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SYNTHESIS AND ANTITUMOR ACTIVITY OF A PORPHYRIN DERIVATIVE IN PHOTODYNAMIC THERAPY

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A photosensitizer 5, 15-di (p-benzoato) porphyrin (DBPP) was synthesized and characterized. UV-Vis absorption spectra indicated that compound DBPP had a characteristic long wavelength absorption peak at 630 nm and a singlet oxygen quantum yield of 0.39. Fluorescence spectra showed that DBPP had strong emission peaks at 633 nm and 698 nm that can be used in fluorescence imaging. To investigate the photodynamic ability of DBPP against human esophageal cancer (Eca-109 cells) and human bladder (T24 cells), cell viability of DBPP *in vitro* were studied. Without light activation, DBPP was nontoxic to Eca-109 cells or T24 cells, however, upon light activation, DBPP exhibited significant photo-toxicity. These results suggested that compound DBPP could be a promising drug candidate for photodynamic therapy in cancer.

Key words: photosensitizer; photodynamic therapy; DBPP; cancer.

INTRODUCTION

As a novel phototherapy, photodynamic therapy (PDT) has been widely used in a variety of oncological, cardiovascular and dermatological diseases (Zhang LJ, *et al*., 2014; Dougherty TJ, *et al*., 1998; Dougherty TJ, 1995). PDT is a non-invasive treatment that involves the accumulation of a photosensitizer (PS) and reactive oxygen species (ROS) in malignant tissue (Serra A, *et al*., 2010 b; Wu D, *et al*., 2015; Lu K, *et al*., 2014). After irradiation with laser light in certain wavelength, the activated photosensitizer can generate singlet oxygen ($^1O_2$) or other reactive oxygen species (Zhang LJ, *et al*., 2014; Bacellar IO, *et al*., 2015; Serra AC, *et al*., 2002 a). The oxidation reaction will then happen in tumor tissue and initiate oxidative damage to cell membranes or proteins until the cell death. $^1O_2$ as a critical factor in PDT could induce type II photochemical reaction and cause cellular toxicity through apoptosis or necrosis (Zhang L-J, *et al*., 2015; Li JW, *et al*., 2015; Buytaert E, *et al*., 2005). An ideal photosensitizer for PDT requires strong absorption in long wavelengths (600 nm to 800 nm) to ensure well tissue penetration (Huang Z, 2005; Wainwright M, 2008). High selectivity is equally important so that can make photosensitizer kill the diseased tissue and have no damage to normal cells around them. In addition, a suitable photosensitizer also need low dark toxicity and strong photocytotoxicity, easy to acquire, high yield of $^1O_2$ and rapidly metabolic capability from the body. The 5, 15-diarylporphyrins derivatives have great promises and efficacy for some cancers treatment due to their absorption in long wavelengths (Serra A, *et al*., 2010 b; Banfi S, *et al*., 2012). A substantial effort has been put into the development of photosensitizers over the past few decades by many scientists (Orlandi VT, *et al*., 2013; Pereira NAM, *et al*., 2011), and numerous efforts in our laboratory have been directed toward the synthesis of new potential photosensitizers related to 5, 15-diarylporphyrins. The present work aims to investigate the PDT effect of compound DBPP, including the synthesis and characterization, photophysical properties, singlet oxygen quantum yields and photodynamic activities *in vitro*.

EXPERIMENTAL

Materials and Methods

All the chemicals and reagents were commercially purchased and used as supplied. All reactions involving air-sensitive reagents were taken under an atmosphere of nitrogen with magnetic stirring. Dichloromethane (DCM) was

dried by distillation over CaH_2. ^1H NMR spectrum spectrum was recorded at room temperature on a Bruker 400 MHz spectrometer, and was reported in ppm relative to TMS and referenced to the solvent indicated. ESI-mass spectrum was carried out on a Micromass triple quadrupole mass spectrometer. UV-Vis absorption spectra were recorded on an ultraviolet visible spectrophotometer (Model V-530, Japan). Fluorescence spectrum was measured on a fluorescence spectrophotometer (FluoroMax-4, France). Melting point was obtained on a "stuart" Bibby apparatus and was uncorrected. Column chromatography was performed with silica gel H (200-300 mesh).

