROS has to be tested not only in fluid media but also in cellular environments. To this end, intracellular ROS content can be measured by using a fluorescent probe, such as 2´,7´-dichlorfluorescein-diacetate (DCFH-DA). This probe, after diffusing into cells, is deacetylated by esterases and then oxidized to the fluorescent 2´,7´-dichlorfluorescein (DCF) in the presence of ROS (mainly hydrogen peroxide and lipid hydroperoxides). DCF fluorescence is in general detected by fluorescence microscopy, flow cytometry or with a fluorometer. All the ZnPc-based photosensitizers studied so far in cell cultures showed high ROS generation quantum yields, revealing that they behave as competent PSs for PDT applications (Yu W et al., 2018; Nag et al., 2018; Wang et al., 2017; Lu et al., 2016). In spite of this property, it should be taken into account that tumor hypoxia might reduce the antitumor efficacy of PDT due to the activation of the hypoxia-inducible factor 1α (HIF-1α). In this regard, it was demonstrated that combined treatments of ZnPc with a HIF-1α inhibitor improved PDT efficacy in human carcinoma cells (Weijer et al., 2016; Broekgaarden et al., 2016b).

The intracellular PS location, which determines both the primary site of photodamage and the mechanisms of cell death, is markedly influenced by the structural characteristics of the PS and the type of cell. In addition, cellular uptake of ZnPcs is a time- and concentration-

dependent process. These fluorescent dyes are distributed homogeneously in the cytosol, as shown by confocal microscopy studies, and no evidence of nuclear localization has been detected. The mechanism of ZnPcs internalization seems to be dependent on the vehicule used to dissolve the PS. In this sense, Soriano et al. (2013) demonstrated that caveolin-mediated endocytosis was responsible for internalizing a ZnPc dissolved in dimethylformamide, while an endocytic pathway dependent on clathrin mediated the incorporation of ZnPc included in liposomes.

By using organelle-specific fluorescent probes, ZnPcs have been mainly found in mitochondria, lysosomes, endoplasmic reticulum (ER), plasma membrane or Golgi apparatus (GA) (Figure 2). Despite the variety of studies on the main sites of intracellular accumulation of ZnPcs, it is still not very simple to establish a clear relationship between the subcellular localization and the structural characteristics of these PSs. For instance, by working with an unsubstituted ZnPc, Fabris et al. (2001) showed that a liposomal formulation of the PS mainly localized in GA and plasma membrane after 2 h of incubation with transformed fibroblast, whereas both GA and mitochondrial localization were found after 24 h of exposure. Soriano et al. (2014), using a liposomal formulation of the same PS in HeLa cells, reported a similar intracellular localization after short incubation periods. Although these results suggested that the incubation time is a variable that must be considered in subcellular localization studies, Cristobal et al. (2006) found that a ZnPc/liposomal formulation localized in GA, but not lysosomes or mitochondria, after either 3 or 18 h of incubation with lung carcinoma cells.

The localization of a ZnPc dissolved in dimethylformamide or incorporated into a network of titanium dioxide was studied by Lopez et al. (2010) after 24 h of incubation in different cell lines. These authors reported an exclusive mitochondrial or lysosomal localization of the PS tested depending on the cell line studied. In 2013, Shao et al., with the

aid of specific organelle-probes, concluded that the unsubstituted ZnPc solubilized in the Cremophor EL formulation mainly localized in mitochondria, lysosome and ER after 24 h of incubation with hepatocellular carcinoma cells. Based on the dissimilar findings found with different formulations of unsubstituted ZnPc, it is possible to conclude that either the intrinsic properties of each formulation, the type of cell, the incubation time or the fluorescent probes employed to detect subcellular organelles could influence the results obtained.

Regarding studies performed with substituted ZnPcs, Wood et al. (1997) showed that while anionic and cationic polysubstituted ZnPcs localized in lysosomes, neutral ZnPcs accumulated in GA. Afterwards, a great number of reports showed that either mono or polysubstituted ZnPcs target mainly to mitochondria and/or lysosomes, and in a lower degree to ER. In this regard, the mitochondrial and lysosomal localization of mixed sulfonated ZnPc (ZnPcSmix) has been shown in different cell types (Manoto et al, 2012; Manoto el al, 2013; Mfouo-Tynga et al., 2013; Sekhejane et al, 2014). The same distribution pattern was observed by Li et al. (2012) working with ZnPc-$(Lys)_n$, a group of amphipathic monosubstituted ZnPcs conjugated to different numbers of lysine residue. Therefore, both mitochondrial and lysosomal localization have been reported for either negatively or positively charged PSs. Similarly, independently of the charge or the hydrophobic/hydrophilic nature of the PS, just a lysosomal accumulation was reported for hydrophobic ZnPcs, such as sulfur-linked octaalkylamino substituted ZnPc (Rumie Vittar et al., 2008), methoxy (Yslas et al., 2007) or amine-terminated monosubstituted ZnPcs (Peng et al., 2017), and hydrophilic ZnPcs, as cationic sulfur-linked (Gauna et al., 2011) or arginine substituted ZnPcs (Wang et al., 2017).

