Picolyl Porphyrin Nanostructures as a Functional Drug Entrant for Photodynamic Therapy in Human Breast Cancers

Betsy Marydasan,†‡ § Rajshree R. Nair, † P. S. Saneesh Babu, † Danaboyina Ramaiah,‡⁺ and S. Asha Nair*†⁺

†Cancer Research Programme, Rajiv Gandhi Centre for Biotechnology, Trivandrum 695014, Kerala, India
‡Chemistry Department, CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat 785 006, India

Supporting Information

ABSTRACT: The major challenge in photodynamic therapy (PDT) is to discover versatile photosensitizers (PSs) that possess good solubility in biological media, enhanced singlet oxygen generation efficacy, and photodynamic activity. Working in this direction, we synthesized a picolylamine-functionalized porphyrin conjugate, compound 1, and its zinc complex compound 2. Compound 1 forms spherical structures in methanol, whereas compound 2 exhibited vesicular structures. Compared to the existing PSs like foscan and photofrin, compound 2 exhibited a high singlet oxygen generation efficiency and triplet quantum yield. The complex also showed good water solubility, and its PDT activity was demonstrated through in vitro studies using MDA-MB 231 breast cancer cells. The mechanism of biological activity evaluated using various techniques proved that the active compound 2 induced predominantly singlet oxygen-triggered apoptosis-mediated cancerous cell death. Our results demonstrate that zinc insertion in the picolyl porphyrin induces an enhanced triplet excited state, and the singlet oxygen yields quantitatively and imparts excellent in vitro photodynamic activity, thereby demonstrating their pertinence as a nanodrug in future photobiological applications.

INTRODUCTION

Cancer is a group of diseases involving abnormal growth of cells which tend to invade in an uncontrolled way and will eventually affect human life. According to the World Health Organization, by the year 2035 cancer will form a global “tidal wave” and will swallow 24 million people’s life. To date, many attempts were carried out globally to improve the cancer treatment techniques. Compared to traditional cancer treatments like surgery, radiotherapy, chemotherapy, and so forth, photodynamic therapy (PDT) remains well chosen as an emerging modern technique because it significantly reduces side effects and improves target specificity.¹ This treatment modality involves the combined effect of light and a chemical substance called a photosensitizer (PS). The PS together with molecular oxygen generates highly reactive oxygen species (ROS) which results in the elimination of cancerous cells.² A group of sensitzers mainly based on porphyrins, phthalocyanines, BODIPY, chlorins, squaraines, methylene blue, and so forth have been evaluated for photodynamic treatment as they generate singlet oxygen effectively.³⁻⁵ Even though the discovery of PSs such as foscan, photofrin that are currently in cancer clinics has been considered as landmarks in the history of PDT, these sensitizers possess certain drawbacks like low singlet oxygen productivity, poor water solubility, as well as photosensitivity. For the last few decades, the key problem in the field of PDT has been to overcome the disadvantages of the existing PSs and to invent new functionalized PSs that exhibit enhanced singlet oxygen generation efficiency, solubility in biological media, and high photodynamic efficacy.⁶⁻⁸ Development of functionalized porphyrins possessing nanoscale dimensions has been an interesting research topic as these materials could find various applications ranging from artificial light harvesting systems to therapeutic agents in the biomedical field.⁹⁻¹⁴ Among these, the synthesis of the porphyrin arrays in the nanometric size possesses the most challenging aspect because their synthesis, growth of size, and complexity render their development tedious and inefficient. Interestingly, the supramolecular self-assembly being a versatile alternative technique, offers the quick construction of one, two, and three dimensional nanometric architectures having discrete structure, property, and functionalities.¹⁵⁻¹⁹ Recently, nanotechnology has taken the lead to extensively enhance the photodynamic activity in cancer treatment. The self-assembly of porphyrins remains as a successful process till date as it self-associates in the solution through weak intermolecular forces. Such weak forces render flexibility to the self-assembled nanostructures providing control over their size as well as...
Scheme 1. Picolyl Porphyrin Derivatives Compound 1 and 2

geometry which is important in terms of sustaining suitable host molecules such as dyes or drugs within these structures.20,21 These porphyrins when activated by a light stimuli can actively produce smart agents like singlet oxygen which could have the potential to destroy the cancerous cells without affecting the normal cells.26 In this context, the development of functionalized porphyrin, which can form nanostructures that can hold guest molecules with significant singlet oxygen efficiency, photostability, and good solubility in the biological medium is quite demanding for their photodynamic therapeutic applications and plays a key role in drug delivery. Of late, our group has been a part of developing efficient PSs based on aza-BODIPYS, squaraines, and porphyrin systems for PDT.22–24

