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Anticancer Activities of a Metal-Free Phthalocyanine on MCF-7 and MDA-MB-231 Cells and Singlet Oxygen Production as a Photosensitizer in PDT

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Abstract: Cancer, which is often described as an uncontrollable rapid proliferation of cells, is currently the leading cause of death in the world together with cardiac disease. Therefore, the main purpose of the current research work was to study the anticancer effects of a first-time-synthesized phthalocyanine (Pc) as photosensitizer in PDT against cancer and evaluate its effects on human cells in vitro. Quantum yields of singlet oxygen photogeneration were in air using the relative method with standard-ZnPc as reference and DPBF as chemical quencher for singlet oxygen. The concentration of DPBF was prepared almost 3 x 10^{-5} molar to avoid chain reactions induced by DPBF in the presence of singlet oxygen. Solutions of Pc as sensitizer (absorbance = 2.0 at the irradiation wavelength) containing DPBF were prepared in the dark and irradiated in the Q band region using the setup described. DPBF degradation at 417 nm was monitored with UV-Vis spectrophotometry. For in vitro studies, nine different MFPc-1 concentrations (0.2 µM- 0.4 µM- 0.8 µM- 1.6 µM- 3.2 µM- 6.4 µM- 12.8 µM- 25.6 µM- 51.2 µM) applied to MCF-7 and MDA-MB-231 breast cancer cell lines for 24 hours and MTT assay was carried out. After determination of optimum concentration, mitotic index, and apoptotic index values of cell lines were determined with administration of these concentrations. Singlet oxygen quantum yield (Φ_{Δ}), which is a measure of the efficiency, of MFPc-1 was found 0.50, although MFPc-1 is being metal-free phthalocyanine. For in vitro studies after the application of different concentrations to MCF-7 and MDA-MB-231 for 24 hours, the optimum concentration was determined as 12 µM for both cell lines by the MTT assay. After application of the determined optimum concentration for 24, 48 and 72 hours, there was a significant decrease in the mitotic index values and significant increase in the apoptotic index values of both MCF-7 and MDA-MB-231 breast cancer cell lines.

Keywords: Metal-free phthalocyanine, singlet oxygen, in vitro, breast cancer, cell kinetic.

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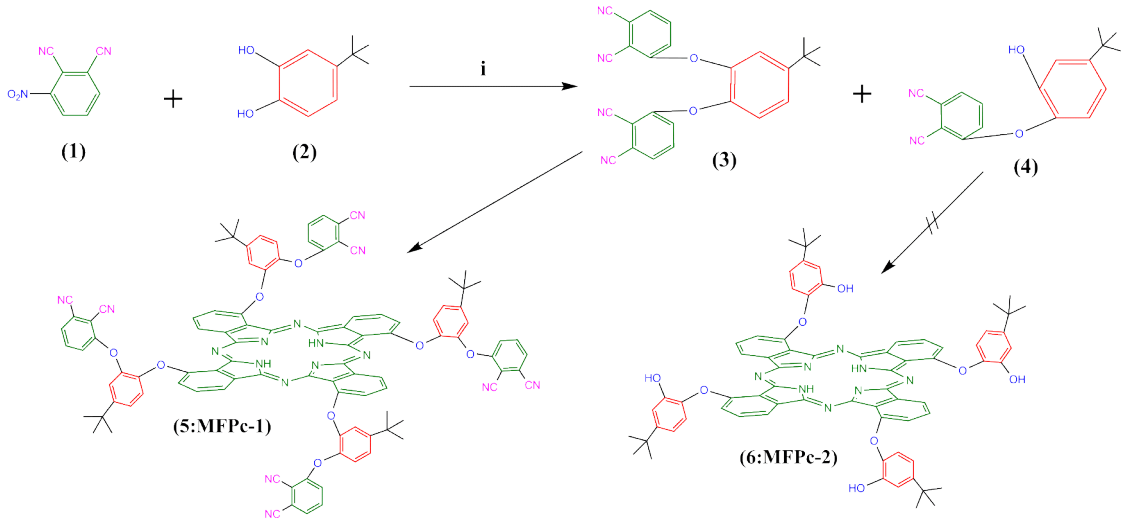
INTRODUCTION