Synthesis of the 5, 15-di (p-benzoato) porphyrin (DBPP)

Dipyrromethane (compound **1**, 392 mg, 2.68 mmol) and 4-(methoxycarbonyl) benzaldehyde (222 mg, 2.7 mmol) were dissolved in anhydrous DCM (520 mL) in a round bottom flask. Trifluoroacetic acid (TFA, 0.12 mL, 1.61 mmol) was added dropwise via springe under nitrogen and the solution was stirred at room temperature for 3 h. 2, 3-dichloro-5, 6-dicyano-1, 4-benzoquinone (DDQ, 736 mg, 3.22 mmol) was added and the solution was stirred for a further 3 h. Triethylamine (4 mL) was added to neutralize the reaction mixture (Mikroyannidis JA, *et al*., 2011). The solvent was removed with a rotary evaporator and the crude product was purified by a silica gel column (DCM : PE = 1 : 1). The first fraction was collected to give a purple red solid 5, 15-di (p-methyl-benzoato) porphyrin (compound **2**, 323 mg, 41.7%).

Compound **2** (556 mg, 0.96 mmol) was dissolved in a mixture of tetrahydrofuran (THF) and methanol (200 mL, V / V = 1 : 1). 2 M KOH (48 mL, 96 mmol) was added and the solution was heated to reflux for 14 h. The solvent was removed with a rotary evaporator and 1 M HCl was added to neutralize the pH to 3 - 4. The precipitate was filtered by Buchner funnel and the filter cake was washed with H_2O. The solid residue was dried under vacuum to give a purple solid (500 mg, 94.5%). Mp > 300 °C. ^1H NMR (400 MHz, DMSO-d_6, ppm) δ: 10.70 (s, 2H), 9.70 (d, J = 4.7 Hz, 4H), 9.07 (d, J = 4.7 Hz, 4H), 8.44 (q, J = 7.9 Hz, 8H), -3.26 (s, 2H)。 ESI-MS (M+1): 551.2.

PHOTO-PHYSICAL AND PHOTOCHEMICAL MEASUREMENTS
Absorption and emission spectra

UV-Vis absorption spectra of compound DBPP were recorded with ultraviolet visible spectrophotometer. DBPP was dissolved in dimethyl sulfoxide (DMSO) to solutions at different concentrations (range from 1 μM to 10 μM). The absorbance of DBPP between 300 nm and 800 nm was measured at these concentrations. Fluorescence spectra were measured with fluorescence spectrophotometer. Compound DBPP was dissolved in DMSO to get 2 μM a solution. Slits were kept narrow to 1 nm in excitation and 1 or 2 nm in emission. Right angle detection was used. All the measurements were carried out at room temperature in quartz cuvette with path length of 1 cm.

Determination of the quantum yield for singlet oxygen generation

The singlet oxygen quantum yields (Φ_Δ) of generation was measured in DMF by chemical oxidation using 1, 3-diphenylisobenzofuran (DPBF) as the scavenger. Typically, 3 mL DMF solution containing 20 μM DPBF and 0.5 μM DBPP was mixed and irradiated with 635 nm light at the laser intensity of 5 mW/cm^2. The reaction was monitored spectrophotometrically by measuring the decrease in optical density every 1 min at an absorbance maximum of 410 nm of DPBF. The natural logarithm values of absorption of DPBF at 410 nm (Ln $[DPBF]_0/[DPBF]_t$) were plotted against the irradiation time (Tang W, *et al*., 2016), where $[DPBF]_0$ and $[DPBF]_t$ represent absorbance at time 0 and at time t, respectively. The singlet oxygen generation rate of compound DBPP was calculated by a first-order linear leastsquares model, the rate constant was converted into Φ_Δ using methylene blue as a standard.

IN VITRO EXPERIMENTS
Cell line and culture conditions

Human esophageal cancer cell line (Eca-109 cells) and human bladder cell line (T24 cells) were obtained from the type culture collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in normal RPMI-1640 culture medium (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS, BioChrom, Cambridge, UK) in 5% CO_2, 21 % O_2 at 37 °C in a humidified incubator. Cells in the exponential phase of growth were used in each experiment. All cell culture related reagents were obtained from commercial suppliers.