Herein, we report a porphyrin compound 1, modified with picolylamine groups and its zinc complex compound 2. We successfully enhanced the intersystem crossing efficiency as well as water solubility of the porphyrin conjugate through zinc metal ion insertion both in the core and in the periphery. The compound 2 has the ability to undergo self-assembly to form vesicles in methanol whereas the ligand forms spherical structures. Further, we have evaluated the photodynamic efficacy of the compound 2 employing in vitro studies using MDAMB 231 breast cancer cells. These studies revealed that the compound 2 with five zinc metal ions showed better photocytotoxicity when compared to compound 1. The mechanism of biological activity associated with this PS has been evaluated by FACS analysis, chromatin condensation, tetramethylrhodamine methyl ester (TMRM) assay, PARP cleavage, and cytochemical staining methods which indicate that the most active porphyrin compound 2 could effectively destroy the cancerous cells through predominantly the apoptotic pathway.

**RESULTS AND DISCUSSION**

We synthesized porphyrin compounds 1 and 2 employing the modified Lindsey’s method and they were characterized through various spectral as well as analytical methods (Scheme 1, Figures S1 and S2 Supporting Information).25 The compound 1 showed a porphyrin chromophoric Soret absorption band at 416 nm followed by Q bands in the near-infrared region. In contrast, compound 2 showed a red-shifted band at 423 nm and relatively weak bands at 555–600 nm. Fluorescence spectra of the porphyrin compound 1 exhibited a maximum at 650 nm whereas the zinc complex compound 2 showed emission maxima at 610 and 655 nm having a fluorescence quantum yield of (Φ_F) 0.026 ≤ 0.09 for these derivatives (Figure S3, Supporting Information).26 Among the synthesized porphyrin compounds, compound 2 exhibited good solubility in aqueous media.

We were interested to develop the nanostructures of these porphyrin derivatives by varying the solvent polarity. Interestingly, we found out that the porphyrin compounds 1 and 2 forms different self-assembled structures in methanol. The morphological analysis of the nanostructures formed from the compounds 1 and 2 were carried out using different microscopic techniques like AFM (atomic force microscopy), SEM (scanning electron microscopy), TEM (transmission electron microscopy), and DLS (dynamic light scattering). The compound 1, which does not possess zinc metal ions, forms uniform spheres with a size of 500 ± 50 nm whereas the compound 2 showed formation of highly stable vesicular structures in methanol with an average size of 250 ± 50 nm (Figure 1). The formation of the vesicular structures was further evidenced through the DLS experiments, zeta potential, and AFM analysis (Figures S4 and S9 Supporting Information).

Further, we were interested to understand the PDT efficacy of the self-assembled nanostructures formed from the porphyrin compounds 1–2. The nanosecond laser flash photolysis technique was performed in order to establish the transient intermediates formed by the compounds. We monitored the transient absorption spectra of 1–2 after laser pulse excitation (λ_{ex} 355 nm) which showed an absorption maximum at 440 and 470 nm with bleach at the ground state absorption region. The transient intermediate of these compounds showed a first-order process having a lifetime of 10–12 μs and these were confirmed by saturating the solutions with molecular oxygen. To quantify the triplet excited state
quantum yields (ΦΔ) of 1–2, we followed a triplet–triplet energy transfer method by using [Ru(by)₃]²⁺ as the standard and β-carotene as the energy acceptor. We quantified the triplet quantum yield of 0.64 ± 0.03 for the compound 1, whereas a quantum yield of 0.97 ± 0.02 was observed for the compound 2 (Figures 2 and S5, Supporting Information).