A series of cationic ZnPcs conjugated to a bifunctional peptide were only found in lysosomes (Sibrian-Vazquez et al., 2007). On the other hand, Ge et al. (2013) reported the design and synthesis of an specific mitochondria-targeted ZnPc substituted with a quaternary

ammonium salt on the periphery, whereas Muli et al. (2008), based on the properties of rhodamine B to target mitochondria, described the synthesis and characterization of asymmetric ZnPc-rhodamine B conjugates for selective mitochondrial targeting. Duan et al. (2010) also showed a selective affinity to the mitochondria of a non-ionic 2-(dimethylamino)ethylthio ZnPc. In addition, the effect of the formulation on the intracellular localization of ZnPcs should not be neglected. In this sense, Lan et al. (2016) described a restricted mitochondrial localization or a mitochondrial/lysosomal localization for a ZnPc substituted with sulfonated quinolineoxy groups and formulated either with Cremophor EL or PBS. It should also be considered that the exclusive mitochondrial localization of ZnPc-based PSs found in several reports responds to the employment of just a specific mitochondrial probe (Oluwole et al., 2017; Ke et al., 2017; Huang et al., 2016; Lo et al., 2007; Alexandratou et al., 2005).

Our group demonstrated a lysosomal localization of a water-soluble tetrasubstituted ZnPc replaced with sulfur-linked cationic aliphatic chains in the human nasopharynx KB carcinoma cell line (Marino et al., 2010). The same cationic phthalocyanine targeted lysosomes and mitochondria in murine melanoma cells (Valli et al., 2019), reinforcing the concept that the intracellular location may vary according to the cell line studied. We also reported a preferential lysosomal localization of a lipophilic ZnPc (Pc9: tetrakis-[(2-dimethylamino)ethylsulfanyl]phthalocyaninatozinc(II)) loaded either into liposomes or poloxamine micelles in KB cells (García Vior et al., 2013; López Zeballos et al., 2013). Since both formulations showed different phototoxic potencies (IC_{50} values were in the range of 0.21-0.47 μM for Pc9-loaded into liposome formulations and 9.5-22 nM for Pc9-micelles formulations), the results herein obtained suggested that PS location was independent of the vehicle used and other factors, such as a differential uptake, could explain the variation in potency. At comparable intracellular concentrations, Garcia et al. (2016) demonstrated that

the intracellular fate of the PS did depend on the nature of the vehicle used. In this case, when the location of an unsubstituted ZnPc internalized in liposomes or BSA was studied, ZnPc:BSA preferentially located in GA, while the liposomal formulation was mostly located in the cellular membrane. In the last years, as interest in the role of ER in phototoxic cell death increased, various studies focused on this organelle as possible localization site. However, it must be taken into account that while lysosomal and mitochondrial probes have been extensively used for the study of ZnPcs, ER probes have been employed to a lesser degree. As mentioned before, Shao et al. (2013) verified the ER location of a ZnPc/Cremophor formulation by using an ER fluorescent Tracker. Likewise, when Manoto et al. (2012) demonstrated the mitochondrial and lysosomal distribution of ZnPcSmix in different tumor cell types, they also verified the absence of co-localization in the ER with a specific probe. Ongarora et al. (2012b), after studying the subcellular distribution of a series of cationic ZnPcs in tumor cells, showed that although these compounds localized in multiple sites within the cell (such as mitochondria, lysosomes, ER and GA), the most active ones -containing a PEG group- were mainly found within the ER, suggesting that pegylation could favor the intracellular localization in the ER. Fujishiro et al. (2018) also reported that a cationic ZnPc carrying N-methyl-pyridinium groups was observed in greater amounts in the ER, Golgi, and lysosomes, whereas Kiew et al. (2017), working with a ZnPc conjugated to poly-L-glutamic acid, revealed a high co-localization in lysosomes and partial co-localization with the ER and mitochondria. In our laboratory, we explored the intracellular distribution of a micellar formulation of a lipophilic ZnPc in CT26 colon cells, and found preferential photosensitizer incorporation into lysosomal vesicles and ER cisterns, but not mitochondria (Chiarante et al., 2017).

In summary, since the subcellular localization of PSs is directly related to the mechanisms involved in cell oxidative damage, we strongly believe that a rigorous study of

the subcellular localization of ZnPcs, using a complete battery of organelle-specific probes, is an important step to achieve a clear understanding of the type of cell death.

3.2. Mechanisms of cell death

The photo-oxidative damage caused by PDT in tumor cells may activate different modalities of cell death, which can act independently, simultaneously or in combination, depending on the nature of PS, the administered dose, the cell type and the light exposure dose. Accordingly, for each tumor cell line treated under specific experimental conditions with a particular type of PS, the intracellular localization of the PS strongly determines the mode of cell death. The most frequent cellular responses induced in 2D or 3D cultures after PDT treatment include apoptosis, necrosis and autophagy (Butyaert et al., 2007; Agostinis et al., 2011; Mroz et al., 2010) (Figure 2). More recently, but only in 2D cultures, paraptosis has been described as a type of cell death induced by PSs that target the ER for photodamage, but it has not been described yet for ZnPcs (Kessel, 2018; Kessel and Oleinck, 2018; Kessel, 2019). Apoptosis has been recognized as a frequent mechanism associated to the photo-oxidative damage induced by PDT (Buytaert et al., 2007) and has been extensively characterized after ZnPc-induced photodamage. This cell death programme is mediated through the activation of either death receptor or mitochondrial-dependent pathways (extrinsic and intrinsic pathways, respectively), usually regulated by caspases (Grütter, 2000; Kaufmann and Hengartner, 2001; Galluzzi et al., 2012).