Phthalocyanine (Pc) was discovered by accident in 1907 as a by-product in the synthesis of o-cyanobenzamide (1). Pcs are macrocyclic compounds that consist of 4 pyrrole subunits that are linked by azomethine bridges in a 16-membered ring with 18π electron system which is responsible for the intense blue/green color (2). Pcs can be modified to suit a specific application either by changing the peripheral group and non-peripheral group or by changing the central metal (3). Therefore, they are useful in a wide range of areas such as in medicinal and material science as gas sensors (4) photodynamic therapy sensitizers (5) nonlinear optical materials (6). In medicine, Pcs were used in clinical photodiagnosis due to photosensitizer properties (7,8). For PDT, the first official approval and clinical were concurrently reported in 1993 and 1996, and it can be alternative

due to be a safe in cancer treatment, respectively (9-11). PDT has advantages over other cancer treatment modalities, such as surgery, radiotherapy, and chemotherapy. PDT consist of light, photosensitizer, and cancer tissue which is based on the generation of singlet oxygen ($^1O_2$-$^1\Sigma_g$) and a Pc can do this as a photosensitizer that leads to cancerous cell death upon the wavelength of irradiation between 650 and 900 nm (12-15).

In the present study, we have obtained Pc(MFPc-1) substituted with electron-donating group including di-tert-butyl while 3,3′-(4-tert-butyl-1,2-phenylene)bis(oxy)diphthalonitrile was recrystallized from methanol. This is not surprised since the first accidental synthesis of Pcs by Braun and Tcherniac (16). Here, we report the production of singlet oxygen as possible photosensitizer based on PDT. We also present here antiproliferative effect of the MFPc-1 obtained on cancer cells (17,18).



Scheme 1. Synthetic pathway. **i**: DMSO, K_2CO_3, 3 days, room temperature.

EXPERIMENTAL SECTION

Materials and Methods

3-nitrophthalonitrile **1** and 1,2-dihydroxy-4-tert-butylbenzene **2** were purchased from Alfa Aesar and Sigma-Aldrich, USA, respectively. The materials, instruments and methods that are used for synthesis, photophysical (fluorescence quantum yield) and photochemical studies (singlet oxygen quantum yield) as previously published in the literature (19-25).

Synthesis

A reaction was started so that can obtain 3,3′-(4-tert-butyl-1,2-phenylene)bis(oxy) diphthalonitrile **3** (24,25) from 4-tert-butylbenzene-1,2-diol **2** (2.40 g, 14.45 mmoL) and 3-nitrophthalonitrile **1** (5.00 g, 28.90 mmoL)) in the present anhydrous potassium carbonate (11.98 g, 86.68 mmol) in dimethylsulfoxide (15 mL) under Ar atmosphere, after stirring for 4 h at 60 °C. The reaction mixture was stirred at room temperature for 3 days and was subsequently poured into water (500 mL). The beige to yellow powdery product was filtered off. It was observed to give a green color in a flat-bottomed flask while being crystallized from ethanol (150 mL), yielding a MFPC-1. Then, it was purified via column chromatography using a gradient of tetrahydrofuran and methanol as the eluent, respectively. Pc was obtained as a deep green powder with mp > 200 °C and is soluble in THF, DMSO, and DMF. The other data such as FTIR and UV-Vis spectrum are given as follows.

Yield 0.021 g. UV–Vis (DMSO), λ_{max}/nm (logε): 724 (4.67), 695 (4.67), 660 (4.35), 632 (4.25), 320 (4.68). FT-IR (ATR), μ_{max}/cm^{-1}: 2958 (Ar–CH), 2906/2865(Aliph. –CH), 2237 (C≡N), 1575 (C=C), 1275/1245 (C–O–C). ^{1}H-NMR (DMSO-d$_6$), δ,/ppm: 7.85–7.00 (36H, Ar–H), 1.35 (36 H, t-butyl). Anal. calcd. for $C_{104}H_{74}N_{16}O_8$: C, 74.54; H, 4.45; N, 13.37 found: C, 74.58; H, 4.45; N, 13.45%. MALDI–TOF–MS: m/z calcd. 1674.59 amu; found 1678.00 amu $[M+4H]^+$.