Dark toxicity

Eca-109 cells and T-24 cells (1×10^4 cells/well) were cultured in RPMI-1640 medium with 10% (v/v) FBS, collected with

0.25% (w/v) trypsin (Liao P, *et al*., 2016), and seeded in 96-well plates at 6 × 10^3 cells per well for 24 h with concentrations range from 0.1 µM to 20 µM of compound DBPP. Maximum DMSO concentration in the cell culture medium was < 0.3% v/v. Cell toxicity analysis was performed at the end of the incubation time. 3- (4, 5 - dimethyl- 2-thiazolyl) - 2, 5- diphenyl - 2H - tetrazolium bromide (MTT) dissolved in phosphate buffered saline (PBS, 5 mg/mL) was added (10 µL per 100 µL of medium) to all wells and the plates were incubated at 37 °C with 5% CO_2 and 100% relative humidity for another 4 hours. After this time, the medium was then discarded and 200 µL DMSO was added to each well. The MTT test was performed using an ELISA plate reader (Bio-Rad, California, USA).

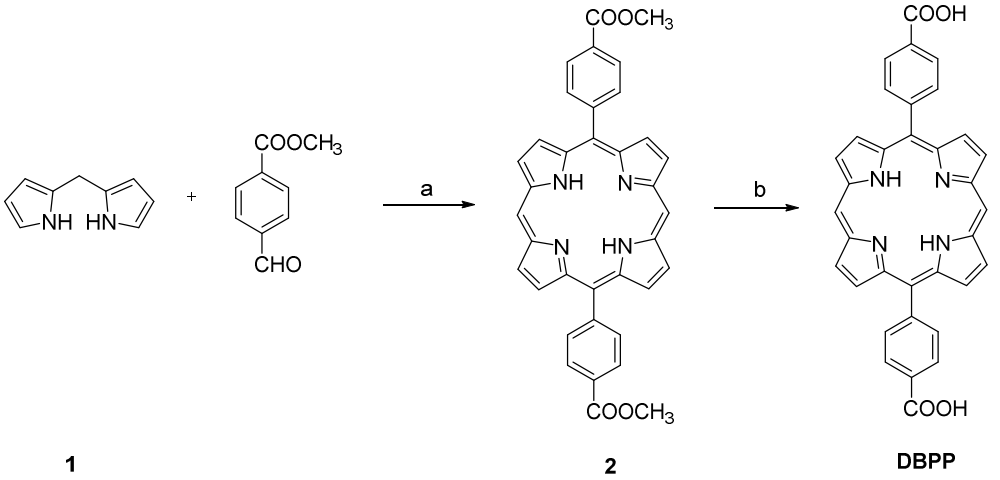
Photo-toxicity

Cells were prepared as described above. Thereafter, the media containing compound DBPP were removed and cells were washed with fresh PBS for three times. Then cells were irradiated with light doses range from 1 to 16 J/cm^2 (fluence rate 25 mW/cm^2) using an Nd: YAG laser at 650 nm. A plate similarly treated but not exposed to light was used as reference for the dark cytotoxicity in the same experimental conditions. Experiments were conducted in quadruplicate and repeated thrice. MTT solution (5 mg/mL) was added after a further incubation for 24 h to measure the cell phototoxicity at 570 nm.

RESULTS AND DISCUSSION

Synthesis of the compound DBPP

The photosensitizer DBPP was prepared from 4-(methoxycarbonyl) benzaldehyde in a multistep synthetic sequence (**Scheme 1**). Dipyrromethane (compound **1**) was synthesized as the literature (Mikroyannidis JA, *et al*., 2011), it was then reacted with 4-(methoxycarbonyl) benzaldehyde in DCM under the existence of TFA. To the reaction solution DDQ was added and stirred for 3 h, then neutralized with Et_3N to give compound **2** as a purple red solid in 41.7% yield. Compound **2** was hydrolyzed with 2 M KOH in THF - MeOH under refluxing to give compound DBPP in 94.5% yield after purification by column chromatography. DBPP was characterized by ^1H NMR spectrum, mass spectrum, UV-Vis spectrum and fluorescence spectrum.



Scheme 1. Reagents and reaction conditions: (a) TFA, DCM, room temperature, 3 h; then DDQ, 3 h; then Et_3N, 1 h, 41.7 %. (b) 2M KOH, THF - MeOH (V/V = 1 : 1), reflux 14 h, 94.5 %.