3.2.1. Death receptor-dependent apoptosis: although the extrinsic pathway of apoptosis can contribute to the PDT-induced apoptotic response (Butyaert et al., 2007), few evidence of the involvement of this pathway after photodamage triggered by ZnPcs are found. The activation of the receptor death machinery has been suggested by Machado et al. (2009) who showed that Fas dimerization induced by p38MAPK activation in tumor cells treated with a ZnPc favors the formation of the Fas-FADD death complex needed to activate caspase 8. On

the other hand, the decrease of Fas levels reported by Xia et al. (2011), after PDT treatment with a tetra-α-(4-carboxyphenoxy) ZnPc, suggested indeed an alteration of the death receptor complex assembly. Therefore, it seems that more than a direct involvement of this pathway, some molecular signals induced after ZnPcs PDT could be responsible for the activation or inhibition of an extrinsic apoptotic response.

3.2.2. Mitochondrial-dependent apoptosis: since mitochondrial homeostasis is essential for cell viability, the damage to this organelle after ZnPcs PDT has been extensively studied taking into account both the mitochondrial localization of PSs and a possible downstream effect due to the injury initiated at other subcellular locations. Caspases activation, deregulation in the expression levels of Bcl-2 family proteins, reduction of mitochondrial membrane potential, cytosolic release of cytochrome c, PARP cleavage and chromatin condensation, among others, have been characteristics widely explored following ZnPcs PDT (Figure 3). Thus, after treatment, activation of caspases 8 and 9, leading to the cleavage of procaspase 3, has been reported in different cell types (Shao et al., 2013; Wang Y et al., 2014; Doustvandi et al., 2017; Wang Y et al., 2018a). A time- and dose-dependent increase of the executioner caspase 3 has been described after irradiation of both unsubstituted and substituted ZnPcs (Fabris et al., 2006; Shao et al., 2012; Kuzyniak et al., 2017). Interestingly, an apoptotic caspase-independent mechanism has been described after irradiation of a water-soluble octakis(3-aminopropyloxy) ZnPc in a human breast adenocarcinoma cell line (Rumie Vittar et al., 2010). In this work, the authors showed that ZnPc PDT induces the translocation of the apoptosis-inducing factor (AIF) to the nucleus, a mediator involved in DNA fragmentation in a caspase-independent manner (Figure 3). Also noteworthy, Doustvandi et al. (2017), after exploring the apoptotic response induced by a ZnPc at different light doses, suggested that an increase in light dose could switch a caspase-independent apoptotic mechanism to a caspase-dependent one.

Regarding the involvement of other caspases, some insights have been reported about the role of caspase 2 after PDT with ZnPcs. Thus, Cristóbal et al. (2006) demonstrated that caspase 2 was rapidly activated after treatment with a ZnPc liposomal formulation exclusively located in GA and suggested that this activation preceded the disorganization of the organelle to trigger an apoptotic response. Later, Mfouo-Tynga et al. (2014), by using ZnPcSmix, showed that the proteolytic caspase 2 cleaves and activates Bid into its truncated form (tBid) to promote mitochondrial permeabilization. Other studies with substituted ZnPcs also demonstrated that Bid cleavage occurs after irradiation (Rumie Vittar el al., 2010; Marino et al, 2013; Chiarante et al., 2018), although, in most reports, caspase 8 and lysosomal proteases have been identified as the main enzymes responsible for Bid fragmentation (Butyaert el al, 2007; Kessel and Oleinick, 2018) (Figure 4).

The unbalance in the expression levels of Bcl-2 family proteins unquestionably plays a role in the mitochondria-mediated cell death (Figure 3). A down-regulation of antiapoptotic Bcl-2 proteins (Machado et al., 2009; Dai et al., 2016; Doustvandi et al., 2017; Zamani et al., 2018) and up-regulation of some apoptotic proteins, such as Bak (Liu et al., 2017) and Bax (Machado et al., 2009; Dai et al., 2016; Zamani et al., 2018), have been commonly informed after PDT treatment with different formulations of ZnPcs in a wide variety of cell types. In some cases, although Bax levels remained unchanged after irradiation (Marino et al., 2013; Kuzyniak et al., 2016), a clear translocation of this protein from cytosol to mitochondrial membrane suggested its involvement in the opening of mitochondrial pores (Rello-Varona et al., 2008; Acedo et al., 2014). As a consequence of changes in Bcl-2 proteins, ZnPcs PDT treatment leads to the loss of mitochondrial membrane potential regardless of the chemical nature of the PS employed (Alexandratou et al., 2005; Huang et al., 2005; Shao et al., 2013; Ge et al., 2013) and the release of apoptogenic molecules, such as cytochrome c, to the cytosol (Medina et al., 2009; Marino et al., 2013; Soriano et al., 2014). The cytosolic release

and nuclear translocation of other mitochondrial apoptogenic species, such as AIF, have also been demonstrated after irradiation of some substituted ZnPcs (Rumie Vittar et al., 2010; Wang Y et al., 2018a).