Photophysical Studies

Fluorescence quantum yield (Φ_F)

Relative Φ_F was calculated by a comparative method using a standard reference, using Equation 1.

$$\Phi_F = \Phi_{F(Std)} \frac{F \times A_{Std} \times n^2}{F_{Std} \times A \times n^2_{Std}} \quad \text{(Eq. 1)}$$

Photochemical Studies

Singlet oxygen quantum yield (Φ_Δ)

Singlet oxygen quantum yield was also determined by chemical procedure according to to Eq. (2).

$$\emptyset_A = \frac{\emptyset_A^{Std} \times R_{DPBF} \times I_{abs}^{Std}}{R_{DPBF}^{Std} \times I_{abs}} \quad \text{(Eq. 2)}$$

Cell Studies

Cell culture

The MCF-7 and MDA-MB-231 cells used in the experiments were purchased by our research laboratory from American Type Culture Collection (ATTC Manassas, VA, USA). Cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100-unit mL^{-1} penicillin and 100 µg mL^{-1} streptomycin at 37 °C and 5% CO_2 (26).

Cell viability

The antiproliferative effect of MFPc-1 on MCF7 and MDA-MB-231 cells were determined by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Cells were plated in 96-well plates with $3x10^4$ cells/200 µL medium for 24 h at 37 °C. MFPc-1 was applied to the cells at 0,2 µM- 0,4 µM- 0,8 µM- 1,6 µM- 3,2 µM- 6,4 µM- 12,8 µM- 25,6 µM- 51,2 µM concentrations for 24 hours. At the end of the experimental period the medium in each well was removed and 40 µL MTT solution (5 mg/mL in phosphate buffered saline/PBS) was added into each well and cells were incubated at 37 °C for 4 h. Then, 16 µL of dimethylsulfoxide (DMSO) was added into each well and cells were shaken thoroughly for 1 h on a shaker. The absorbance of the samples was measured against a background control as a blank using an Elisa reader at 450-690 nm (18).

Mitotic index (MI)

For evaluation of MI, cells were planted in 24-well plates. Each well contained $3x10^4$ cells for both cell lines. Cells were incubated 24 hrs after cell seeding. Cells were treated with 12.8 µM concentration for 24, 48 and 72 hours. At the end of this experimental period, for fixation, Carnoy fixative was used and the cells were made clear with the Feulgen method and stained with Giemsa. For analyzing MI, metaphases, anaphases and telophases stages of cell division were evaluated. For calculating of MI, approximately 3000 cells were counted (27).

Apoptotic index (AI)

The AI represents the percentage of fragmented nuclei. DAPI (6-diamidino-2-phenylindole) was used to determine the apoptotic nucleus. It is a blue fluorescent dye. Cells were cultured in 6-well culture dishes and fixed with methanol: FTS mixture after the experimental process until staining was performed. Washing was carried out to remove the dye. For washing, PBS was used. A fluorescent microscope was used to identify apoptotic cells. For calculating of AI, approximately cells in 100 microscopic fields were evaluated (27).

Statistics

All parameters of cell kinetics were evaluated according to the controls and each other. Therefore, in order to analyze the results, one-way Anova test, Dunnett's test and Student's t-test were used. These statistical analyses were performed using SPSS statistics software (V22.0 IBM, Armonk, NY, USA). In the tests $p < 0.05$ level of significance was accepted.

