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Imidazole Metalloporphyrins as Photosensitizers for Photodynamic Therapy: Role of Molecular Charge, Central Metal and Hydroxyl Radical Production

Pawel Mroz^{1,2}, Jayeeta Bhaumik³, Dilek K. Dogutan³, Zarmeneh Aly^{1,4}, Zahra Kamal^{1,4}, Laiqua Khalid^{1,4}, Hooi Ling Kee⁵, David F. Bocian⁶, Dewey Holten⁵, Jonathan S. Lindsey³, and Michael R. Hamblin^{1,2,7,*}

¹Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA

²Department of Dermatology, Harvard Medical School, Boston, MA

³Department of Chemistry, North Carolina State University, Raleigh, NC

⁴Aga Khan Medical College, Karachi, Pakistan

⁵Department of Chemistry, Washington University, St Louis, MO

⁶Department of Chemistry, University of California, Riverside, CA

⁷Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA

SUMMARY

The in vitro photodynamic therapy activity of four imidazole-substituted metalloporphyrins has been studied using human (HeLa) and mouse (CT26) cancer cell lines: an anionic Zn porphyrin and a homologous series of three cationic Zn, Pd or InCl porphyrins. A dramatic difference in phototoxicity was found: Pd cationic > InCl cationic > Zn cationic > Zn anionic. HeLa cells were more susceptible than CT26 cells. Induction of apoptosis was demonstrated using a fluorescent caspase assay. The anionic Zn porphyrin localized in lysosomes while the cationic Zn porphyrin localized in lysosomes and mitochondria, as assessed by fluorescence microscopy. Studies using fluorescent probes suggested that the cationic Pd porphyrin produced more hydroxyl radicals as the reactive oxygen species. Thus, the cationic Pd porphyrin has high potential as a photosensitizer and gives insights into characteristics for improved molecular designs.

Keywords

structure function relationship; photophysics; phototoxicity; metalloporphyrins; apoptosis; fluorescence microscopy; reactive oxygen species; hydroxyl radicals

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Correspondence: Michael R. Hamblin, PhD, Tel 617-726-6182, Fax 617-726-8566, e-mail hamblin@helix.mgh.harvard.edu.

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INTRODUCTION

Photodynamic therapy (PDT) is an emerging therapy for many diseases including cancer, neovascular and hyperproliferative disorders and infections. The therapy relies on the triple interaction between a non-toxic photosensitizer (PS) or dye molecule, harmless visible light of an appropriate wavelength to be absorbed by the PS, and molecular oxygen [1]. Following the absorption of light (photons), one of the electrons of the ground state PS is boosted into a higher-energy orbital to form the first excited singlet state. This is a short-lived (nanoseconds) species that can lose its energy by emitting light (fluorescence) or by internal conversion into heat. The excited singlet state PS may also undergo the process known as intersystem crossing whereby the spin of the excited electron inverts to form the relatively long-lived (microseconds to milliseconds) excited triplet-state that has electron spins parallel. The PS excited triplet can undergo three broad kinds of reactions that are usually known as Type I and Type II. In a Type I reaction, the triplet PS can gain or donate an electron from a neighboring molecule. In a Type II reaction, the triplet PS can transfer its energy directly to molecular oxygen (ground state triplet) to form reactive singlet oxygen [2].

Many of the traditional PS that have been used clinically are based on the porphyrin or tetrapyrrole nucleus found in such preparations as hematoporphyrin derivative [3]. There is a considerable amount of interest in designing and synthesizing new PS including porphyrinoid structures, with improved characteristics for investigation as possible new PDT drugs [4,5]. These desired improved properties include: being pure characterizable compounds, having large absorption peaks in the red and near-infrared regions of the electromagnetic spectrum, and having high uptake in cancer or other pathogenic cells [6]. For PS designed to kill cancer or other mammalian cells, it has been found that the intracellular localization of the PS is another important parameter, with PS that localize in mitochondria tending to be more powerful in killing cells than those PS that are found in other locations such as lysosomes [7].

The molecular characteristics of the PS such as charge, lipophilicity and asymmetry govern the localization and uptake of the compounds by various cell types, and also determine the pharmacokinetics, biodistribution and localization of the PS at the target site. Photosensitizers with constitutive cationic charges such as quaternary ammonium groups have been shown to be highly effective PS both against cancer cells [8] and against microorganisms [9] compared to negatively charged, neutral, or even basic-amino groups.

A range of seemingly disparate tetrapyrrole structures containing metal chelates have been investigated as PS in clinical and pre-clinical PDT applications. Zinc(II) and aluminum(III) phthalocyanines have been studied as both unsubstituted compounds [10] and with varying numbers of sulfonate groups attached to the benzo substituents [11]. Silicon(IV) is chelated in the phthalocyanine known as PC4 [12]. Tin(II) is chelated into an etiopurpurin (SnET₂) otherwise known as Purlitin [13]. Indium(III) is present in a chelate of pyropheophorbide methyl ester known as MV6401 [14]. The rare earth metal lutetium(III) is chelated into the novel macrocycle known as texaphyrin to produce motexafin lutetium [15]. Palladium(II) is chelated into two bacteriochlorin compounds, bacteriopheophorbide (TOOKAD or WST-09) [16] and its taurine derivative (Stakel or WST-11) [17], which have been used for vascular targeted PDT.

In this report we studied a set of four hydrophilic porphyrins: a zinc porphyrin bearing a net negative charge on the imidazole-bearing peripheral substituent and a homologous set of net positively charged imidazolium-substituted porphyrins that contain central zinc(II), palladium (II), and indium(III) metal ions [18]. The four porphyrins are shown in Figure 1. These porphyrins were chosen as model structures to study relationships between the charge and metal substituent, since porphyrin based photosensitizers are the most widely studied

compounds in PDT and hematoporphyrin derivative and protoporphyrin IX are the two most broadly used photosensitizers in the clinical setting. The rationale to choose the imidazole pendant groups was as a means of varying the overall charge without a dramatic change to the structure. Dramatic differences were observed in the effectiveness of the compounds as PS against mouse and human cancer cells, and these differences depended on the charge, central metal atom, and production of hydroxyl radicals as the reactive oxygen species (ROS).