3.2.3. Lysosomal membrane permeabilization: PSs mainly located in lysosomes are expected to permeabilize lysosomal membrane upon light activation and promote mitochondrial injury through the release of lysosomal proteases into the cytosol (Figure 4). The contribution of lysosomal enzymes to the apoptotic response following photodamage has been earlier described (Reiners et al., 2002; Cirman et al., 2004; Ichinose et al., 2006). In particular, the first reports of ZnPcs affecting lysosomal integrity involved fluorescence microscopy studies. These works showed either a loss of the fluorescent punctuate staining of the specific LysoTracker Green probe after irradiation of different tumor cells (Yslas et al., 2007; Rumie Vittar et al., 2008; Manoto et al., 2013) or the lost of the acridine orange probe from intact acidic lysosomes (Marino et al., 2013; Sun et al., 2017; Chiarante et al., 2018). Studies performed in our laboratory either with a cationic (Marino et al., 2013) or a lipophilic (Chiarante et al., 2018) ZnPc located in lysosomes revealed that PDT treatment promotes the cytosolic release of the lysosomal protease cathepsin D in a ROS-dependent manner, since the expression levels of cathepsin D significantly diminished in the presence of antioxidants. We further showed that Bid cleavage was inhibited in the presence of a Pepstatin A, a cathepsin D inhibitor, supporting a role of cathepsin D in Bid activation (Marino el al., 2013). In addition, the reduction of caspase 8 activity observed after incubation of ZnPc-treated with Pepstatin A supported the involvement of cathepsin D in caspase 8 activation (Chiarante et al., 2018) (Figure 4).

3.2.4. ER stress: as a key organelle involved both in the folding and trafficking of newly synthesized proteins as well as in the maintenance of Ca^{2+} homeostasis, ER photodamage after PDT can contribute to an apoptotic cell death (Buytaert et al., 2007; Moserova and

Kralova, 2012). In the field of ZnPcs, Yu L et al. (2018) reported the ER localization of a biotinylated glutathione-responsive ZnPc that triggers ER stress after light irradiation. Studies performed in our laboratory with a lipophilic ZnPc also showed that ER stress induced by PDT can be propagated to the mitochondrial apoptotic pathway (Chiarante et al., 2018). In both reports, an increase of intracellular Ca^{2+} concentration and higher expression levels of ER stress marker proteins, such as BIP/GRP78 and CHOP/GADD153, were demonstrated after photoactivation (Figure 5). Remarkably, we also demonstrated that calpains, activated by the high cytosolic Ca^{2+} concentrations reached after ER stress, contribute to Bax proteolytic damage (Chiarante et al., 2018). This Bax fragmentation, as it was shown in other apoptotic processes, could favor the formation of a more apoptotic fragment (Toyota et al., 2003).

3.2.5. Necrosis: the first works addressing the mechanism of tumor necrosis showed areas of necrotic degeneration in tumor tissues treated by PDT with unsubstituted ZnPcs (Milanesi et al., 1990; van Leengoed et al., 1994; Ruck et al., 1996; Winsborrow et al., 1997). Necrotic regions were also described following *in vivo* PDT treatment with more complex formulations of ZnPcs (Allémann et al., 1997; Hu et al., 1998; Porthilo et al., 2013). Since tumor necrosis was defined by the analysis of histological tumor sections or the macroscopic appearance of PDT-treated tumors, it should be taken into account that these necrotic areas do not actually reflect the mechanism of cell death. Indeed, in cell cultures, necrosis is characterized by several features, including extensive plasma membrane damage, cell swelling, release of cellular contents to the environment, swelling of cytoplasmic organelles and moderate chromatin condensation (Galluzzi et al., 2007). In the field of PDT, although several of the typical characteristics of necrosis were identified in tumor cell lines after treatment with ZnPc formulations, sometimes the necrotic process might be secondary to a late apoptosis (de Oliveira et al., 2010; Broekgaarden et al., 2015). In addition, it is generally

considered that the necrotic cell death induced by photodamage predominates at high doses of PDT (Oleinick et al., 2002). In this regard, Yslas et al. (2007), by working with a 0.5 μM concentration of a substituted ZnPc, showed that PDT treatment can induce either necrosis or an apoptotic cell death depending on the light dose used. Thus, while an apoptotic pathway occurs mainly at a light dose of 11 J/cm^2, necrosis predominates at 29 J/cm^2. Similarly, Acedo et al. (2014) demonstrated that the simultaneous administration of a ZnPc and a cationic porphyrin changes the mechanism of cell death from apoptosis to necrosis when the light dose increases from 2.4 to 3.6 J/cm^2. Interestingly, our group reported a dual apoptotic and necrotic response triggered by a sulfur-linked cationic ZnPc located both in lysosomes and mitochondria in melanoma cells. In this case, although necrosis increased at high light doses or PS concentrations, this type of cell death was also detected simultaneously with the activation of an apoptotic response after irradiating cells with 340 mJ/cm^2 (Valli et al., 2019).