Further, we were intrigued to analyze the efficacy of photosensitized singlet oxygen generation by the compounds 1–2, as singlet oxygen plays a key role in photodynamic activity. To quantify the singlet oxygen generation efficiency, we used both luminescence and chemical trap methods. In the chemical trap method, we used 1,3-diphenylisobenzofuran (DPBF) as the singlet oxygen trapping agent, whereas in the luminescence method, we monitored the singlet oxygen luminescence at 1273 nm. In the DPBF-trap method, we irradiated compound 1 in methanol after adding DPBF using a mercury lamp (200 W) having a long pass filter (515 nm) for 0–60 s. We monitored the decrease in absorbance at 414 nm, which corresponds to the dye (1)–sensitized photo-oxidation of DPBF. From the graph, the decrease in absorbance of DPBF with and without irradiation is shown in Figure 2A. The singlet oxygen quantum yields by comparing optically matched solutions of the standard, hematoporphyrin (Hp), were quantified from the emission intensities of 1–2 and the reference compound Hp. For the compound 1 without any zinc ions, we obtained a singlet oxygen quantum yield of 0.59 ± 0.02. It is interesting to note that the compound 2 having Zn²⁺ metal ions showed a singlet oxygen quantum yield of 0.95 ± 0.03 and these results were in absolute agreement with the chemical trap method. These results lucidly indicate that by incorporating the zinc ions at suitable positions of the porphyridin ligand, we could enhance the singlet oxygen generation efficiency because of the increased spin–orbit coupling interactions (Figure S7 Supporting Information). Interestingly, singlet oxygen generation efficiency and triplet quantum yield of compound 2 was found to be higher than the currently existing PSs such as foscarn and photofrin.

Investigation of in Vitro Photobiological Properties.
To evaluate the photodynamic efficacy of the porphyrin nanostructures of 1–2, we employed photocytotoxicity studies using three different cancer cell lines such as breast (MDA-MB-231), cervical (SiHa), and colon (SW 620). As a standard colorimetric assay for determining cellular proliferation, we employed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT is yellow in color, which is reduced to purple formazan by cellular reductase enzymes present in living cells. The effectiveness of the PS to inhibit the biological function of tumor cells can be quantified by measuring the absorbance of formazan formed in the region 500–600 nm. The IC₅₀ value, which is a direct measurement of cytotoxicity of a sensitizer, was calculated under different cases such as dark and light conditions. We had screened both PSs in the three cell lines under similar conditions and the growth inhibition percentage of cells in the presence of compound 1–2 with and without irradiation is shown in Figure 4. Among the three cancer cell lines, MDA-MB-231 breast cancer cell line showed the least photocytotoxicity of 12 and 7 μM for compound 1 and 2, respectively, which stands ideal for potent PSs compared to other cell lines. Cytotoxic studies revealed that both PSs are essentially noncytotoxic in the absence of light but interestingly upon light activation it showed high photocytotoxicity. It is apparent that compound 2 having zinc ions showed better photocytotoxicity than compound 1 in all the three cell lines. It is interesting to note that compound 2 having zinc metal ions has been identified as a lead molecule because it exhibits significant photophysical properties and photocytotoxicity. Therefore, we investigated the detailed photobiological activity pertaining to lead porphyrin 2 to understand the mechanism underlying its biological activity.

To understand the localization of compound 2 and cellular uptake in MDA-MB 231 cells, we employed confocal microscopy technique. It was interesting to observe that when MDA-MB 231 cell lines were incubated with 7 μM of 2, the cells readily showed an uptake of the sensitizer within 1 h. The localization of 2 was confirmed by Hoechst dye as the counter stain (Figure 5). The merged images of the MDA MB 231 cells with both compound 2 and Hoechst dye at different conditions showed intense and punctate fluorescence outside the nucleus indicating its presumed cytoplasmic association.

To unravel the mechanism behind the PDT activity and cellular damage induced by compound 2, we evaluated the Annexin V-FITC/PI assay using the flow cytometric analysis. Annexin V (tagged with FITC) has a high affinity for phosphatidylserine (which is present on the inner leaflet of lamp and NIR detector. The singlet oxygen generations (ΦΔ) were quantified from the emission intensities of 1–2 and the reference compound Hp. For the compound 1 without any zinc ions, we obtained a singlet oxygen quantum yield of 0.59 ± 0.02. It is interesting to note that the compound 2 having Zn²⁺ metal ions showed a singlet oxygen quantum yield of 0.95 ± 0.03 and these results were in absolute agreement with the chemical trap method. These results lucidly indicate that by incorporating the zinc ions at suitable positions of the porphyridin ligand, we could enhance the singlet oxygen generation efficiency because of the increased spin–orbit coupling interactions (Figure S7 Supporting Information). Interestingly, singlet oxygen generation efficiency and triplet quantum yield of compound 2 was found to be higher than the currently existing PSs such as foscarn and photofrin.