MATERIALS AND METHODS

Synthesis of Imidazole Porphyrins

General Procedures—Absorption spectra and fluorescence spectra were collected at room temperature. Porphyrins were analyzed in neat form by laser desorption mass spectrometry (LD-MS) [19] and by matrix-assisted laser-desorption ionization mass spectrometry (MALDI-MS) using the matrix 4-hydroxy- α -cyanocinnamic acid. In both LD-MS and MALDI-MS analyses, positive ions were detected. Solvents were dried according to standard procedures. All chemicals were used as received from commercial sources.

5-(1,3-Diethylimidazol-2-ium)-10-phenylporphinatoindium(III) chloride (2-InCl)

—Following a general procedure [20] porphyrin **3-Zn** [18] (23 mg, 0.035 mmol) in ethanol (EtOH) (7 mL) was treated with concentrated aqueous HCl (7 mL). The resulting mixture was refluxed for 21 h under argon. The reaction mixture was then cooled to room temperature, whereupon the volatile solvent was removed. The resulting suspension was poured into a large volume of ethyl acetate and water. Saturated aqueous NaHCO₃ solution was added slowly to the aqueous-organic mixture. The organic layer was separated, and the aqueous layer was extracted twice with ethyl acetate. The combined organic extract was washed with saturated aqueous NH₄Cl solution and water. The organic layer was separated, dried (Na₂SO₄) and concentrated to give free base porphyrin **4** as a dark purple solid. Data for **4**: LD-MS obsd 452.2, calcd 452.2 (C₂₉H₂₀N₆); λ_{abs} (methanol, (MeOH)) 402, 500, 534, 574, nm; λ_{em} (MeOH) 625, 700 nm. A solution of porphyrin **4** (~0.035 mmol) in anhydrous DMF (7 mL) was treated with InCl₃ (0.30 g, 1.4 mmol). The reaction mixture was heated to reflux for 40 h under argon. The crude reaction mixture was concentrated. The residue was chromatographed [silica, CH₂Cl₂/MeOH in a gradient of (9:1) to (4:1)] to afford porphyrin **4-InCl** as a green solid. Data for **4-InCl**: MALDI-MS 595.8; λ_{abs} (MeOH) 410, 598 nm; λ_{em} (MeOH) 620 nm. The entire sample of **4-InCl** (~0.035 mmol) was treated with EtI (0.28 mL, 3.5 mmol) in DMF (2.0 mL), and the resulting mixture was heated at 65 °C for 3 days. The reaction mixture was then concentrated and chromatographed twice [(1) silica, CH₂Cl₂/MeOH (9:1); (2) neutral alumina, CH₂Cl₂ → CH₂Cl₂/MeOH (9:1)] to afford a solid residue. The solid residue was purified by preparative size-exclusion chromatography (3 × 30 cm) eluted with HPLC-grade tetrahydrofuran (THF). A final chromatographic procedure [alumina, CH₂Cl₂/MeOH in a gradient of (9:1) to (3:1)] afforded the title compound as a dark green solid (9.0 mg, 33% overall yield, assuming an iodide counterion): MALDI-MS obsd 656.8, calcd 657.1 (C₃₃H₂₇ClInN₆, lacking a counterion); λ_{abs} (MeOH) 411, 601 nm; λ_{em} (MeOH) 620, 670 nm.

Photophysics

The absorbance and fluorescence spectra, fluorescence quantum yields and excited-state singlet lifetime measurements of **1-Zn**, **2-Zn**, **2-Pd**, and **2-InCl** were investigated at room temperature in a 3:1 mixture of THF and MeOH. The lifetimes of the lowest energy triplet excited state were measured at room temperature and at 77 K on compounds in ethanol. Details of the measurements, including determination of molar absorption coefficients utilized herein to prepare solutions of known concentration, are given elsewhere [26].

Cell Culture

HeLa (human cervical squamous carcinoma cells) [21], and CT26 (murine colon adenocarcinoma) [22] were obtained from ATCC (Manassas, VA) and cultured in RPMI1600 medium (Gibco Invitrogen, Carlsbad, CA) with L-glutamine and NaHCO₃ supplemented with heat inactivated fetal bovine serum 10% (vol/vol), penicillin (100 U/mL) and streptomycin (100 µg/mL) (all from Sigma, St Louis, MO) at 37 °C in 5% CO₂ humidified atmosphere in 75 cm² flasks (BD Falcon, San Jose, CA). When the cells reached 80% confluence, they were washed with PBS (Sigma) and harvested with 2 mL of 0.25% Trypsin-EDTA solution (Sigma). Cells were centrifuged and counted in trypan blue (Sigma) and plated at 5000 cells/well in flat-bottomed 96 well plates (Fisher). Cells were allowed to attach for 24 h.

Light Source

Illumination of cells utilized a non-coherent white light source (Lumacare, Newport Beach, CA) fitted with a light guide containing a band pass filter (λ 400–700 nm) adjusted to give a uniform spot of 4 cm in diameter with an irradiance of 100 mW/cm² as measured with a power meter (model DMM 199 with 201 Standard head, Coherent, Santa Clara, CA). For the experiments assessing the production of intracellular ROS we used 405 nm laser light (Nichia Corp, Detroit, MI).

PDT Experiments

The PS were dissolved in DMSO at a concentration of 5 mM and stored in the dark at room temperature. PS were added at different concentrations to cells in fresh complete medium for 24 h incubation periods. The DMSO concentration in the medium was less than 0.5%. After incubation the medium was replaced with 200 µL of fresh medium and PDT with white light was performed. For experiments where the light dose was varied, fluences of 0 (dark toxicity), 2, 4, 8, 10, 12, 16, and 20 J/cm² were used, and 9 wells (one group) were illuminated at one time. Controls were cells with no treatment and cells with light alone at the highest fluence. For experiments where the porphyrin concentration was varied (concentrations between 100 nM and 24 µM) a fixed fluence of 10 J/cm² delivered at the same irradiance was used. Here additional groups of cells were incubated with all the concentrations of porphyrins but no illumination was used to determine the dark toxicity of the PS. At the completion of the illumination the plates were returned to the incubator for a further 24 h, before further studies. We used a MTT colorimetric assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) a tetrazolium salt that measures mitochondrial reductase activity and correlates well with colony-forming assays as a measure of cell viability, as has been described previously [23–26]. The absorbance for MTT assay was read at 560nm.