Fabris et al. (2001) showed that necrosis represents the main mode of death of transformed fibroblasts incubated with a liposomal formulation of an unsubstituted ZnPc mainly localized in the GA and plasma membrane after short periods of incubation, whereas after 24 h of exposure, morphological changes typical of apoptosis were observed when the Pc was found in GA and also in mitochondria. Soriano et al. (2014) reported a necrotic cell death induced after PDT with a ZnPc located in the plasma membrane, but the induction of a process of regulated necrosis (necroptosis) when the PS is located in the GA. Kim et al. (2014) developed a liposomal delivery system to localize an hydrophobic ZnPc selectively into the plasma membrane and showed that a substantial membrane disruption occurs upon irradiation, leading to a necrosis-like cell death. Thus, in a broad sense, the occurrence of PDT-induced apoptosis or necrosis may depend on several factors, including the PS used and its intracellular localization, the light dose, incubation times, the cell type and even the nature of the formulation. About the influence of the vehicle, de Oliveira et al. (2010), after

evaluating the phototoxic activity of different ZnPcs liposomal formulations containing cholesterol, showed that the phototoxicity is totally dependent on the presence of cholesterol and cell death of irradiated tumor cells is consistent with necrosis. Later, García et al. (2016) revealed different mechanisms of cell death for ZnPcs, depending both on the delivery vehicle and the PS intracellular location, being apoptosis dominant for ZnPcs formulations preferentially accumulated in GA, and necrosis for those located in cell membrane.

3.2.6. Autophagy: this process displays a dual role, since it may induce either a survival response or contribute to a death pathway (Butyaert et al., 2007; Kessel and Oleinik, 2018). In the field of ZnPcs, Yu et al. (2019) demonstrated that PDT treatment with an unsubstituted ZnPc and the autophagy inhibitor 3-methyladenine inhibited tumor growth in a model of tumor metastasis, indicating that autophagy indeed exerts a protective role. On the other hand, Mfouo-Tynga et al. (2018b), after PDT treatment of breast cancer cells with a novel formulation of ZnPcSmix and gold nanoparticle encapsulated dendrimers, showed an up-regulation of ULK-1, an autophagy gene that has been reported to contribute to cellular damage and death.

4. Photodynamic effect of ZnPcs in 3D cultures and *in vivo* assays

In order to evaluate the contribution of the cellular environment, tumor vasculature and the immunological response in PDT efficacy, more complex tumor models, that resemble more closely what actually happens in the treatment of oncological diseases, are required. To this end, 3D spheroids, chicken chorioallantoic membrane and animal tumor assays have been employed to examine the antitumor efficacy of ZnPcs.

4.1. Multicellular tumor spheroids (MCTS)

These 3D cell aggregates represent models intermediate in complexity between 2D cultures and *in vivo* tumors (Sutherland et al., 1970). A few works employing MCTS showed that photoactivation of ZnPcs could efficiently reduce cell viability. Studies performed in our laboratory with a micellar formulation of a lipophilic ZnPc revealed that although an efficient phototoxic response was observed after treatment of colon tumor spheroids, almost a 40 times higher IC_{50} value was obtained in 3D cultures with respect to 2D cell monolayers (Chiarante et al., 2017). Similar differences in the phototoxic response were observed by Manoto et al. (2015) between lung tumor spheroids and 2D cultures. Both groups also demonstrated the induction of an apoptotic response in MCTS (Chiarante et al., 2017; Manoto et al., 2013; 2015). In addition, the influence of tumor spheroids size has been explored. In this sense, it has been reported that MCTS with a size of 500 μm are more resistant to PDT as compared to MCTS with a size of 250 μm. Since oxygen pressure and nutrients gradually decrease from the spheroid surface to the core, and PDT is an oxygen dependent therapy, the resistance to PDT seen in spheroids might be due to the reduced amount of oxygen found in the inner region of larger MCTS (Madsen et al., 2006; Manoto et al, 2015). Furthermore, a different gene expression profile was observed after ZnPcSmix PDT of tumor cells grown as monolayers or MCTS, suggesting that different mechanisms of action would be involved in 2D or 3D cell photodamage (Manoto et al., 2017b).

Tumor spheroids were also employed to evaluate the efficacy of different carriers to transport lipophilic ZnPcs. For example, natural membrane vesicles derived from tumor cells loaded with ZnPc (Lee et al., 2015) or nanocarriers prepared via ZnPc deposition on TiO_2 (Flak et al., 2017) exhibited high potential as drug delivery agents and behaved as efficient phototoxic agents in tumor spheroids.

4.2. Chicken chorioallantoic membrane assays

As an alternative *in vivo* model, tumor cells can be inoculated on the chicken chorioallantoic membrane of fertilized eggs to evaluate the antitumor and antiangiogenic effect of different compounds (Uchida et al., 1987; Shoin et al., 1991). A total occlusion of chorioallantoic membranes vessels and improved photodynamic properties were observed by Chin et al. (2014) after photoactivation of a substituted ZnPc. Obata et al. (2015) demonstrated a significant growth inhibition of B16-F10 tumor cells transplanted to chorioallantoic membranes of chick-embryos irradiated with trifluoroethoxy-ZnPc formulations conjugated with β-cyclodextrin. Likewise, Kuzyniak et al. (2016; 2017), by working with substituted ZnPcs, showed a significant reduction of tumor growth and changes in the vascular network of chorioallantoic membranes.