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the plasma membrane). Upon initiation of apoptosis phosphatidylserine on the inner leaflet is translocated to the outer leaflet allowing its binding and thereby serving as a sensitive probe to identify apoptotic cells.30 Generally Annexin V-FITC is used in combination with propidium iodide (PI), another fluorophore, to differentiate viable and dead cells. An intact membrane prevents PI from binding to the nucleic acid content and thereby distinguishes apoptotic and necrotic cells (Figure 6).

We observed that most cells showed no apoptotic and necrotic changes upon PDT treatment with 2 (7 μM) in the absence of light suggesting it to be nontoxic for normal cells. This indicates that compound 2 were noncytotoxic towards the MDA-MB-231 cells in the absence of light. However, upon photo-irradiation, the percentage of cells at the lower right quadrant (Q4), that is, in the early apoptotic stage (i.e., externalization of phospholipid phosphatidylserine but not membrane leakage, Annexin V-FITC+/PI−) increased from 12.5 to 51.3%, when the concentration of compound 2 increased from 3.5 to 7 μM. These results clearly depict that the porphyrin conjugate 2 induces cancer cell death predominantly through the apoptosis mechanism.

Cell death by apoptosis was further evaluated by employing the TMRM staining experiment by fluorescence imaging. The PDT of MDA-MB-231 cells with compound 2 at 3.5 μM resulted in about 28% reduction in membrane potential, whereas at 7 μM, 64% reduction in mitochondrial membrane potential was observed. Alternatively, the light and dark controls showed only 7 and 9% decrease in mitochondrial membrane potential. These observations confirm that compound 2 induces cell death during the PDT treatment mainly through the apoptotic pathway (Figure 7A).

Further, we confirmed the membrane damage through the chromatin condensation technique using fluorescence imaging with Hoechst.31−34 Hoechst could stain only the viable cells resulting in blue fluorescence and the membrane damage can be easily monitored through the decrease in fluorescence. It was found that MDA-MB-231 cells showed 22% chromatin condensation after PDT with compound 2 at 3.5 μM, whereas it showed a 56% decrease in fluorescence at 7 μM. In contrast, in light and dark controls only 11.5 and 12% chromatin condensation was observed, respectively, which clearly indicated a gradual concentration dependent elimination of cancerous cells due to the PDT activity of the porphyrin conjugate 2 (Figure 7B).

Figure 4. Histogram depicting the cytotoxicity of the compound 1 and 2 in light (A,B) and the dark (C,D) in MDA MB 231 (red), SiHa (blue), and SW 620 (pink) cell lines showing the percentage growth inhibition upon irradiation using Waldmann PDT 1200 L (570 nm, 50 mJ/cm², 15 min).

Figure 5. Uptake of 2 by MDA-MB-231 cells. Red fluorescence in MDA-MB-231 cells after treatment with 2 (7 μM). Merged image showing MDA-MB-231 cell nucleus with a vital nuclear dye DAPI (blue) and 2 (red).
Another hallmark study on apoptosis is that it employs the cytochemical double staining method using AO and EB. The apoptotic cells and the condensed nuclei are stained selectively by EB (red), whereas the control cells had only taken up AO (green). This could be clearly evidenced from the lower panels shown in Figure 7C. PDT with 2 in MDA-MB-231 cells at 3.5 μM resulted in about 8% EB staining. At 7 μM of compound 2, we observed 72% EB staining, whereas in light and dark controls only 1 and 1.5% staining was observed respectively. The double staining method using compound 2 revealed that it could effectively induce apoptosis through cell membrane permeabilization and chromatin condensation.