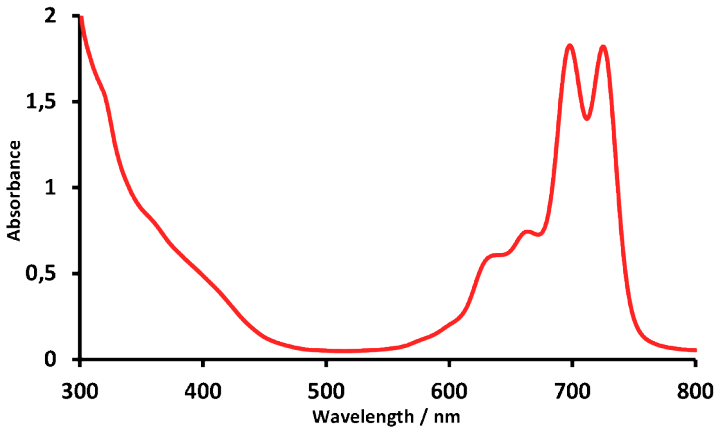


Figure 1: UV-Vis spectrum for MFPc-1 in DMSO.

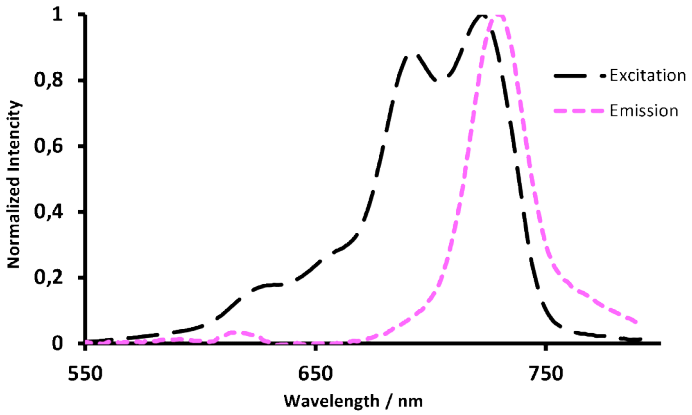


Figure 2: The excitation spectrum (long dashed line, black), emission spectrum (square line, pink), excitation wavelength 617 nm.

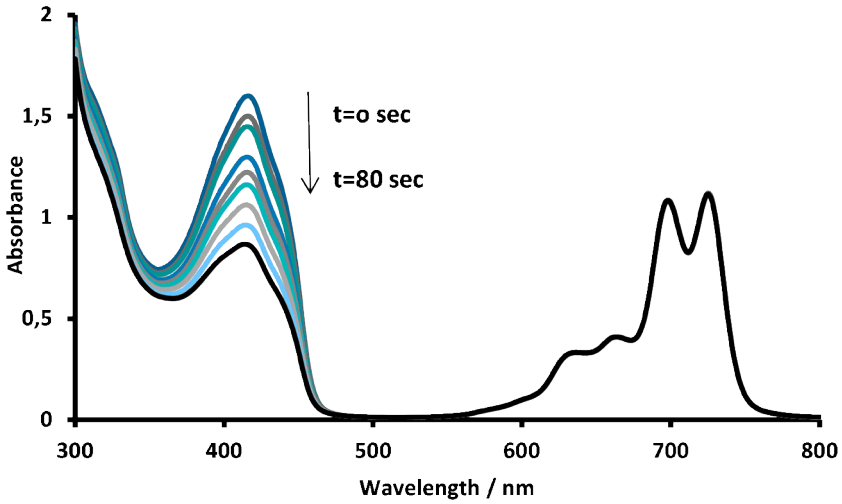


Figure 3: Singlet oxygen generation spectrum at 417 nm.

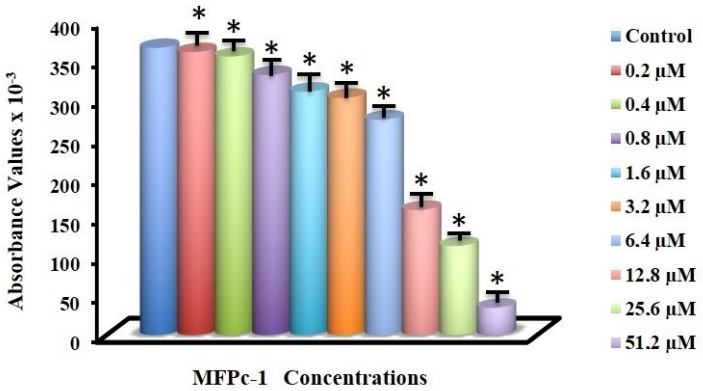


Figure 4: Absorbance values of mitochondrial dehydrogenase activity of MCF-7 cells treated with different MFPc-1 concentrations for 24 h (450-690 nm) ($p<0.05$).