UV-Vis absorption and fluorescence spectra

The UV-Vis absorption of compound DBPP were measured in DMSO with different concentrations and the spectra were given in **Fig 3a**. Absorption spectrum of compound DBPP displayed an intense Soret band at 409 nm, and four less intense Q band at 504 nm, 539 nm, 576 nm and 630 nm, respectively. The molar extinction coefficients were shown in **Table 1**. The ideal photosensitizer for using in PDT should have a strong absorbance with a high extinction coefficient in the long - wavelength (600 nm ~ 800 nm) region, where the maximum penetration of tissue by the light. Some of the currently approved PDT photosensitizers have low absorbance in the optical window for photosensitizer excitation, but compound DBPP showed a strong absorption at 609 nm with a high molar extinction coefficient (ε) of 163300 M^{-1}. cm^{-1}, suggesting that compound DBPP have respond to long wavelength light and would be a potential photosensitizer in PDT (Zhang LJ, *et al*., 2014). When excited at 409 nm, compound DBPP showed strong emission peaks at 633 nm and 698

nm (**Fig 3b**), which could be applied to targeted fluorescence imaging and photo-diagnosis (Vankayala R, *et al*., 2015).

Table 1. Molar absorption coefficients of compound DBPP

Wavelength (nm)	Molar absorption coefficient ε (M^{-1}. cm^{-1})
409	163300
504	7800
539	4300
576	3500
630	1300

Singlet oxygen quantum yield

The generation of singlet oxygen by DBPP was measured after exposure to 635 nm light at the laser intensity of 5 mW/cm^2 in the presence of DPBF. The disappearance of DPBF spectra were monitored at 410 nm using UV–Vis spectrophotometer (**Fig 4a**). The value of singlet oxygen quantum yield generation (Φ_Δ) was determined using the following equations (Zhang L-J, *et al*., 2015).

$$\Phi_\Delta{}^S = \Phi_\Delta{}^R (k^S I_{aT}{}^R / k^R I_{aT}{}^S) \quad (1)$$

$$I_a = I_0 (1\text{-}e^{-2.3A}) \quad (2)$$

Where *S* and *R* indicate the sample and reference compound, respectively. I_a is defined as the total amount of light absorbed by DBPP. *A* is the corresponding absorbance at irradiation wavelength. The absorption intensity of DPBF at 410 nm continuously decreased when the irradiation time range from 0 min to 8 min. The natural logarithm values of absorption of DPBF at 410 nm (Ln $[DPBF]_0/[DPBF]_t$) were plotted against the irradiation time (**Fig 4b**) , straight lines were obtained for the sensitizers, and the slope for compound DBPP was obtained after fitting with a linear function (correlation coefficient R > 0.999). The Φ_Δ of compound DBPP in DMF was 0.39.

Dark toxicity

Compound DBPP was tested at different concentrations (0.1 μM to 60 μM) for 24 h without light irradiation (**Fig 5**). As shown in **Fig 5**, DBPP did not show any significant dark cytotoxicity at concentrations up to 0.1 μM. The survival rate of Eca-109 cells or T24 cells was greater than 80% for concentrations of 10 μM or less as assessed by MTT assay. When concentration was 20 μM, a rapid increase in cytotoxicity was detected. Raising the concentration from 20 μM to 60 μM, the cell viability was decreased sharply at the same light dose. That means 20 μM or more DBPP induced a significant amount of nonviable cells and this concentration can't be used for PDT.

Photo-toxicity

Fig 6 showed light dose dependence effect on Eca-109 cells and T24 cells after incubation with different concentration of compound DBPP and irradiation at different light dose. The cell viabilities were decreased with the increase of light dose after incubation with the same concentration DBPP. Similarly, irradiation at the same light dose, the cell viability was decreased with the increase of concentration DBPP. Without the photosensitizer, the exposure of Eca-109 cells and T24 cells to light did not affect cell survival (data not given). Low light doses (1 J/cm^2) caused moderate damage even at the highest concentration tested. By raising the light dose to 8 J/cm^2, about 40% lethality was achieved which was judged insufficient. Cell viability below 30% was observed in the presence of 16 μM DBPP with the maximum light dose (16 J/cm^2). Considering the balance between dark toxicity and photo damage, it was suggested that 16 μM TPMC and 16 J/cm^2 were suitable parameters for effective photodynamic studies. IC_{50} values (the concentration of photosensitizer that provides 50% inhibition of cell growth) of compound DBPP at 16 J/cm^2 light dose for Eca-109 cells and T24 cells were 12.16 μM and 7.13 μM, respectively.