4.3. Animal tumor models

Pharmacokinetics studies and the *in vivo* efficacy of ZnPcs have been investigated in different murine tumor models. After systemic administration, the phthalocyanine content in tumor and normal tissues has been usually determined by spectrofluorometric analysis. Reddi et al. (1990) evaluated the pharmacokinetic properties of ZnPc in mice bearing transplanted MS-2 fibrosarcoma and showed an improved selectivity of tumor targeting when low density lipoproteins, instead of liposomes, were employed as drug delivery systems. The slow clearance of ZnPc by the tumor also suggested that PDT effectiveness can be maintained at relatively long time intervals after administration. As a result, an efficient tumor response was obtained when irradiation was performed at 70 h after ZnPc administration. Shopova et al. (1992) also showed the maintenance for 72 h of the concentration of a liposomal ZnPc formulation in tumor tissues of hamsters bearing induced or transplanted rhabdomyosarcoma. Similar results were obtained with zinc(II)-naphthalocyanines (ZnNcs) in a Lewis lung carcinoma model (Shopova et al., 1994).

Different chemical modifications and vehicles have been employed to improve pharmacokinetics in mice, including increased tumor targeting and lower liver and spleen retentions. A study performed with unsubstituted ZnPc and octapentyl or octadecyl ZnPc derivatives in fibrosarcoma-bearing mice showed that the maximal concentration of phthalocyanines in tumors was reached at 24 h post-injection. In addition, a slight increase in the efficiency and selectivity of tumor targeting was obtained upon increasing the length of the alkyl chains protruding from the macrocycle (Fabris et al., 1997; Jori and Fabris, 1998). Thus, both derivatives showed an approximately 50-fold higher concentration in the tumor than in the peritumoral tissue (Fabris et al., 1997). As most of the hydrophobic photosensitizers, Pcs were accumulated in large amounts in the liver and were eliminated from the organism through the bile-gut pathway (Jori, 1990; 2004). When the biodistribution of two substituted ZnPcs was studied, the monosulphonated analogue $ZnPcF_{12}S_1$, which exhibited high phototoxicity in murine tumors, displayed faster hepatic clearance and lower retention by the spleen than the perfluorinated $ZnPcF_{16}$ (Allémann et al., 1997). Camerin et al. (2010) reported the *in vivo* PDT efficiency of a gold NP loaded with a hydrophobic ZnPc derivative (C11Pc). This formulation exhibited good tumor selectivity after 24 h of injection (Camerin et al., 2010). Later, Camerin et al. (2016) described a different delivery vehicle based on the co-self-assembly of C11Pc and a polyethylene glycol derivative (PEG) onto gold NPs. The incorporation of PEG on the particle surface enhanced the half-life of the conjugate in the serum, improving the *in vivo* photodynamic therapy of amelanotic melanoma. The pharmacokinetic studies showed that the retention time of the conjugates both in the serum and in the tumor increased as compared with NPs functionalized with C11Pc alone. The conjugates were eliminated via the bile-gut pathway without observable toxicity. Milla et al. (2009) also showed that a liposomal formulation of a trifluoromethylbenzyloxy ZnPc was accumulated in spleen, liver and duodenum. In spite of

these findings, the absence of hepatic toxicity after treatment with substituted ZnPcs was demonstrated by monitoring the hepatic cytochrome P450 activity and the levels of serum glutamic-pyruvic transaminase (Larroque et al., 1996; Yslas et al., 2010). The lack of renal toxicity was also assessed by Yslas et al. (2010) by checking creatinine and urea levels.

Light sources employed in PDT with ZnPcs include lasers between 670 and 700 nm, the wavelengths corresponding to their maximum absorption. ZnPcs and light dose-dependent phototoxic action was demonstrated in different *in vivo* models (Reddi et al., 1990; Allémann et al., 1997). Yslas et al. (2009) evaluated the effect of a tetra-methoxy ZnPc liposomal formulation, employing different light doses. The highest PDT efficacy in a mouse mammary adenocarcinoma model was obtained for the maximal light dose evaluated in the study (210 J/cm^2). Doses mostly employed *in vivo* for a variety of ZnPcs are in the range of 150-400 J/cm^2.

In vivo studies demonstrated that, not only the photochemical properties of ZnPcs, but also tumor drug uptake, are important factors for an effective PDT. For instance, different PS tumor concentrations and phototherapeutic effects were observed *in vivo* for ZnNcs liposomal formulations that exhibited similar quantum yields of $^1O_2$-formation *in vitro* (Shopova et al., 1994). Furthermore, maximum concentrations of ZnPcs were reached 24 h after intravenous PS administration in different tumor models (Shopova et al., 1992; Allémann et al., 1997; Jori and Fabris, 1998; Yslas et al, 2009; Camerin et al., 2010). In general, the photosensitizing effect of ZnPcs *in vivo* was demonstrated by a decrease of tumor volume (Larroque et al., 1996; Yslas et al., 2010; Camerin et al., 2010) and an increase in the survival time of treated animals (Shopova et al., 1992; 1994). In addition, apoptotic pathway (Fabris et al., 1997; Jori and Fabris, 1998; Yslas et al, 2009, 2010; Xu et al., 2014) and random necrosis (Fabris et al., 1997; Jori and Fabris, 1998; Rumie Vittar et al., 2008) have been identified as the main mechanisms responsible for cell death.

