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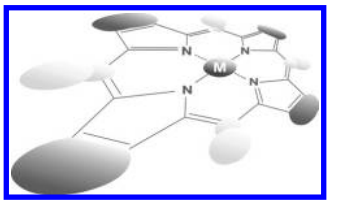

Phototoxic effects of pyropheophorbide-a from chlorophyll-a on cervical cancer cells

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ABSTRACT: Photodynamic therapy (PDT) is a promising modality in both the curative and palliative treatment against a variety of experimental and naturally occurring human cancers. At present, chlorophyll *a* derivatives are extensively used for the synthesis of photosensitizers (PSs) for PDT of tumors. In the present study, chlorophyll-a was extracted from the blue-green algae *Spirulina platensis* by refluxing with acetone. The extract was further acid treated to obtain methylpheophorbide-a (MPa), which was then refluxed in collidine and methylpyropheophorbide-a (Mppa) was obtained. After that, Mppa was converted to pyropheophorbide-a (Ppa) by treatment with 50% sulfuric acid. Finally, phototoxicity and dark toxicity of purified Ppa in two different cell lines, TC-1 and CaSki, were examined by MTT assay. The results suggest that Ppa is more toxic to TC-1 cell line than CaSki cell line. *In vivo*, the photosensitizing efficiency of Ppa was also higher than those of unloaded PS. These results indicate the potential of Ppa in PDT.

KEYWORDS: photodynamic therapy, photosensitizer, pyropheophorbide-a, algae.

INTRODUCTION

Photodynamic therapy (PDT) of cancer has emerged as a highly effective treatment for oncological diseases [1–3] and it has been successful in the management of a wide variety of solid, malignant tumors [4]. The dual selectivity of PDT is produced by both a preferential uptake of the photosensitizer by the diseased tissue and the ability to confine activation of the photosensitizer to this diseased tissue by restricting the illumination to that specific region [5]. Basic principle of PDT is a photogeneration of highly reactive singlet oxygen (strong oxidizer that causes necrosis of the tumor tissue) *via* excitation energy transfer from photosensitizer [6].

Since first clinically approved PS (photosensitizer), Photofrin, was known to have some drawbacks such as prolonged skin phototoxicity, low absorption at longer wavelength, and not single compound, *etc*, researchers faced challenges to find new photosensitizers [7]. To find optimal photosensitizers, numerous compounds have been synthesized and examined including porphyrins, chlorins, phthalocyanines and purpurins [8, 9]. Among them, some chlorin derivatives, called second generation PS with promising physicochemical properties and high PDT efficiency were found [10–12]. Some of them such as Foscan, LS11 and Radachlorin, were approved for clinical use [13]. 3-Devinyl-3-(1-hexyloxyethyl) pyropheophorbide-a (HPPH), photochlor, is one promising derivative of pyropheophorbide-a (Ppa), which is under phase II

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clinical trial [14]. It showed a superior PDT effect in some tumor of animal [15]. Also, many studies were performed on Ppa-conjugates with other molecules [16, 17], but almost no studies for PDT efficacy of free Ppa have been reported. In addition, Ppa is a potential candidate as PS for PDT because it can be prepared easily as a single compound that generates singlet oxygen in high yield and has high absorption intensity at longer wavelength (665 nm) [18].

A few cervical cancer cell lines such as TC-1, HeLa, and SiHa cells were included for evaluating PDT on the inhibition of cell growth after Radachlorin treatment on the cells [19]. While the MTT assay results of SiHa were consistent with TC-1, HeLa showed a cell line-dependent Radachlorine/PDT-resistant trend compared to the other cells. It was also demonstrated that the mechanisms involved in the death of human cervical cancer cells triggered by PDT with Photogem exerts its antitumor activity primarily by inducing necrosis rather than by inducing apoptosis [20]. While Ppa is a well-known photosensitizer, no application for TC-1 and CaSki cell lines yet. In this study, we report the relationship of the enhanced cytotoxicity of Ppa in the cervical cancer cells and its potential relationship to the clinical outcome following PDT. We examined *in vitro* phototoxic effects of Ppa that is prepared from Mppa obtained from *Spirulina platensis* (a blue-green algae) with and without light on two different cervical cancer cell lines. We also evaluated antitumor PDT effects in TC-1 cell-bearing mice including treatment variables.