Photosensitizer Uptake

5×10^3 HeLa cells were plated per well in a 96 well plate and allowed overnight to attach. The next day **2-Zn**, **1-Zn** or **2-Pd** was added and incubated for 24 h. After incubation, the medium was removed and 200 µL of 1M NaOH/1%SDS was added and cells were incubated overnight. The fluorescence (**2-Zn** and **1-Zn**) was measured using excitation at 402 nm and detection at 641 nm with a plate-reader (Molecular Devices, Sunnyvale CA), while the absorption was measured at the Soret maximum (λ 402 nm). Unfortunately the 2-Pd compound is non-fluorescent therefore the uptake had to be measured by the means of absorption. The protein per sample was measured with bicinchoninic acid protein assay [27]. Fluorescence (for 1-Zn and 2-Zn) and absorption standard curves (for 1-Zn, 2-Zn and 2-Pd) were prepared by adding known amounts of **2-Zn**, **1-Zn** or **2-Pd** solutions to pre-prepared cell lysates in NaOH/SDS and incubated for 24 h. On the following day the fluorescence and absorption was measured as described above.

Apoptosis Assay

The induction of apoptosis by imidazole-porphyrin mediated PDT was measured by a fluorescence assay using Ac-DEVD-AFC (BD Pharmingen, San Jose, CA), a caspase fluorescent substrate [28]. The results were normalized to the content of protein in the sample. Briefly HeLa cells were treated with PDT sufficient to kill 80% of the cells. Following PDT, samples were collected at 2, 4, 6, 12, and 24 h and centrifuged. The pellet was resuspended in 100 μ L of lysis buffer [29] containing protease inhibitor and subjected to 3–4 cycles of freezing and thawing. Then 50 μ L of each sample was transferred to separate wells and 50 μ L of 2X reaction buffer was added together with Ac-DEVD-AFC (final concentration 50 μ M). Samples were incubated in the dark for 1 h at 37 °C, and fluorescence was measured in a plate reader (λ_{ex} 400 nm, λ_{em} 505 nm). The protein per sample was measured with bicinchoninic acid protein assay.

Fluorescence Microscopy – Intracellular Localization

5×10^5 HeLa cells were plated on 35mm dishes and allowed overnight to attach. The next day 5 μ M **2-Zn** or **1-Zn** in culture medium was added and incubated for 24 h. Cells were washed in PBS and 5 μ g/mL of lysotracker or mitotracker (LysoTracker Green DND-26, MitoTracker Green FM, Molecular Probes Invitrogen) was added and incubated for 30 min at 37 °C. Cells were again washed in PBS and 5–10 min later a Leica DMR confocal laser fluorescence microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) with excitation by a λ 488 nm argon laser and emission with either a λ 525 nm $\pm$ 10-nm bandpass filter, or a λ 580-nm longpass filter and a 63 $\times$ 1.20 NA water immersion lens was used to image the cells at a resolution of 1024 $\times$ 1024 pixels. Images were acquired using TCS NT software (Version 1.6.551, Leica Lasertechnik, Heidelberg, Germany). The intracellular localization of 2-Pd compound by confocal microscopy could not be identified due to the lack of fluorescence of this compound.

Detection of photodamage by fluorescent probes

We used the fluorescent probes, acridine orange (AO) and rhodamine 123 (Rho 123) (both from Sigma, St Louis, MO) to detect the location of PDT associated intracellular damage. 5×10^5 HeLa cells were plated on 35mm dishes and allowed overnight to attach. The next day 5 μ M **2-Zn** or 0.5 μ M **2-Pd** in culture medium was added and incubated for 24 h. Cells were washed in PBS and 5 J/cm² of white light was delivered. Immediately after PDT 0.5 μ M of AO or 0.5 μ M Rho 123 was added to the cells and incubated for 5 min in complete medium at 37 °C. Next the cells were washed and the intracellular localization of the dye was observed by confocal microscopy. Both probes were excited with Ar-laser 488-nm and emission wavelengths were AO (green fluorescence 525 nm $\pm$ 10-nm, or red fluorescence 580-nm longpass) and Rho 123 (525 nm $\pm$ 10-nm) [30]. As controls we used HeLa cells that were incubated with either AO or Rho 123 but did not receive PDT.

Intracellular ROS

HeLa cells were incubated with 5 μ M **2-Zn**, **1-Zn** or **2-Pd** for 24 h, and on the next day 5 μ g/mL of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA, Molecular Probes Invitrogen) in complete medium was added and incubated for 30 min at 37 °C. Cells were then washed with PBS, and 5 J/cm² of 405 nm laser light (Nichia Corp, Detroit, MI) was delivered. Blue light was used to minimize the photoactivation of dichlorodihydrofluorescein by small amounts of contaminating dichlorofluorescein that has been shown to be able to act as a PS when green light is used [31]. In similar experiments, 10 μ M of 3'-(4-hydroxyphenyl)fluorescein (HPF, Molecular Probes Invitrogen) was added and incubated for 1 h in complete medium at 37 °C. After the incubation, the cells were washed

with PBS and 5 J/cm² of white light was delivered. The cells were imaged immediately after PDT with the confocal microscope (488-nm excitation, 530-nm detection).

Cell-free experiments were also performed with HPF in 96 well plates. The dyes were aliquoted at the final concentration of 5 μ M per well in PBS, and HPF was added to each well at the final concentration of 10 μ M. Four wells formed one experimental group. For hydroxyl radical quenching experiments 100 mM mannitol was added. All groups were illuminated simultaneously and white light was delivered in sequential doses of 5 J/cm². After each dose the fluorescence was measured by fluorescence plate reader (488-nm excitation, 520-nm detection).