It is interesting to mention that, when the interval of time between drug and light administration was reduced to 1 h or 3 h, signs of vascular damage were reported. For example, Bremner et al. (1999) evaluated the effects of water-soluble ZnPcs containing neutral, positive and negative side-chains in a murine fibrosarcoma model and showed that tumor growth delay was greater with a 1 h than with a 24 h time interval, being the positive-charged ZnPc the most effective sensitizer. In this study, a reduction in blood flow was shown to be an important factor in PDT. The effect of PDT on vessels was evaluated by Fingar et al. (1993) in Sprague-Dawley rats. Release of eicosanoids, vessel constriction, venule leakage and increased tumor interstitial pressure, as well as tumor reduction, were observed with monosulfonated and tertiary butyl substituted ZnPcs after light exposure 24 h post photosensitizer injection. Vascular damage after PDT treatment of a murine melanoma with ZnPc was also reported by Camerin et al. (2010), who confirmed damage of blood capillaries and endothelial cells by electron microscopy. Nevertheless, increased expression of vascular endothelial growth factor (VEGF) and higher microvessel density were detected in animals exposed to PDT. VEGF up-regulation was proposed to be associated with PDT-induced hypoxia (Bhuvaneswari et al., 2007; Das et al., 2010), and may suggest a side effect of PDT promoting tumor recurrence after treatment (Xu et al., 2014).

Novel carriers to transport and efficiently activate ZnPcs have been developed and tested *in vivo*. Lanthanide-dope upconversion nanoparticles coupled with a β-carboxy ZnPc (Wang M et al., 2014) and other synthetic materials, such as layered double hydroxides bound to unsubstituted ZnPc or to the octasulfonate ZnPc, showed to be efficient PDT agents for tumor growth inhibition (Liang et al., 2014b; Li et al., 2017). A tumor-pH-responsive photosensitizer, prepared by conjugating ZnPc with 2,4,6-tris (N, N-dimethylaminomethyl) phenoxy, showed not only to ablate tumor cells in 4T1 cells-bearing mice, but also presented

fluorescence imaging of tumor sites, suggesting that this Pc may be employed for tumor treatment and theranostics (Yan et al., 2018a).

To sum up, all the *in vivo* studies provided strong evidences for the clinical application of ZnPcs and its derivatives formulations in PDT for cancer treatment. As it was mentioned, critical parameters must be adjusted to obtain a proper PDT efficacy, such as the optimal PS dose, the interval between photosensitizer administration and time of irradiation, light dose and light dose rate.

5. Toxicity studies and clinical trials

The first clinical trials were performed with the ZnPc tetrasulfonate ($ZnPcS_4$) for use in veterinary medicine. $ZnPcS_4$ was initially administered to Swiss Webster mice to assess acute toxicity. Doses >100 mg/kg produced renal tubular nephrosis, resulting in acute toxicity and mortality. Based on these data, a phase I clinical trial of $ZnPcS_4$-based PDT against spontaneous tumors in dogs was started at a dose of 0.25 mg/kg and irradiation with 675 nm light (100 J/cm^2) 24 h after PS intravenous injection (Borgatti-Jeffreys et al., 2005). This study demonstrated tumor response using doses of 0.25 mg/kg and no adverse effects were observed with $ZnPcS_4$ doses up to 2 mg/kg. Then, a new phase I clinical trial with an enlarged cohort of dogs was performed (Borgatti-Jeffreys et al., 2007). The PDT efficacy was confirmed in both mesenchymal and epithelial tumors. Another study showed partial responses or complete remissions of squamous cell carcinoma of the head and neck in dogs and cats treated with $ZnPcS_4$-PDT with no prolonged cutaneous photosensitization or adverse effects (Lucroy, 2006).

In parallel, a repeated-dose toxicity study of ZnPc-based-PDT was performed in Wistar rats. The intravenous administration of di-sulfo-di-phthalimidomethyl ZnPc, known as Photocyanine, and irradiation with a 670 nm laser light induced liver abnormalities and

pigmentation in several important organs at 4 mg/kg/day. The no-observed-adverse-effect levels were 1.0 mg/kg in Wistar rats (Zhang et al., 2006) and 1.5 mg/kg in dogs (Liu et al., 2007). Characterization studies with Photocyanine were performed in order to determine optimal light dose and drug dosage in a murine sarcoma S180 model. An inhibitory effect on tumor growth was observed for 2 mg ZnPc/kg when irradiation doses using a 670-nm laser increased in the range of 36-144 J/cm^2. Vertical resection of irradiated tumors showed that the depth of local tissue necrosis was 5.7-7.5 mm (Xue et al., 2011).