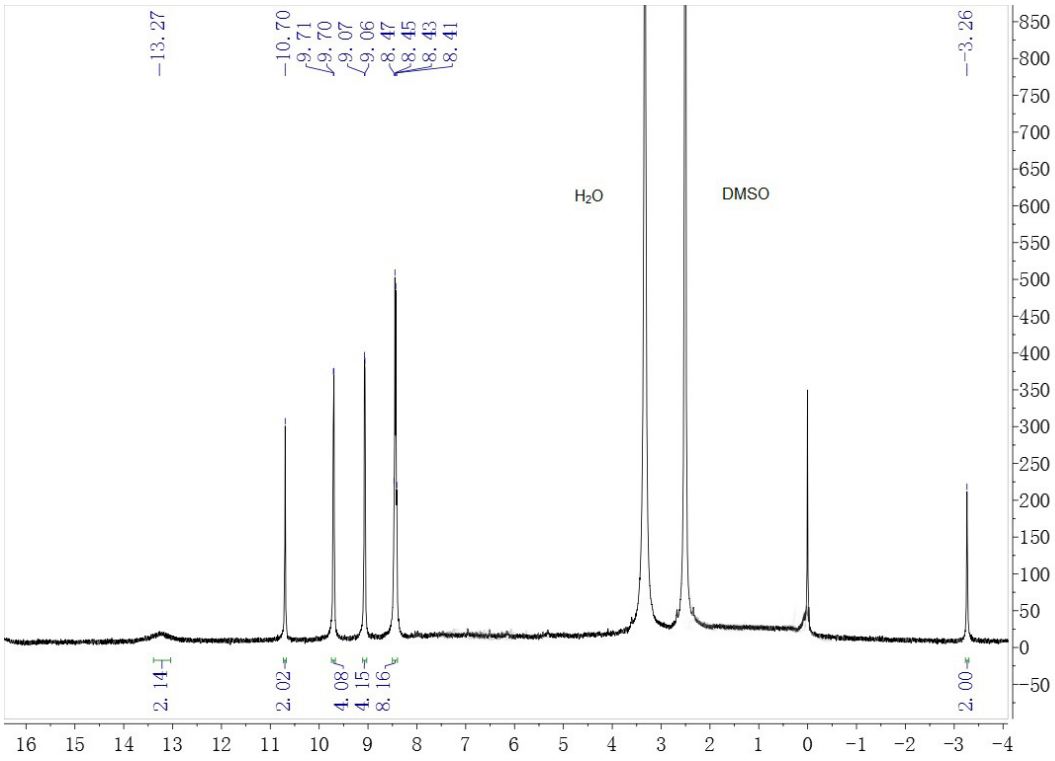


Fig 1. ^{1}H NMR spectrum of compound DBPP in DMSO- d_6.