RESULTS

Preparation of Porphyrins

The four porphyrins examined in this study are shown in Figure 1. Each porphyrin is of the *trans*-AB-type wherein a phenyl group is located at one meso-position, an imidazolium substituent is located at the *trans* meso-position, and the flanking meso-positions lack substituents. Porphyrins **1-Zn** and **2-Zn** are zinc(II) chelates, whereas **2-InCl** is a chloro-indium(III) chelate, and **2-Pd** is a palladium(II) chelate. The imidazolium group in each porphyrin is dialkylated. In porphyrin **1-Zn**, the alkyl groups are 4-sulfobutyl units, whereas ethyl groups are present in porphyrins derived from **2**. The synthesis of porphyrins **1-Zn**, **2-Zn**, and **2-Pd** has been reported [18]. Porphyrin **2-InCl** was prepared as follows (Figure 1). Known porphyrin [18] **3-Zn** was treated with conc. aqueous HCl in refluxing EtOH [20] to afford the free-base porphyrin **4** that lacks the 2-trimethylsilylethoxymethyl protecting group. Refluxing a mixture of porphyrin **4** and InCl₃ in DMF for 40 h afforded indium-chelated porphyrin **4-InCl**. Porphyrin **4-InCl** was heated with EtI in DMF at 65 °C for 3 days, whereupon diethyl imidazolium–porphyrin **2-InCl** was obtained as a green solid. The overall yield for the three steps starting from the porphyrin **3-Zn** to the resulting indium imidazolium–porphyrin **2-InCl** was 33% (assuming an iodide counterion).

Photophysics

The photophysical properties of the four porphyrins in a number of media and at room temperature and 77 K have been characterized separately [32]. Salient characteristics are summarized in Table 1. Figure 2 shows optical spectra of the compounds in tetrahydrofuran/methanol, where aggregation is minimal. The fluorescence follow the same trends as the lifetimes of the lowest energy singlet excited state (*S*₁) in the order **2-Pd** < **2-InCl** < **1-Zn** ~ **2-Zn**. These trends reflect a progressive increase in the yield of intersystem crossing from *S*₁ to the lowest energy triplet excited state (*T*₁): **2-Pd** (>0.99) > **2-InCl** (0.98) > **2-Zn** (0.80) ~ **1-Zn** (0.80). The *T*₁ lifetime is much shorter for **2-Pd** (10 μ s, 295 K; 2 ms, 77 K) than for **1-Zn** and **2-Zn** (~5 ms, 295 K; ~60 ms, 77 K), which reflects in part a ~1000-fold greater radiative (phosphorescence) rate constant for **2-Pd**. The *T*₁ lifetimes are considerably reduced in air-saturated solutions at 295 K (e.g., 300 ns for **2-Pd**) and give rate constants for quenching by O₂ (e.g., 1.7 $\times 10^9$ M⁻¹s⁻¹ for **2-Pd**) that are comparable to those for a variety of porphyrins [33]. These values indicate that the quenching process for each porphyrin in air-saturated solution occurs near the diffusion limit (for triplet-triplet energy transfer) and is basically quantitative (because of a sufficient number of effective collisions), despite the much shorter *T*₁ lifetime for **2-Pd** compared to the other sensitizers.

PDT experiments

We initially used a concentration of 5 μ M of the compounds **1-Zn** and **2-Zn** incubated with cells for 24 h and illuminated with broadband white light. Figure 3A depicts the light-dose dependent loss of mitochondrial activity in the two cell lines for **1-Zn**, and Figure 3B shows

the phototoxic dose response with **2-Zn**. In both cell lines **2-Zn** (Figure 3B) was highly effective at inactivating mitochondria of cancer cells, while **1-Zn** had no effect (Figure 3A). In the case of the human HeLa cells (Figure 3B) 5 μM of **2-Zn** and 20 J/cm^2 killed 95% of the cells (almost reaching the level of detection of the assay), while HeLa cells were completely unharmed after 5 μM of **1-Zn** and 20 J/cm^2 . The mouse colon carcinoma line CT26 was less sensitive to **2-Zn**-mediated PDT than HeLa cells. In case of **2-Pd**, the preliminary experiments suggested that 5 μM concentration is too high for use in the fluence response experiments, since even a very low fluence resulted in 100% cytotoxicity. Therefore in the subsequent experiments, we progressively reduced the concentration of **2-Pd** in the incubation until the value of 0.5 μM was reached. Figure 3C demonstrates a good light-dose dependent inactivation of mitochondria relationship with both cell lines, and that HeLa cells are significantly more sensitive than are CT26.

Because PDT-mediated inactivation of mitochondria was not observed in any cell line using **1-Zn** as a PS at 5 μM , we decided to use an increasing range of concentrations of all the PS and deliver only one constant fluence of white light (10 J/cm^2) in order to be able to derive meaningful comparisons of the effectiveness of the different compounds as PS. Because PS may exhibit some dark toxicity when used at high concentrations, a dark control was used for each data point. Dark toxicity was negligible (greater than 85% survival at the highest porphyrin concentration (data not shown for clarity). Figure 3A and 3B show the PS dose-dependent inactivation of mitochondria of the four compounds (the InCl compound **2-InCl** was by this time available) for both cell lines. The data are best presented in double-log plots because both the percentage of inactivated mitochondria and the concentrations used spanned a large range of values. As expected from the previous experiment, **2-Pd** was highly phototoxic to both cell lines with measurable inactivation of mitochondria being observed at concentrations as low as 100 nM and maximal effect reached at 500 nM. Next in the order of effectiveness was **2-InCl** where concentrations between 250 nM and 1 μM showed a dose-dependent phototoxicity in both cell lines with the HeLa cells again being more susceptible. The cationic porphyrin **2-Zn** was effective at concentrations between 2 and 10 μM , while the anionic compound **1-Zn** did show some phototoxicity when used at concentrations between 10 and 50 μM .