6. ZnPc-PDT combined strategies

In the last years several works have focused on the employment of combined therapies in order to overcome the high failure rate of single-agent or single-regimen therapy. Multiple drug combinations for cancer aim to exploit the additive or synergistic effects obtained from the action of two species, resulting in an improved antitumor efficacy, reduced side effects, and retarded drug resistance. Thus, PDT coupled with chemotherapy agents has proven to be a promising combination treatment for some kinds of tumors (Brodin et al., 2015). The encapsulation of ZnPc and doxorubicin (DOX) into nanovehicles linked to folic acid achieved a synergistic chemotherapy-PDT in tumor cells overexpressing folic acid receptors (Liang et al., 2015; Flak et al., 2017; Huang et al., 2018). ZnPc and DOX were also conjugated through acid-labile and redox-responsive disulfide linkers in polymeric micelles. The observation of DOX fluorescence in the nucleus and ZnPc in the cytoplasm demonstrated that acidic and reducing intracellular environments could trigger the release of drugs by cleaving the linkers (Gao and Lo, 2018). A synergistic cytotoxicity was therefore found for certain DOX:ZnPc ratios in tumor-bearing mice. ZnPc was also covalently conjugated with DOX by the sequence Thr-Ser-Gly-Pro. In this case, although the conjugation of ZnPc with DOX led to the inhibition of the photodynamic activity of the PS

and reduced DOX cytotoxicity, the peptide was sensitive to the activity of a protease highly expressed on cancer-associated fibroblasts. As a consequence, an enhanced cytotoxicity was observed upon cleavage and illumination (Ke et al., 2017). Synergistic anticancer effects of PDT and chemotherapy were also demonstrated by the combination of ZnPc with docetaxel (Conte et al., 2013) or paclitaxel (Wang Y et al., 2018b). In addition, the simultaneous administration of two PSs, such as ZnPc and a cationic porphyrin, led to a potentiated antitumor effect in mice bearing amelanotic melanoma cells (Acedo et al., 2014). ZnPc-PDT has also been efficiently combined with a Bcl-2 inhibitor (Liu et al., 2017), an autophagy inhibitor (Yu et al., 2019), a cell cycle inhibitor (Yan et al., 2018b) or an immunostimulant agent (Marrache et al., 2013). In addition, the combination of the autophagy inhibitor 3-methyladenine with ZnPc-PDT as an strategy for osteosarcoma treatment down-regulated the expression levels of death ligand-1 (PD-L1), inhibiting tumor growth in a model of tumor metastasis (Yu et al., 2019). Recent studies have pointed out the role of PD-1 and its ligand PD-L1 as the most critical immune checkpoint blocking T-cell response and promoting tumor growth (Herbst et al., 2014; Dermani et al., 2019). Thus, further studies of combined therapy with immune checkpoint blockade and ZnPc-PDT will broaden the possibility of enhancing the immune anti-tumor response.

When ZnPcs were incorporated into systems employed for hyperthermia generation, such as carbon nanohorns and gold nanorods, enhanced PDT efficiency was obtained. In these systems, laser irradiation was useful to simultaneously activate the PS and hyperthermia surfaces (Zhang et al., 2008; Freitas et al., 2017). Similarly, magnetohyperthermia and PDT with ZnPcs incorporated into magnetic nanoemulsions or nanoparticles led to significant tumor regression (Primo et al., 2008; Bolfarini et al., 2012; Feuser et al., 2015). Therefore, the development of multiple drug combinations or new

strategies will undoubtedly contribute to finding better therapeutic options for cancer treatment in the near future.

7. Conclusions

Based on the properties that should posses an ideal PS, phthalocyanines have been widely employed as second generation sensitizers. In particular, many efforts have been made to develop medicinal Pcs with therapeutic efficacy. In the field of ZnPcs, the photophysical and photochemical properties, subcellular localization, phototoxic activity, mechanisms of cell death and improved targeting to tumor tissues have been the main topics explored. The simultaneous localization of some ZnPcs in lysosomes/mitochondria or lysosomes/ER seems to be optimal for cell photodamage and its potential as therapeutic agents. Although the most extensive mode of cell death characterized so far has been apoptosis, it is necessary to continue investigating the contribution of other death modalities, such as necrosis, autophagy or even paraptosis, a type of cell death not yet studied for ZnPcs. The search of new possible mechanisms of action may consequently contribute to novel applications for single or combined ZnPcs treatments. Up to this moment, there is an immediate need to increase the number of clinical trials evaluating ZnPcs. As a result, it would be possible to confirm preclinical studies and orientate future research. PDT has much to offer to cancer treatment due to its unique combination of tumor cytotoxicity, vessel occlusion and immune response with minimal side effects. A deeper understanding of the effects of PDT with ZnPcs could enable in the future the improvement of currently used protocols and the treatment of an increased number of oncological diseases.

Conflict of interest

The authors declare no conflict of interest.

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Legends to Figures

Figure 1. Chemical structure of ZnPc (A), α-tetrasubstituted ZnPc (B) and β-tetrasubstituted ZnPc (C)

Figure 2. Main intracellular sites of ZnPcs localization. After irradiation, ZnPcs promote the generation of ROS that triggers different phototoxic cellular responses.

Figure 3. Mitochondria membrane permeabilization (MMP) induced by deregulation in the expression levels of Bcl-2 family proteins leads to activation of either caspase-dependent and independent apoptosis.

Figure 4. Contribution of GA photodamage and lysosomal membrane permeabilization (LMP) to the activation of the mitochondrial-dependent apoptosis.

Figure 5. Involvement of ER stress induced by photoactivated ZnPcs in the mitochondrial apoptotic pathway.