ROS-mediated cell death has been widely attributed to be associated with PDT. Hence, we were interested to study the cellular oxidative stress in complex 2-based PDT using a 5- (and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA) assay.²⁵⁻³⁷ CM-H₂DCFDA, when it is in its reduced state, emits no fluorescence, whereas when cellular oxidation occurs, it is hydrolyzed by esterases to releases the acetate moieties and thereby emitting a green fluorescence. When MDA MB 231 cells were treated with compound 2 at 3.5 μM and irradiated, followed by the addition of CM-H₂DCFDA, we observed a fluorescence of 34.7% in cells. Interestingly on increasing to 7 μM of compound 2, the cells showed about 58.9% of CM-H₂DCFDA fluorescence (Figure 8). In contrast, light and dark control showed negligible fluorescence. This result clearly indicates that PDT with compound 2 induced a significant subcellular ROS generation leading to oxidative stress in MDA-MB-231 cells.

Cell proliferation is a tightly regulated process following a cyclic pattern and hence termed as the cell cycle. In the cell cycle pattern, there are different checkpoints to ensure the quality of DNA replication and chromosome distribution. In
In our study, we have analyzed the cell cycle pattern by treating the cells with compound 2. Interestingly, we found about 31% of the G2/M arrest after treatment, which appeared significantly as exhibited in Figure S8 in the Supporting Information. Moreover, the PARP cleavage, a 116 kDa nuclear poly(ADP-ribose) polymerase represents the hallmark of DNA damage-induced apoptosis. We observed that there was no PARP cleavage after treatment with compound 2 (7 μM) in the absence of light, which again confirms the noncytotoxic nature of 2 in the absence of light (Figure 9). However, upon photo-irradiation with 2-induced PARP cleavage at concentrations of 1.75, 3.5, and 7 μM, thus confirming that compound 2 induces cell death via apoptosis.

**CONCLUSIONS**

In summary, we have designed and synthesized picolylamine-linked porphyrin derivatives 1–2 and have developed their self-assembled structures which were investigated through various spectral and microscopy techniques. Of the picolyl porphyrin systems synthesized, compound 2 exhibited the vesicular structure in methanol with an average size of 250 ± 50 nm, whereas the compound 1 forms spherical structures. We established the photodynamic activity of these porphyrin nanostructures through the triplet excited state as well as the singlet oxygen generation efficacy. Excited state studies revealed that zinc insertion in the picolyl porphyrin system compound 1, both the core and peripheral positions, enhances its intersystem crossing efficiency and showed a triplet excited state quantum yield of 0.97 and a singlet oxygen generation efficiency of 0.92. Moreover, the in vitro PDT investigations of the porphyrin conjugates using the breast cancer cell line MDA-MB 231 cancerous cells revealed that compound 2 with five zinc metal ions showed better photocytotoxicity having an IC50 value of 7 μM. The investigation on the mechanism of biological activity showed that the cell death of these breast cancer cells mainly occurred through the apoptotic pathway which was evidenced through the TMRM assay, chromatin condensation, and AO/EB assay. Interestingly, the conjugate 2 showed a cellular uptake within an hour and an intracellular ROS generation of about 59%. Moreover, the cell cycle pattern showed the cell cycle arrest in the G2/M phase and the mechanism of cell death happened to be via apoptosis as confirmed through PARP cleavage. These results demonstrate that Zn2+ ion insertion in the picolyl porphyrin helps in enhancing the efficiency of singlet oxygen generation and photodynamic activity. Henceforth these systems can act as versatile sensitizers for future potential applications in the field of PDT and cancer therapeutic regimes.

**EXPERIMENTAL TECHNIQUES**

**General Methods.** We used a Shimadzu UV-3101 and 2401PC UV-vis-NIR scanning spectrophotometer for monitoring absorption spectra, whereas the emission spectra were monitored using a SPEX-Fluorolog F112X spectrofluorimeter. The NMR spectra (1H and 13C) were recorded on 300 and 500 MHz Bruker advanced DPX spectrometers using TMS as the standard. The emission quantum yields of the compounds 1–2 were measured by the relative method using hematoporphyrin (Φf = 0.01) as the standard. The triplet excited state studies were employed with a nanosecond laser flash photolysis system which is an Applied Photophysics model LKS-20 laser kinetic spectrophotometer having a OCR-12 Series Quanta Ray Nd:YAG laser. We fixed the analyzer and the laser beams at right angles to each other with an energy of 50 mJ on exciting at 355 nm. Cytotoxicity studies were measured with a Waldmann PDT 1200 L having a 1200 W
lamp (200 J cm\(^{-2}\), 50 mW cm\(^{-2}\)), which can excite in the region 600–720 nm.