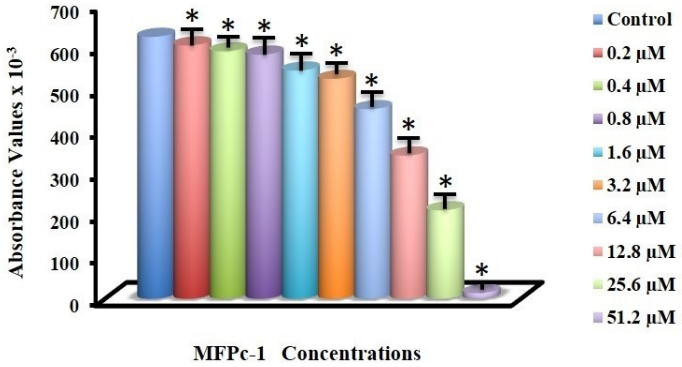


Figure 5: Absorbance values of mitochondrial dehydrogenase activity of MDA-MB-231 cells treated with different MFPc-1 concentrations for 24 h (450-690 nm) ($p<0.05$).

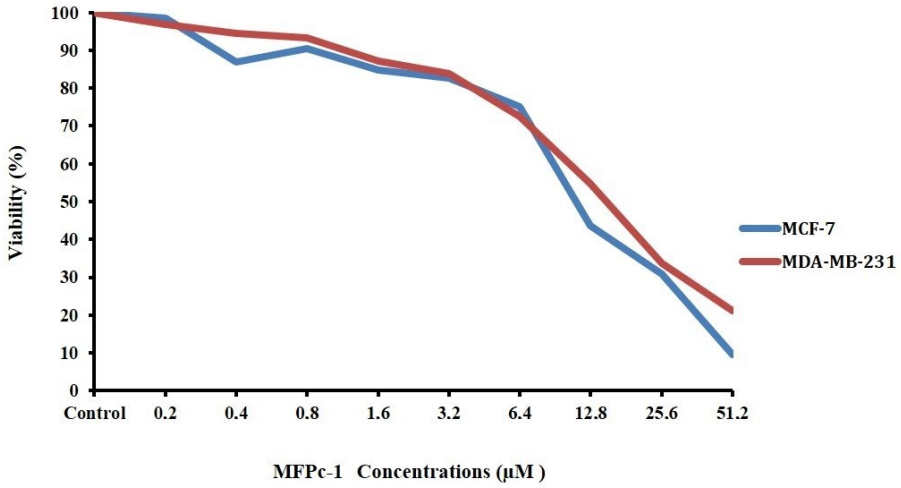


Figure 6: Percent viability values of MCF-7 and MDA-MB-231 cells treated with different concentrations of MFPc-1 for 24 hours ($p<0.05$).

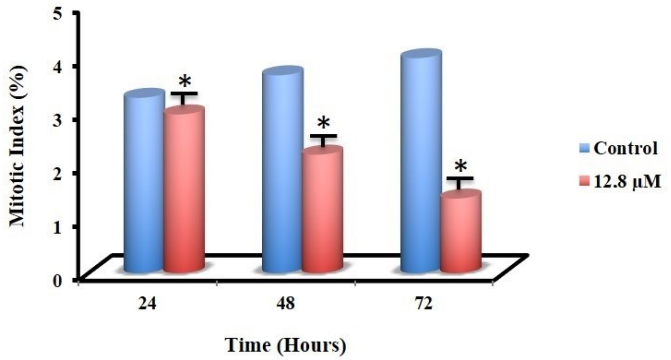


Figure 7: Mitotic index values of MCF-7 cells treated with 12.8 µM concentration of MFPc-1 for 24, 48 and 72 h ($p<0.05$).