Based on the presented results we compared the relative phototoxicity of three porphyrins in HeLa cells on a photon absorbed per cell killed basis (Table 2). The relative effectiveness as a function of photon absorbed by killed cell was calculated by dividing 1/LD50 by (integrated absorbance $\times$ relative uptake). The relative phototoxicity of **2-Pd** compound was almost 25 times higher compared to **2-Zn** compound and over 78 times higher in comparison to **1-Zn** compound. There was only a small difference between **1-Zn** and **2-Zn** compounds in relative effectiveness (3 times).

Although an incubation time of 24 h was used throughout these experiments, it was not clear that this length of incubation time was necessary. In addition, differences in the rate of uptake might provide insight into the wide difference in activity between **2-Zn** and **2-Pd**. Therefore for **2-Zn** and **2-Pd** in HeLa cells we used a single concentration of 5 μM for **2-Zn** and 500 nM for **2-Pd**, together with a single fluence of 10 J/cm^2 of white light and varied the incubation time. As can be seen in Figure 4A, there was increasing cell death measured as a function of mitochondria inactivation with increasing incubation time up to 6 h, but no further increase between 6 and 24 h incubation time. Interestingly the porphyrin **2-Pd** led to phototoxicity at an earlier time point than homologous compound **2-Zn**.

We sought to understand whether differences in the effectiveness of the **2-Zn** and **2-Pd** compounds could be related to the induction of apoptosis after illumination. It is known that many PS cause the death of cancer cells upon illumination by initiating the process of apoptosis via pathways involving mitochondrial damage leading to cytochrome *c* release, caspase

activation and nuclear fragmentation. Figure 4B shows that indeed both **2-Zn** and **2-Pd** did cause caspase activation and that the peak of caspase activity occurs earlier with **2-Pd** than with **2-Zn**. The peak value is similar for both PS and it was concluded that differences in mode of cell death did not account for the large difference in phototoxicity.

Photosensitizer Uptake Experiments

Traditionally the cell uptake of PS is measured by extraction of cells after incubation with PS and quantification of the dissolved PS in the cell extract by fluorescence spectrophotometry. In the case of these four compounds, porphyrins **2-Zn** and **1-Zn** had the expected fluorescence consistent with their structure, while **2-InCl** had very low fluorescence and **2-Pd** had no detectable fluorescence due to high intersystem crossing rates. We compared the uptake of **2-Zn** and **1-Zn** with increasing concentration in the incubation medium by fluorescence extraction in HeLa cell line. There was a dose dependent increase in cell uptake for both PS as seen in Figure 4C. The uptake of **1-Zn** was lower than that seen for **2-Zn**, but did not appear to be as low as might be expected from the lack of phototoxicity observed with **1-Zn** at these concentrations. This observation suggests that **2-Zn** is a more efficient PS on a molecule per cell basis than is **1-Zn**.

Since **2-Pd** is essentially non-fluorescent, we used the absorption of cell extracts to measure the uptake. To determine the validity of this measurement we calculated the correlation ($r^2=0.995$, $p<0.0001$) between the uptake values measured by fluorescence and by absorption for **1-Zn** and **2-Zn**. Figure 4D shows that the relationship of the uptakes measured by absorption and fluorescence (Figure 4C) for **1-Zn** and **2-Zn** were the same. We concluded that the absorption assay was reliable and could be used to determine the uptake of non-fluorescent **2-Pd**. As can be seen in Figure 4D, there was no significant difference between the cell uptakes of **2-Zn** and **2-Pd** suggesting that the large differences in phototoxicity seen for the two porphyrins were not due to differences in uptake.

Fluorescence microscopy

Because the zinc porphyrins had sufficient detectable fluorescence we were able to carry out confocal microscopy experiments to determine their intracellular localization with the use of green fluorescent intracellular tracers for lysosomes and mitochondria. The anionic **1-Zn** localizes mainly in lysosomes as can be seen from Figure 5; substantial overlap exists between the red porphyrin fluorescence and the green lysotracker fluorescence (Figure 5A) while no overlap is seen with red porphyrin fluorescence and green mitotracker fluorescence (Figure 5B). By contrast the cationic **2-Zn** shows overlap with both green lysotracker (Figure 5C) and with green mitotracker (Figure 5D), indicating that its localization is more mitochondrial than that of **1-Zn**. However in case of **2-Zn** we observed a “patchy” accumulation of the dye in the mitochondria. In some cells a perfect overlap was observed and in the others only minimal accumulation of the dye was seen. Figure S1 presents additional fields of view that provide additional evidence for “patchy” distribution of **2-Zn** dye.

Detection of photodamage by fluorescent probes

Because **2-Pd** compound does not have any measurable fluorescence we could not image its intracellular localization directly. However, we employed the fluorescent probes, such as acridine orange (AO) that labels lysosomes and rhodamine 123 (Rho 123) that labels mitochondria [34], to determine the location of intracellular damage caused by PDT with **2-Pd** and for comparison by PDT with **2-Zn**. AO has been shown to emit two colors of fluorescence; a red fluorescence that is typical of aggregated dye in lysosomes and a green fluorescence typical of the disaggregated dye when bound to nucleic acids such as nuclear DNA [35]. The typical labeling patterns for AO and Rho 123 for control, non-treated HeLa cells and cells that received PDT mediated by **2-Zn** and **2-Pd** are shown in figure 6. After

delivering 5J/cm² of white light we observed significant change in the pattern of probe accumulation. Instead of well demarcated lysosomes containing AO we observed dispersed fluorescence throughout the cells, with significant translocation of the AO to the nucleus (probably in nucleosomes), where upon binding to DNA it also produced green fluorescence. The nuclear relocalization was more pronounced in the case of **2-Pd** compared to **2-Zn** where the overall PDT damage was also more severe (see the presence of many extracellular blebs). The characteristic structure of mitochondria outlined by accumulation of Rho 123 in control HeLa cells was even more dramatically altered by PDT damage. We observed strong green fluorescence throughout the cells after PDT treatment suggesting the disruption of mitochondria and relocalization of the probe. Interestingly Rho123 also appeared to have relocated into nucleosomes after mitochondrial damage as might be expected of a lipophilic cationic dye. Both **2-Zn** and **2-Pd** damaged both lysosomes and mitochondria, but it may be that the relative damage to mitochondria was more pronounced for **2-Pd** compared to **2-Zn**. These results support our hypothesis that the one of the factors that is responsible for better PDT effect of **2-Pd** over **2-Zn** is preferential localization and damage to mitochondria than lysosomes.