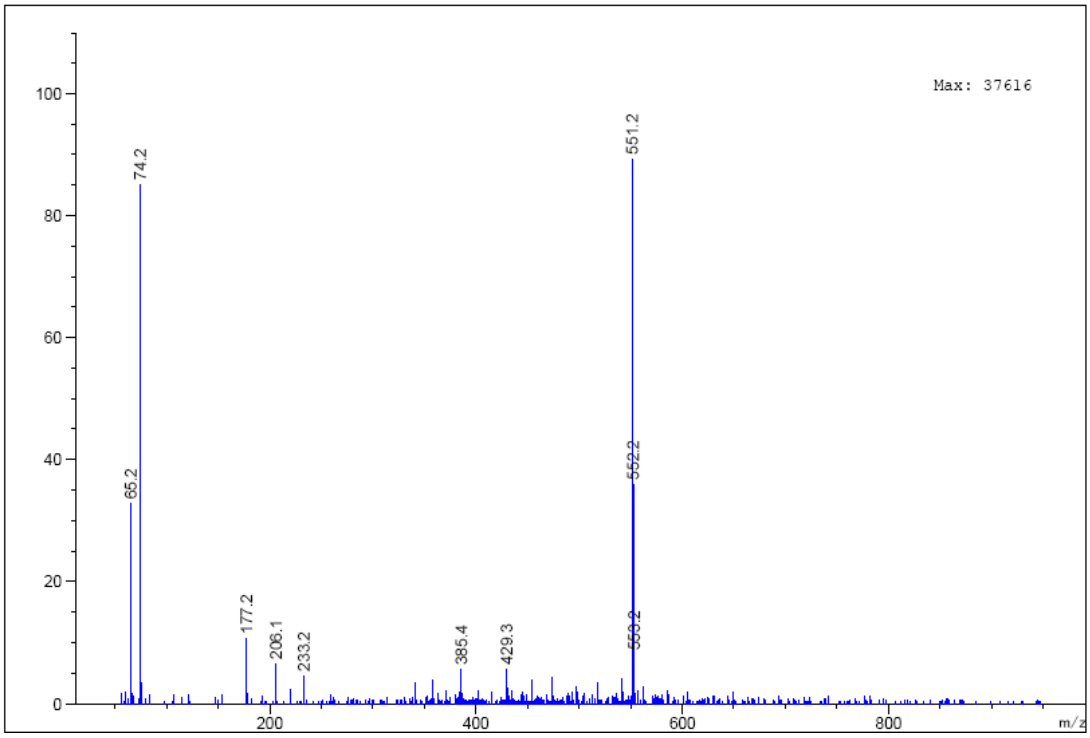


Fig 2. Mass spectrum of 5, 15-di (p-benzoato) porphyrin (DBPP) in acetonitrile

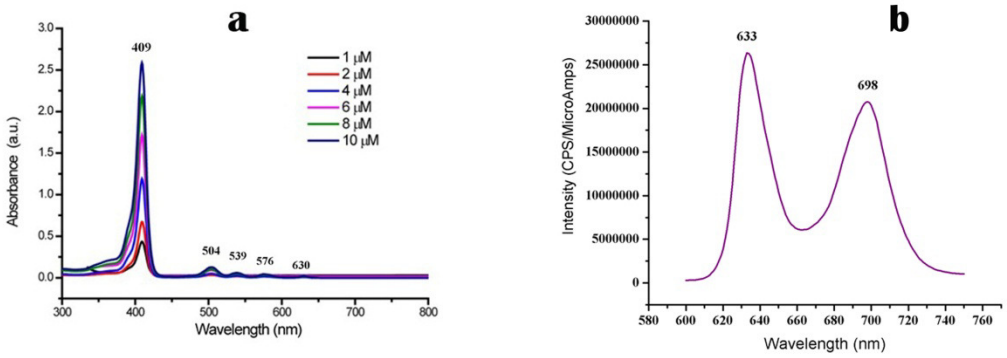


Fig 3. Spectra of compound DBPP. (a) UV-Vis absorption spectra of compound DBPP at different concentrations (DMSO). (b) Fluorescence spectrum of compound DBPP, excition wavelength is 409 nm (2 μM in DMSO).

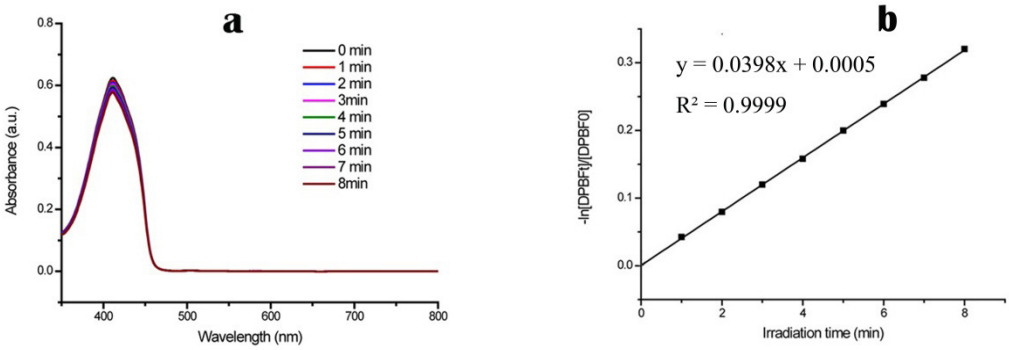


Fig 4. The singlet oxygen quantum yield (Φ_Δ) of compound DBPP. (a) Photodecomposition of DPBF by $^1O_2$ after irradiation of compound DBPP in DMF. (b) First-order plot for the photodecomposition of DPBF photosensitized by compound DBPP