MATERIALS AND METHODS

Chemical reagents

All reagents and solvents used in this study were purchased from Sigma-Aldrich. Structures of PS were characterized by ^{1}H NMR, MS, and UV-vis spectroscopy at NCIRF, Seoul National University and at Integrative Research Support Center, The Catholic University of Korea. Mpa was prepared from *Spirulina plantesis* algae (purchased from DIC LIFETEC Co., Ltd, Japan) by extraction with acetone. Mppa was obtained from Mpa by refluxing in collidine for 2.5 h. At last Ppa was prepared as reported previously [21]. Briefly, Mppa (1 g) was dissolved in 20 mL of sulfuric acid (50%) and stirred at room temperature for 2.5 h. The reaction mixture was diluted with cold distilled-water and extracted with dichloromethane (200 mL). The organic layer was washed with distilled water two times and evaporated by rotary evaporator. The residue was purified on silica gel (60–230 mesh, Merck, Germany) by column chromatography using 2–5% methanol in dichloromethane. Finally, Ppa was obtained in yield of 88%. R_f: 0.25 (5% methanol in dichloromethane). UV-vis (CH_2Cl_2): λ_{max}, nm ($M^{-1}.cm^{-1}$) 667.7 (43162.1), 539.5 (22223.3), 511.7 (18963.6), 414.2 (80694.4). ^{1}H NMR (500 MHz, $CDCl_3$, TMS int): δ, ppm 9.43 (s, 1H, 5-*meso*-H), 9.32 (s, 1H, 10-*meso*-H), 8.52 (s, 1H, 20-*meso*-H), 7.95 (dd, 1H, 3^1-CH, J = 11.5, 11.5 Hz), 6.20 (dd, H, 3^2-CH_2, J = 17.9, 11.5 Hz,), 5.17 (dd, 2H, 13^2-CH_2, J = 19, 19 Hz), 4.46 (q, 1H, 18-CH, J = 7.3 Hz), 4.29 (d, 1H, 17-CH, J = 9 Hz), 3.64 (q, 2H, 8^1-CH_2, J = 7.5 Hz), 3.61 (s, 3H, 12^1-CH_3), 3.38 (s, 3H, 2^1-CH_3), 3.20 (s, 3H, 7^1-CH_3), 2.71–2.59 (m, 2H, 17^2-CH_2), 2.38–2.23 (m, 2H, 17^1-CH_2), 1.80 (3H, d, 18^1-CH_3, J = 7.3 Hz), 1.66 (t, 3H, 8^2-CH_3 J = 7.5 Hz), 0.48 and -1.70 (brs, each 1H, 21-, 23-NH). EIS-MS: *m/z* 535 $[M]^+$.

Cell cultures

TC-1 cell line positive for HPV E6/E7 was generously provided by Dr. T.C. Wu, at the Johns Hopkins University, MD, USA. TC-1 and CaSki (a new epidermoid cervical cancer cell line) cells were routinely propagated in monolayer cultures in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.22% sodium bicarbonate and penicillin/streptomycin. The cells were cultured in a 5% CO_2 incubator at 37 °C. Additionally, 400 mg/L G418 was added to TC-1 culture media.

Phototoxicity

For viable cell counting, CaSki and TC-1 cells (3×10^3 cells) per well (96 well plate) were treated with Ppa, at concentration range of 0.03, 0.06, 0.125, 0.25, 0.5, 1 and 2 μM, after incubation for 12 h (37 °C, 5% CO_2) in RPMI 1640 containing 10% FBS. Following 24 h incubation, laser irradiation (662 ± 3 nm, 6.25 J/cm^2) was performed. Cell growth inhibition was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 24 h after irradiation. For the MTT assay, 20 μL of 5 mg/mL MTT solution was added to each well and incubated for 4 h. 100 μL of dimethylsulfoxide (DMSO) was added to each well, shaken for 10 s, and the absorbance was measured with an ELISA-reader (Spectra Max 340/Molecular Devices, USA) at 570 nm. Each group consisted of three wells; the means of their values were used as the measured values.