Intracellular ROS

We first used the probe dichlorodihydrofluorescein (DCDHF) for intracellular ROS. This probe is fluorescence activated by many different ROS such as hydrogen peroxide, hydroxyl radicals, and by organic peroxides that are formed by reaction between unsaturated lipids and singlet oxygen. All three tested porphyrins (**2-Pd**, **2-Zn**, **1-Zn**) produced increased DCDHF fluorescence after illumination with 5 J/cm² of blue light (Figure 6) in the order **2-Pd** > **2-Zn** > **1-Zn**; however, the difference between the three compounds did not appear to be sufficient to explain the observed differences in phototoxicity. We then asked whether the new specific fluorescence probe for hydroxyl radicals, HPF [36] might show a major difference between the three porphyrins. As is shown in Figure 6A, there was only significant intracellular fluorescence generated in HeLa cells after white light illumination in the case of **2-Pd**, while **2-Zn** and **1-Zn** did not show a HPF fluorescence increase. In supplementary Figure S2 we show an appropriate set of controls to demonstrate that neither DCDHF or HPF was alone activated by the light used for the porphyrin [31], and that none of the three porphyrins exhibited fluorescence in the green region of the spectrum. In the supplementary Figure S3 we also present micrographs of HeLa cells that were incubated with 50uM concentration of **2-Zn** and 10uM HPF. The results show that even at 10 times higher concentration the production of hydroxyl radicals by **2-Zn** PDT was minimal.

To confirm the hypothesis that illumination of **2-Pd** produced hydroxyl radicals, we repeated the experiments with cell-free solutions of the three porphyrins in PBS (Figure 6B, C and D). There was a light-dose dependent increase in HPF fluorescence from **2-Pd** that was significantly greater than that seen in the case of **2-Zn** and **1-Zn**. Moreover, the light-dependent increase in fluorescence from **2-Pd** and HPF was significantly quenched by 100 mM mannitol. Mannitol is well known as a quencher of hydroxyl radicals [37, 38]. Interestingly the increase in HPF fluorescence was greater in the case of the cationic **2-Zn** than it was for the anionic **1-Zn** (although much less than that for **2-Pd**). The proportion of HPF fluorescence increase that was quenched by mannitol was comparable (about 50%) for all three porphyrins.

DISCUSSION

This report has found a novel structure-function relationship among PDT agents consisting of cationic imidazolium porphyrins with different central metal ions (**2-Pd** > **2-InCl** > **2-Zn**). As calculated in Table 2 the relative effectiveness on a photon absorbed per cell killed basis was that **2-Pd** was twenty-five times more effective than **2-Zn**. There have only been a few reports

of structure-function relationships for porphyrinic compounds with different central metal substituents where the function of interest is PDT effectiveness as PS against cancer cells in vitro. Milanese et al [39] compared a series of *meso*-tetrakis(4-methoxyphenyl)porphyrins with different central metals and found the order of activity was $\text{Cd} = \text{Zn} > \text{free base} > \text{Cu}$. Kolarova et al [40] studied *meso*-tetrakis(sulfonatophenyl)porphyrin and found that the order of phototoxicity was $\text{Zn} > \text{Pd} > \text{free base}$. Rosenfeld et al [41] compared a series of ether derivatives of pyropheophorbide *a* containing different metals and found the order of effectiveness was $\text{InCl} > \text{Zn} > \text{free base} > \text{Cu}$.

As expected the cationic Zn porphyrin **2-Zn** was a much better PS than the anionic Zn porphyrin **1-Zn**. The uptake experiments (both fluorescence and absorption) confirmed that the cationic porphyrin was taken up 4–5 times more readily than the anionic porphyrin. Cationic dyes are thought to be more easily taken up into cells by virtue of better binding to the anionic plasma membrane and therefore more likely to be internalized by endocytotic processes than anionic dyes that do not readily bind to the membrane [42]. Although lipophilic PS also show high uptake into cells, probably by diffusing through lipid bilayer membranes, they are not usually water soluble and require some delivery system such as liposomes, micelles or emulsions to deliver them into cells [43]. The cationic Pd porphyrin **2-Pd** showed the same uptake by the cells as the cationic Zn porphyrin **2-Zn**, and suggested that replacing the divalent zinc ion with the larger but still divalent palladium ion did not make any significant difference to the lipophilicity of the molecule, or to the overall charge on the imidazolium-bearing peripheral substituent. The cationic **2-Zn** was localized in both mitochondria and in lysosomes, while the anionic **1-Zn** was confined to lysosomes. The explanation for this observation lies in the difference in log P values (0.59 for **1-Zn** compared to 3.1 for **2-Zn**) since more hydrophilic PS tend to concentrate in lysosomes and more lipophilic PS concentrate in membrane-rich organelles such as mitochondria and endoplasmic reticulum [44]. Furthermore the net positive charge on **2-Zn** is also likely to encourage the accumulation in mitochondria due to the well known mitochondrial targeting properties of lipophilic cations [45]. There is a substantial body of knowledge on the PDT effect of different sub-cellular localizations of various porphyrins and related compounds such as phthalocyanines. PS that localize in mitochondria are known to be much more phototoxic than PS that localize in lysosomes [46]. Since the three cationic imidazolium porphyrins (**2-Zn**, **2-InCl**, **2-Pd**) differ only in the central metal ion and otherwise are expected to have very similar structures, the only molecular characteristic that potentially could cause differences in subcellular localization is the different axial-ligation behavior of the three metal ions [47]; thus other explanations for the large difference in phototoxicity must exist. The experiments with fluorescent probes for organelle damage showed that both **2-Pd** and **2-Zn** caused damage to both lysosomes and mitochondria, and although **2-Pd** may have been somewhat more mitochondrial than **2-Zn**, this would not seem to explain the large difference in potency.