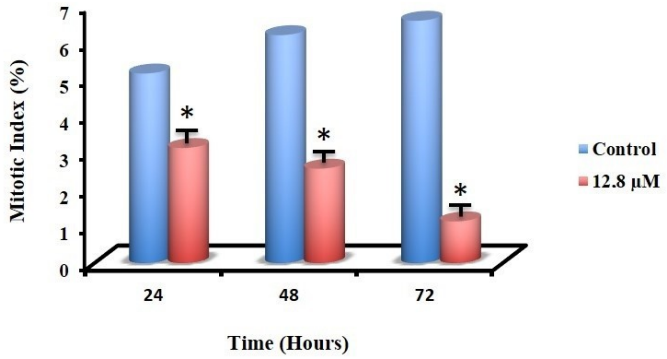


Figure 8: Mitotic index values of MDA-MB-231 cells treated with 12.8 µM concentration of MFPc-1 for 24, 48 and 72 h ($p<0.05$).

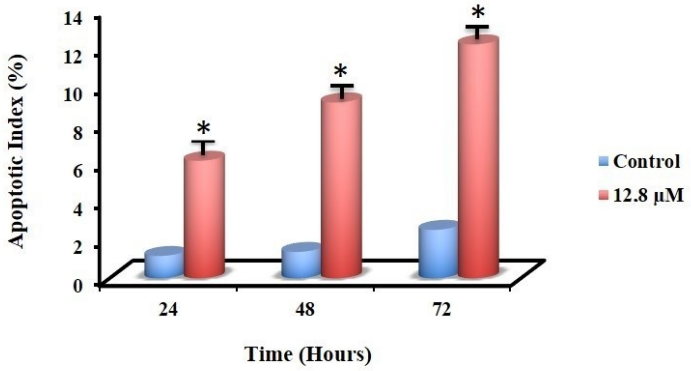


Figure 9: Mitotic index values of MCF-7 cells treated with 12.8 µM concentration of MFPc-1 for 24, 48 and 72 h ($p<0.05$).

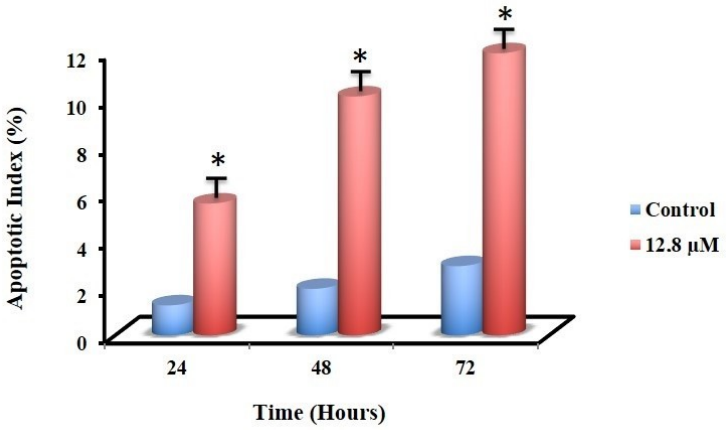


Figure 10: Mitotic index values of MDA-MB-231 cells treated with 12.8 µM concentration of MFPc-1 for 24, 48 and 72 hours ($p<0.05$).

Table 1: The photophysical and photochemical data in DMSO.

Compound	λ_{Abs}	λ_{Ems}	λ_{Exc}	λ_{Stokes}	Φ_F	Φ_Δ	ISC
MFPc-1	695/724	729	690/722	5	0.18	0.50	0.32
Std-ZnPc[a]	672	682	672	10	0.18	0.67	0.15

[a]Data from refs. (28, 29).

RESULTS

We obtained a novel Pc even if it is by chance. Characterization of this Pc was carried out using FT-IR, UV–Vis spectroscopy, mass spectrometry, and elemental analysis. In the UV-Vis spectrum of a Pc, there are two peaks as characteristic called Q band and B (Soret) band. As shown in Figure 1, B band and Q band of MFPc-1 were recorded at 320 nm and at 695/724 nm with D_{4h} symmetry in DMSO at room temperature, respectively. The shape of Q band was typical for metal-free Pcs. On the other hand, the Q band is attributed to π–π* transitions from the HOMO to the LUMO of the Pc ring whereas the B band is observed due to the transitions between the deeper π levels and the LUMO.