In vivo photosensitizing efficacy

The *in vivo* PDT efficacies of Ppa were investigated in 6-week-old C57BL/6 mice with TC-1 tumor implanted in the peritoneum. Groups of five mice were given intravenous injections of several type of Ppa (1.25 or 2.5 mg/kg) or PBS as control when the tumors grew to a volume of 450–500 mm^3. After 6 h of Ppa administration, the mice were anesthetized by an intraperitoneal injection of 50 mg/kg Zoletil 50 (Virbac, France), and the tumors were laser irradiated (UPL-PDT) at a wavelength of 670 ± 10 nm and an interstitial total impact energy of 150 J/cm^2. The mice were kept in an optimal condition, and the tumor volumes were measured every 2 days with calipers.

Tumor volume [V (mm^3)] was calculated as ($L \times W^2$) $\times$ 0.524 (L = length of the longest dimension) and W = width (perpendicular to the long axis).

Western blot

For immunoblots, confluent monolayers of CaSki, HeLa, and TC-1 cells were lysed with cell extraction buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate) (Biosource, CA) in the presence of protease inhibitors. Protein concentration was determined using the BCA Protein Assay (Bio-Rad, Hercules, CA). Electrophoresis was performed on 10–12% SDS-polyacrylamide gels for 1 h, and the gels were then transferred onto PVDF membrane (Millipore, CA). Membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% skim milk for 1 h at room temperature. After blocking, membranes were incubated for 1 h at room temperature or overnight at 4 °C with mouse monoclonal anti-ABCG2 (Santa Cruz, CA), and mouse monoclonal anti-β-actin (Santa Cruz, CA) antibodies in a 1:200–500 dilution buffer (5% skim milk in TBST). Membranes were washed three times with TBST and then incubated with goat anti-mouse IgG-HRP (Zymed, San Francisco, CA) antibodies for 1 h at room temperature. Immunoblots were developed with enhanced chemiluminescence agents according to the manufacturer's instructions (SuperSignal West Pico Chemiluminescent Substrate: PIERCE, Rockford) and exposed to imaging film.

Cellular uptake

CaSki and TC-1 cells (5×10^4 cells/well) were incubated in wells of a 12 well plate overnight at 37 °C with 5% CO_2. The medium was replaced with fresh medium containing Ppa (0.0, 0.01, 0.1, and 1.0 μM). After 1, 3, 6, 12 and 24 h of incubation, the cells were washed twice with 1 mL of PBS and 1 mL of fresh medium was added. Fluorescence of the cells was measured by fluorescence microscopy using an Axiovert 200 MAT apparatus (Zeiss, Gottingen, Germany).

Statistical analysis

Every experiment was conducted in at least triplicate, and the acquired data expressed as the mean ± standard deviation (SD). The statistical comparisons of the cytotoxic effects were performed using Student's *t*-test. The differences were considered significant at a P-value <0.05.

RESULTS AND DISCUSSION

In this study, we prepared pyropheophorbide-a from Mppa by treatment of sulfuric acid based on Burke *et al.*'s protocol [22]. Mppa was prepared from Mpa