The increase in the triplet yield from 0.8 to ~1 on moving from zinc to palladium does not seem to be a sufficient difference to account for the large differences in photodynamic efficiency. We initially employed the general probe for ROS, DCDHF as a means of confirming that the non-fluorescent **2-Pd** was actually taken up into cells, and then tested the novel specific probe for hydroxyl radicals, HPF. This probe has been reported to be activated only by hydroxyl radicals, and not by singlet oxygen, hydrogen peroxide, superoxide, or lipid peroxides [36]. HPF was recently employed to demonstrate that bactericidal antibiotics exert their cytotoxic effect via generation of hydroxyl radicals [48]. We found out that the Pd porphyrin (**2-Pd**) produces more hydroxyl radicals than the Zn porphyrins (**2-Zn** and **1-Zn**) when illuminated inside cells as well as in homogeneous solution. Moreover this effect can be quenched by the hydroxyl-radical quencher, mannitol. These findings may suggest an explanation for the large difference in phototoxicity of the three porphyrins. Many reports have suggested that hydroxyl radicals can be produced during PDT, and that their production is highly toxic to cells.

Photodynamically generated hydroxyl radicals have been reported after illumination of PS such as Photofrin II [37], hematoporphyrin monomethyl ether [38], *meso*-tetrakis[4-(carboxymethoxy)phenyl]porphyrin [49], zinc phthalocyanine [50], bacteriochlorophyll *a* [51], and hypocrellin B [52] among others. Nevertheless, despite these reports, singlet oxygen is accepted as the major mediator of toxicity in PDT [53–56].

There are three possible pathways by which hydroxyl radicals can be formed during PDT. These pathways all derive from Type I photochemistry (electron transfer) rather than Type II photochemistry (energy transfer). Firstly the PS excited triplet state can form a radical anion by accepting an electron from a biological reducing agent present inside cells such as NAD(P)H (eq 1). This radical anion can transfer an electron to molecular oxygen to form superoxide radical anion (eq 2). Superoxide can dismute to hydrogen peroxide either catalyzed by superoxide dismutase or spontaneously (eq 3).

Hydrogen peroxide can undergo Fenton chemistry catalyzed by small concentrations of ferrous iron producing hydroxyl radicals by one-electron reduction of hydrogen peroxide (eq 4), and the oxidized ferric iron can then be reduced back to ferrous iron by superoxide (eq 5); the combination of two reactions is termed the iron catalyzed Haber-Weiss reaction [57].

Secondly superoxide can react with nitric oxide in a very fast reaction to produce peroxynitrite (eq 6). Peroxynitrite can give rise to hydroxyl radicals (eq 7), and its oxidative reactions are very similar to those of hydroxyl radicals [58].

The third possibility is a direct reaction between hydrogen peroxide and the PS radical anion to form hydroxyl radicals and hydroxide anion (eq 8). This is equivalent to the one-electron

reduction of hydrogen peroxide mediated by Fe^{2+} discussed above, but instead the PS radical anion is the reducing agent.

Which of these mechanisms (or others) is the most important in the present situation could be investigated by studies using added ferrous iron and/or iron chelators or added nitric oxide and nitric oxide scavengers, and such studies are underway.

An explanation of why the **2-Pd** porphyrin should produce more hydroxyl radical than the **2-Zn** porphyrin may reside in the greater propensity of the former versus the latter as an electron-transfer acceptor. The average ~ 0.2 eV greater ease of reduction of palladium porphyrins compared to zinc porphyrins [59] is complemented by the ~ 0.2 V higher energy of the triplet excited state (T_1) (Table 1), which should make the T_1 state of **2-Pd** a more potent electron acceptor (oxidant) than **2-Zn** by ~ 0.4 V. Thus, the involvement of such a two-step mechanism using the porphyrin T_1 state as an electron-transfer mediator could complement the modestly higher intersystem-crossing yield in enhancing PDT activity of **2-Pd** versus the other sensitizers. The difference in PDT efficacy of **2-Pd** could be further enhanced by the fact that palladium porphyrins do not coordinate axial ligands to the central metal ion nearly as effectively as zinc porphyrins [47]. Thus, more prolific ligation of **2-Zn**, for example, to entities in the cellular environment may slow or limit diffusion to the desired locale compared to **2-Pd**.

Another, related factor that could be important in determining the photochemical mechanism operating in these compounds is the overall molecular charge. The finding that the cationic **2-Zn** produces more hydroxyl radicals than the anionic **1-Zn** under the same conditions could imply that **2-Zn** is a better electron acceptor than **1-Zn**, because of positive charge. The determination of the difference in reduction potentials of the two zinc porphyrins in vitro, or extrapolating from redox properties in solution (in either aqueous or organic media), are both problematic. However, there are documented examples showing that porphyrins bearing cationic substituents are more easily reduced compared to analogues with anionic substituents [60]. However, such effects would be expected to depend on the extent of conjugation of the peripheral group and the porphyrin macrocycle. Calculations have suggested that the sign of a charge and its position with respect to a tetrapyrrole macrocycle influences redox properties [61]. In this regard, the ability of the chain terminated in the sulfobutyl versus ethyl groups of **1-Zn** versus **2-Zn** to adopt a conformation that places the negative charge close to or over the face of the macrocycle may influence the relative reduction potentials.

These collective characteristics suggest that **2-Pd** could be more predisposed to accept an electron and engage in consequent Type I photochemistry compared to the other porphyrins studied here: a favorable triplet excited-state energy level (and yield), a favorable redox potential imparted by the metal ion, and potential favorable redox and other properties associated with overall positive charge on the compound. There are literature reports concerning the reactive oxygen species produced by the Pd-bacteriopheophorbide PS known as **TOOKAD**. The authors used various experiments and lines of reasoning to attribute the cytotoxic effect (after illumination with near-infrared light) to the primary formation of superoxide and secondary formation of hydroxyl radical produced from hydrogen peroxide reacting with the PS triplet [62].