**Materials.** TMRM, EB, Hoechst, AO, and MTT were purchased from S.D. Fine chemicals, India; Sigma-Aldrich; U.S.A Merk Chemicals, Germany. The reference compounds DPBF and α-carotene were recrystallized using ethanol and chloroform solvent systems. The solvents used for the synthesis and analysis were distilled by following standard protocols.

**Determination of Triplet Excited State and Singlet Oxygen Quantum Yields.** The triplet quantum yield (Φ\(_T\)) of the porphyrin derivatives 1–2 and the quantification of singlet oxygen generation were carried out by a relative method using appropriate references at identical as well as optical conditions as reported earlier.

**Morphological Studies.** The picollylamine-linked porphyrin compounds 1–2 (10\(^{-4}\) M) were taken in methanol and drop casted on the top of the Al grid for SEM analysis, the Cu grid for TEM techniques and mica sheet surface for AFM studies. To record the AFM images, we adopted the tapping mode regime using a NTEGRA (NT-MDT), micro-TiN cantilever tips (NSG10) having a 20–80 Nm\(^{-1}\) spring constant with a 299 kHz resonance frequency. For TEM measurements, we used a high resolution transmission electron microscope (JEOL 100 kV) and for the sample preparations we followed the drop casting method (10\(^{-3}\) (JEOL 100 kV) and for the sample preparations we followed the drop casting method (10\(^{-4}\) M) using a carbon-coated copper grid. A ZEISS EVO MA and LS series model scanning electron microscope was used to record the SEM images with copper grid. A ZEISS EVO MA and LS series model scanning electron microscope (Leica DMI 4000 B, Germany).

**Detection of Cellular ROS Using the CM-H\(_2\)DCFDA Assay.** For cellular ROS stress studies, approximately 10\(^6\) MDA-MB-231 cells were plated in a 60 mm dish with serum-containing media. After 24 h, the cells were treated with 3.5 and 7 μM compound 2 and photo-irradiation was performed using a PDT lamp (530 nm, 570 nM) for 15 min. To one plate, 7 μM compound 2, added without photo-irradiation, was taken as the dark control and one only with PDT treatment was kept as the light control. After 24 h of PDT with compound 2, cellular ROS content was determined using the CM-H\(_2\)DCFDA probe according to the manufacturer’s instructions (Invitrogen), and a flow cytometric analysis was then carried out using FACS Aria II (BD, USA).

**AO/EB Assay.** Apoptotic cell morphology was assessed by the AO/EB assay for 3.5 and 7 μM compound 2 nanoconjugates in MDA-MB 231 cell lines. Approximately 10\(^5\) MDA-MB-231 cells were seeded in 96 well-chambered cover glass plates and incubated for 24 h. PDT treatment was performed as mentioned previously in the TMRM assay. Light and dark controls were taken as previously described above. After 24 h of post-treatment, the cells were rinsed twice with PBS, and the cells were stained with Hoechst dye 33342 (Invitrogen) (5 μg/mL) for 5 min at room temperature. The cells were then washed twice with PBS and visualized under an inverted fluorescence microscope (Leica DMI 4000 B, Germany).

**Hoechst Staining for Analyzing Chromatin Condensation.** To study chromatin condensation, approximately 10\(^5\) MDA-MB-231 cells were seeded in 8 well-chambered cover glass plates and incubated for 24 h. PDT treatment was performed as mentioned previously in the TMRM assay. Light and dark controls were taken as previously described above. After 24 h of post-treatment, the cells were rinsed twice with PBS, and the cells were stained with Hoechst dye 33342 (Invitrogen) (5 μg/mL) for 5 min at room temperature. The cells were then washed twice with PBS and visualized under an inverted fluorescence microscope (Leica DMI 4000 B, Germany).

**Flow Cytometric Analysis of Apoptosis with Annexin V-FITC/PI.** After PDT treatments, the cells were harvested by trypsinization followed by their resuspension in cold PBS. Annexin-V/PI staining was done using an Invitrogen (USA) kit, following the manufacturer’s protocol. Live cells were not stained with Annexin V-FITC/PI (bottom left quadrant). A significant number of cells were stained positive by both Annexin V-FITC and PI after 24 h of post PDT treatment indicating early apoptosis-mediated cell death which has been analyzed by the flow cytometer FACS Aria II (BD, USA).