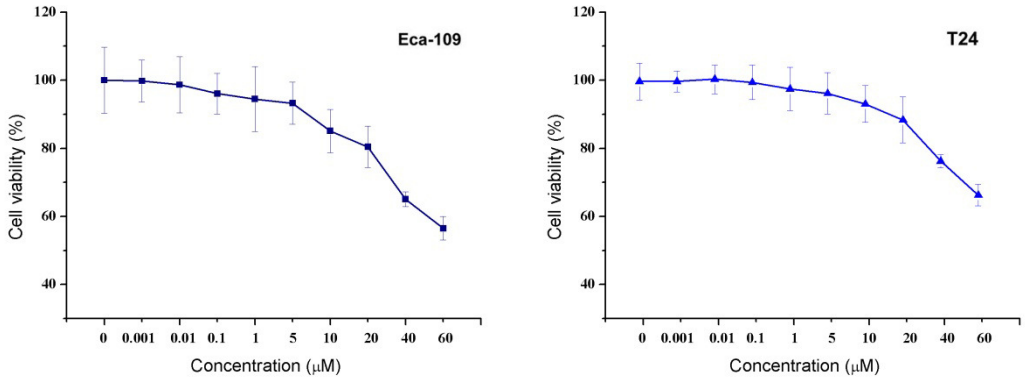


Fig 5. Dark toxicity in Eca-109 cells and T24 cells of compound DBPP at different concentrations. Data correspond to mean values ± SD from three different experiments.

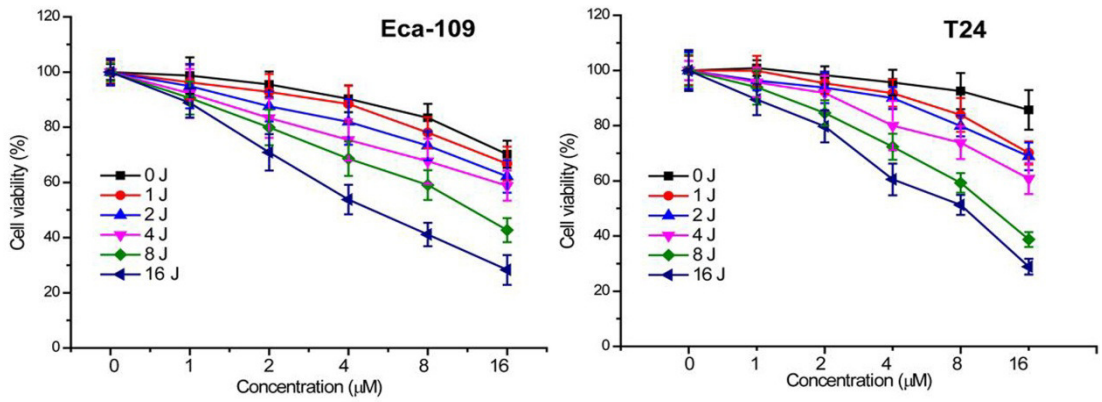


Fig 6. Light dose dependence effect on Eca-109 cells viability and T24 cells viability after incubation with different concentration of DBPP for 24 hours, followed by irradiation. Data correspond to mean values ± SD from at least three different experiments.

CONCLUSIONS

In this paper, the photosensitizer 5, 15-di (p-benzoato) porphyrin (DBPP) were synthesized and characterized, its biophysical properties and photodynamic effects *in vitro* were investigated. DBPP showed strong absorption at 409 nm and had high molar extinction coefficient of 163300 M^{-1}. cm^{-1}, suggesting that DBPP allowed access into deeper pathological tissues (Huang Z, 2005; Ashen-Garry D, *et al*., 2014). When excited at 409 nm, DBPP showed strong emission peaks at 633 nm and 698 nm which could be used in fluorescence imaging and photo-diagnosis. High singlet oxygen quantum yield of 0.39 made DBPP be able to inactivate tumor cells with high efficiency in PDT. In vitro, DBPP showed low dark toxicity and high photo-toxicity to Eca-109 cells and T24 cells, and the cell viability was decreased with the increase of light dose or DBPP concentration. The above results revealed that compound DBPP was a promising drug candidate for PDT application in cancer.

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