In conclusion, we have demonstrated that varying the central metal atom in a series of cationic porphyrins makes a substantial difference in the phototoxicity and that these differences cannot

be easily explained by differences in photophysics, cell uptake or subcellular localization. It is proposed that the explanation resides in the greater propensity of the palladium porphyrin to efficiently generate the highly toxic hydroxyl radical upon illumination, and that this particular ROS is more effective in killing cells than the more commonly studied ROS, singlet oxygen and superoxide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Synthetic scheme and chemical structures.

Figure 2.
Absorption spectra (A) and fluorescence spectra (B) for porphyrin sensitizers in air-saturated tetrahydrofuran/methanol (3/1) at 295 K.

Figure 3.

Broad-band white light-fluence dependent inactivation of mitochondria of HeLa cervical cancer and murine CT26 colon adenocarcinoma cells in culture. (A) **1-Zn** at 5 μ M; (B) **2-Zn** at 5 μ M; (C) **2-Pd** at 0.5 μ M. Concentration dependent phototoxicity of **1-Zn**, **2-Zn**, **2-In-Cl**, **2-Pd** after 10 J/cm² white light delivered to HeLa cells (D) and CT26 cells (E). Cells were incubated with PS for 24 h, illuminated with white light, returned to incubator for 24 h, and a MTT assay was conducted. Each data point is the mean of 9 wells, and bars are SD. All experiments were repeated 2–3 times.

Figure 4.

(A) Effect of varying the incubation time on PDT inactivation of mitochondria of HeLa cells mediated by 5 μM **2-Zn** or 0.5 μM **2-Pd** and 10 J/cm^2 of white light. Regardless of incubation time cells were incubated for 24 h before MTT assay. (B) Time course of apoptosis after PDT mediated by **2-Zn** and **2-Pd**. (C) Cell uptake of **2-Zn** and **1-Zn** as a function of concentration by HeLa cells after 24 h incubation time determined by fluorescence spectrophotometry of cell extracts. (D) Same as panel (C) but including **2-Pd** and uptake determined by absorption spectrophotometry of cell extracts. Data points are means of 8 wells and bars are SD.

Figure 5. Fluorescence micrographs of HeLa cells showing red fluorescence from **1-Zn** (A and B) and **2-Zn** (C and D) overlaid with green fluorescence from lysotracker (A and C) and from mitotracker (B and D). Scale bar is 25 μm .

Figure 6.

Fluorescence micrographs of HeLa cells incubated with 5 μM **2-Zn** or 0.5 μM **2-Pd** and either 0.5 μM of Acridine orange or 0.5 μM Rhodamine 123. Cells were either illuminated or not with 5 J/cm² of white light. Bar is 10 μm .

A

Figure 7.

Fluorescence micrographs of HeLa cells incubated with 5 μ M 1-Zn, 2-Zn or 2-Pd and either 5 μ g/mL of CM-H2DCFDA or 10 μ M HPF. Cells were either illuminated or not with 5 J/cm² of 405-nm laser light (DCFH) or with 5 J/cm² of white light (HPF) (A). White light-dose dependent increase in fluorescence from 10 μ M HPF in solution with 5 μ M 1-Zn (B), 2-Zn (C) or 2-Pd (D) in PBS. Also shown is the effect of quenching with 100-mM mannitol. Data points are means of 4 wells, and bars are SD.

Photophysical Data.^a

Table 1

Cmpd	T (K)	log P ^b	E _S ^c (eV)	E _T ^d (eV)	Φ _f ^e	Φ _p ^f	Φ _{isc} ^g	τ _S ^h (ns)	τ _T ⁱ (ms)	k _q ^j × 10 ⁻⁹ (M ⁻¹ s ⁻¹)
1-Zn	295	0.59	2.12		0.014		0.80	3.0	4.5	5
	77		2.14	1.68		0.0015			64	
2-Zn	295	3.1	2.13		0.015		0.80	3.0	6.1	5
	77		2.14	1.68		0.0021			54	
2-InCl	295	1.95	1.95	1.50	0.0078		0.98	0.24		
2-Pd	295	2.0	2.25	1.84	0.00012		>0.99	0.009	0.01	1.7
	77		2.28	1.89		0.078			2.0	

^a Studies at 295 K utilized compounds in THF/MeOH = 3/1 except that EtOH was used for determination of τ_T at 295 K. Studies at 77 K utilized compounds in frozen EtOH glasses. See [47] for description of the methods used to obtain the values.

^b Partition coefficient between n-octanol and PBS.

^c Energy of the lowest energy singlet excited state taken as the average energy of the Q(0,0) absorption and fluorescence bands.

^d Energy of the lowest energy triplet excited state taken from the position of the T(0,0) phosphorescence band. Since verifiable phosphorescence was not observed for **2-InCl**, the energy was estimated using E_S for this compound and average value of E_S – E_T = 3500 cm⁻¹ from **1-Zn**, **2-Zn**, and **2-Pd**.

^e Fluorescence yield.

^f Phosphorescence yield.

^g Intersystem crossing yield calculated using τ_S and an internal conversion rate of (16.2 ns)⁻¹ for reference compound zinc tetraphenylporphyrin; these values are more reliable than the directly measured yields as discussed in [47].

^h Lifetime of the lowest energy singlet excited state.

ⁱ Lifetime of the lowest energy triplet excited state.

^j Second order rate constant for quenching of the triplet excited state by O₂ derived from the triplet lifetime in air-saturated solution assuming an O₂ concentration of 2 mM. The values have been multiplied by a factor of 10⁻⁹.

Table 2

Comparison of relative phototoxicity of three porphyrins in HeLa cells on a photon absorbed per cell killed basis

Compound	Integrated absorbance (350–700-nm)	Relative uptake (at 1 μ M)	1/LD50 (μ M)	Relative phototoxicity per photon absorbed ³
1-Zn	11.15	1	0.08	0.007
2-Zn	10.7	5	1.2	0.022
2-Pd	6.56	5	18	0.549

¹From Figure 4D²From Figure 3D³Relative effectiveness calculated by dividing 1/LD50 by (integrated absorbance $\times$ relative uptake)