Fluorescence excitation and emission peaks for MFPc-1 are shown in Figure 2 and the data are listed in Table 1. Fluorescence emission peak was observed at 719 nm in DMSO. The Q band in emission spectrum and excitation spectrum, is typical of Pcs (30). While the observed Stokes shift of the MFPc-1 is lower than that of unsubstituted Std-ZnPc. Fluorescence quantum yield value is 0.18 just like a typical metal Pc, this means it is losing less energy.

Singlet oxygen is generated by a Type II reaction and is explained transform to from triplet oxygen energy level with the Jablonski Diagram (Kindly see Supplementary inf. Figure S1, at the end) the generation of singlet oxygen via the Type II process involving a spin coupling interaction with oxygen in its triplet ground-state. The efficiency is known as their singlet oxygen quantum yield (Φ_{Δ}) which is described as the number of molecules of singlet oxygen generated per number of photons absorbed by the sensitizer (here MFPc-1). DPBF is used to determine the singlet oxygen quantum yield which is a good acceptor because it reacts rapidly with singlet oxygen (31). The spectral changes were examined during the photolysis of the MFPc-1 in the presence of DPBF were measured over time (0–80 sec) by UV–Vis spectroscopy. In Figure 3, the spectrum of MFPc-1 shows no change at 417 nm in the Q band, this means the compound is not degraded. The Φ_{Δ} value is 0.50 and is considerably high for metal-free Pcs. This result is considerably good compared to substituted metal-free phthalocyanines published in the literature (32). This may be due to the effect electron-donating of t-*butyl* groups to Pc ring.

Cell proliferation values belong to MCF-7 cells decreased from 365.333 x 10^{-3} to 360 x 10^{-3} for 0.2 µM; to 354.286 x 10^{-3} for 0.4 µM; to 330.857 x 10^{-3} for 0.8 µM; to 309.714 x 10^{-3} for 1.6 µM; to 301.75 x 10^{-3} for 3.2 µM; to 274 x 10^{-3} for 6.4 µM; to 159.25 x 10^{-3} for 12.8 µM; to 113.25x 10^{-3} for 25.6 µM and 34.714 x 10^{-3} for 51.2 µM (Figure 4). Cell proliferation values belong to MDA-MB-231 cells decreased from 622.5 x 10^{-3} to 602.375 x 10^{-3} for 0.2 µM; to 588.25 x 10^{-3} for 0.4 µM; to 580.375 x 10^{-3} for 0.8 µM; to 542.5 x 10^{-3} for 1.6 µM; to 522.625 x 10^{-3} for 3.2 µM; to 450.625 x 10^{-3} for 6.4 µM; to 340.5 x 10^{-3} for 12.8 µM; to 210.25 x 10^{-3} for 25.6 µM and 13.5 x 10^{-3} for 51.2 µM (Figure 5).

After the administration MFPc-1 concentrations for 24 h, cell proliferation values of MCF-7 and MDA-MB-231 cells decreased significantly depending on concentration. The differences between the control and all experimental groups were significant ($p<0.05$). Cell viability values of MCF-7 cell line are 98,54%, 86,98%, 90,56%, 84,78%, 82,6%, 75%, 43,6%, 30,9%, 9,5 for MCF-7 cells; 96,77%, 94,49%, 93,23%, 87,15%, 83,95%, 72,39%, 54,69%, 33,78%, 21,17% for MDA-MB-231 cells respectively for 0,2 µM- 0,4 µM- 0,8 µM- 1,6 µM- 3,2 µM- 6,4 µM- 12,8 µM- 25,6 µM- 51,2 µM concentrations (Figure 6).

Administration of 12,8 µM concentration of MFPc-1 to MCF-7 and MDA-MB-231 cells caused a decrease in mitosis. MI values decreased from 3.27% to 2.96% at 24 h; from 3.69% to 2.21% at 48 h and from 4.01% to 1.39% at 72 h for MCF-7 cells (Figure 7); decreased from 5.14% to 3.12% at 24 h; from 6.18% to 2.56% at 48 h and from 6.57% to 1.13% at 72 h for MDA-MB-231 cells (Figure 8). MI values of MCF-7 and MDA-MB-231 cells decreased significantly with time as a result of MFPc-1 administration. This decrease was statistically significant ($p <0.05$).