**TMRM Assay.** An active mitochondria in cells can be determined by cationic cell permeate fluorescent dye, that is, TMRM. Approximately 10\(^5\) MDA-MB-231 cells were seeded in 8 well-chambered cover glass plates and incubated for 24 h. The stock solution of the compound 2 (10 mM) was prepared and it is further diluted to two different concentrations such as 3.5 and 7 μM in order to carry out the concentration dependent analysis. Compound 2 was then added to the MDA-MB-231 cancerous cell lines at two different concentrations and after 1 h, PDT was obtained. Controls used were light and dark controls. A decrease in the mitochondrial inner membrane potential because of apoptosis causes a decrease in red fluorescence that was measured by a fluorescent microscope (Leica DMI 4000 B, Germany).

**Morphological Studies.** The picollylamine-linked porphyrin compounds 1–2 (10\(^{-4}\) M) were taken in methanol and drop casted on the top of the Al grid for SEM analysis, the Cu grid for TEM techniques and mica sheet surface for AFM studies. To record the AFM images, we adopted the tapping mode regime using a NTEGRA (NT-MDT), micro-TiN cantilever tips (NSG10) having a 20–80 Nm\(^{-1}\) spring constant with a 299 kHz resonance frequency. For TEM measurements, we used a high resolution transmission electron microscope (JEOL 100 kV) and for the sample preparations we followed the drop casting method (10\(^{-4}\) M) using a carbon-coated copper grid. A ZEISS EVO MA and LS series model scanning electron microscope was used to record the SEM images with copper grid for operating conditions at 100–230 V and a frequency of 50–60 Hz with a consumption of 2.5 kVA (single phase). For all the microscopic studies the solvents were subjected to evaporate at room temperature.
taken using appropriate filters by a confocal microscope (Nikon A1R, Japan).

**Cell Cycle Analysis.** For cell cycle analysis, the 10^6 MDA MB 231 cells were seeded in 60 mm culture dishes. Post 24 h of seeding, the cells were treated at different concentrations. Post 24 h PDT treatment cells were trypsinized, harvested, and fixed with 70% ethanol for 1 h and incubated at 4 °C. The fixed cells were given RNase A (100 µg/mL) treatment for 1 h at 37 °C. Then, it was treated with PI (10 µg/mL) for 15 min. The cells were filtered using a cell strainer of 22 µm pore size (BD bioscience, USA). Finally, DNA content of the cells was analyzed using FACS Aria II (BD, USA).

**Immunoblot.** PDT treatment was done as mentioned earlier and incubated for 24 h. The treated cells were scraped out and RIPA lysis buffer added. After 1.5 h in RIPA lysis buffer in a thermomixer, the supernatant was collected and later quantified using the Bradford’s reagent. SDS-PAGE followed by immunoblotting was carried out using 50 µg of total protein and probed using the PARP antibody. Then, probed with horseradish peroxidase-conjugated secondary antibodies and detection was done using the enhanced chemiluminescence method. The images were taken using the Molecular Imager VersaDoc MP4000 (Bio-Rad USA) system.

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**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01380.

**1H NMR, absorption, fluorescence spectra, morphological studies, triplet excited state, singlet oxygen generation experiments, zeta potential, and cell cycle analysis of compound 1 and its zinc complex compound 2 (PDF)**

**AUTHOR INFORMATION**

**Corresponding Authors**

*E-mail: betsydec9@gmail.com* (B.M.).

*E-mail: sasha@rgch.res.in.* Phone: 91-04712529501 (S.A.N.).

**ORCID**

Danaboyina Ramaiah: 0000-0003-1388-4768

S. Asha Nair: 0000-0002-3756-3259

**Present Address**

$^*$Indian Institute of Science Education and Research (IISER) Tirupati, Department of Chemistry, Tirupati, Andhra Pradesh 517507, India.

**Author Contributions**

B.M. and R.R. N. share an equal authorship in this manuscript.

**Notes**

The authors declare no competing financial interest.

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