Administration of 12.8 µM concentration of MFPc-1 to MCF-7 and MDA-MB-231 cells caused apoptotic cell death. AI values increased from 1.16 to 6.12 at 24 h; from 1.37 to 9.16 at 48 h and from 2.52 to 12.18 at 72 h for MCF-7 cells (Figure 9); increased from 1.27 to 5.58 at 24 h; from 1.97 to 10.11 at 48 h and from 2.93 to 11.94 at 72 h for MDA-MB-231 cells (Figure 10). Apoptotic index values of MCF-7 and MDA-MB-231 cells increased significantly with time as a result of MFPc-1 administration. This increase was statistically significant ($p <0.05$).

CONCLUSION

We have explored the photophysical and photochemical properties of a 4-tert-butylbenzene substituted metal-free phthalocyanine. MFPc-1 is a novel material and its findings suggest that MFPc-1 exerts potent anticancer effects and shows that it can potentially be used as a photosensitizer in PDT. In this study, antiproliferative effects of MFPc-1 on MCF-7 cell line derived from estrogen receptor-positive human breast epithelial carcinoma and MDA-MB-231 cell line derived from estrogen receptor-negative human metastatic breast carcinoma were investigated. For this purpose, the anticancer effects of MFPc-1 were evaluated using

different cell kinetics parameters (cell viability, mitotic index and apoptotic index).

Among these parameters, MTT analysis (cell viability) is an effective and fast method used to determine cytotoxicity in vitro. In various studies using phthalocyanine, MTT analysis has been used to determine cytotoxicity (33, 34). In present study as a result of MTT analysis for both cell lines, 12,8 μM concentration was determined as LD_{50} (Lethal Dose 50) that caused the death of half of the cells.

Although the mitotic index parameter is not widely used, it is a laborious and reliable method used in determining cell kinetics. Apoptotic index is one of the frequently used methods in cell kinetics studies. The 12.8 μM concentration used in this present study resulted in a significant reduction in mitotic index values and significant increase in apoptotic index values in both cell lines and similar results were seen.

These parameters showed that MFPc-1 has similar effects in both cell lines. Similar sensitivities to the compound for both cell lines have suggested that the mechanisms causing cell death are independent of the presence of the hormone receptor.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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Supporting Information
For

Anticancer activities of a metal-free phthalocyanine on MCF-7 and MDA-MB-231 cells and singlet oxygen production as a photosensitizer in PDT

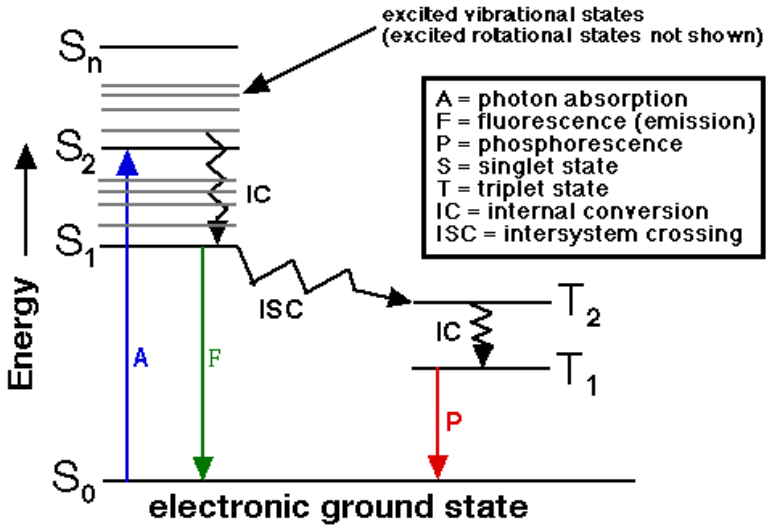

Figure S1: Jablonski diagram.