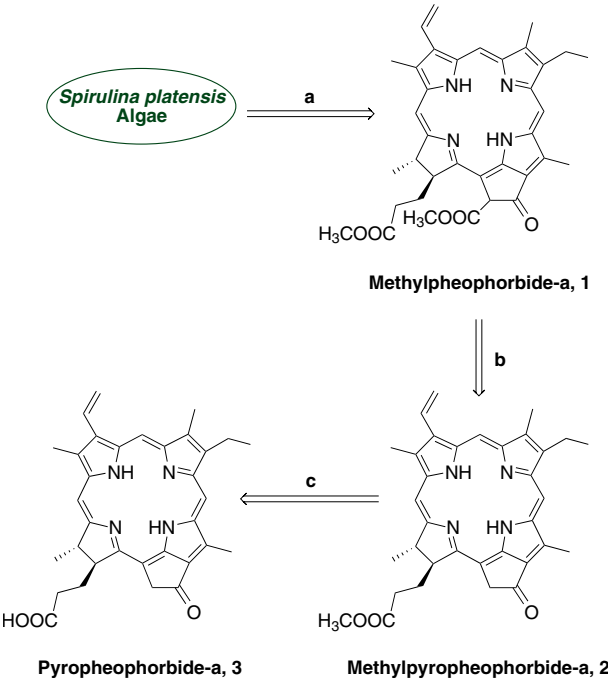

Fig. 1. Preparation of Ppa from *Spirulina platensis* algae. (a) Extraction by acetone three times, treatment of hydrochloric acid in MeOH for 12 h. (b) Reflux in collidine at 110 °C for 2.5 h, evaporation under high vacuum. (c) Treatment of H_2SO_4 (50%) at room temperature for 2 h

by refluxing in collidine as reported by Kenner *et al.* [23], Mpa was obtained from *Spiriluna platensis* blue green algae according to reports published [8, 24] as shown in Fig. 1. After preparing these compounds, all were characterized by ^{1}H NMR, mass spectroscopy, and UV-vis spectrophotometers. As shown in Fig. 2, UV absorption and fluorescence emission of Ppa in DMSO were determined by nano-drop and fluorescence spectrophotometer. Two main absorption peaks of Ppa were observed at 416 and 669 nm wavelength while fluorescence emission peak was observed at 685 nm.

Although *in vitro* studies of Mppa on several cell lines were documented [25], in case of Ppa, very few reports were noted. Also, as reported by Bellnier *et al.* [13], Mppa can be hydrolyzed in *in vitro* and *in vivo* conditions in the presence of plasma, probably by esterase enzymes because of its methoxy group at 17^3 position. Consequently, it is considered less stable than Ppa. As well, Ppa can widely be used for its conjugation with any molecule due to its free reactive carboxylic group at 17^3 position. In addition, it is a well-known potential candidate for photosensitizer due to the photophysical and photochemical properties. For these reasons, in this study, we proposed to test photosensitizing activity of Ppa with and without light on two different cancer cell lines; TC-1 cells, which is a lung epithelial cell immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene, CaSki which is a cervical epidermoid carcinoma cell line.

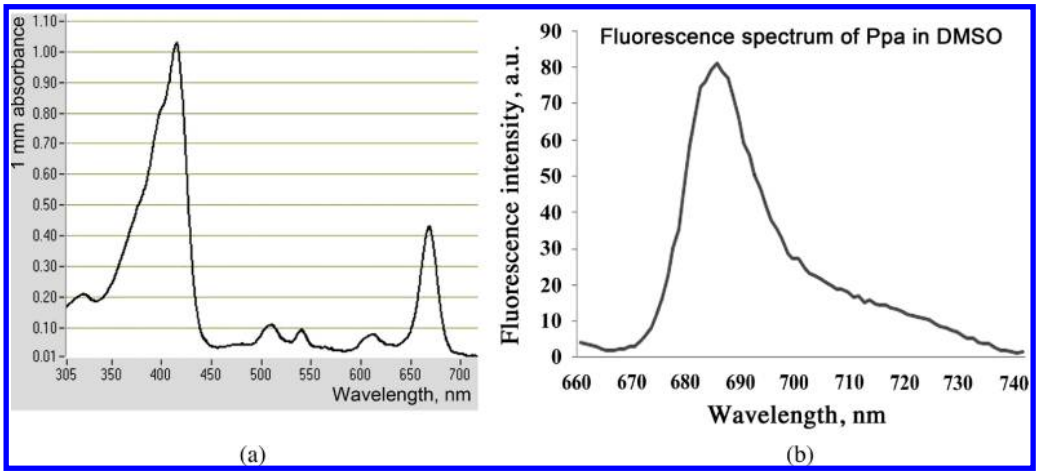

Fig. 2. UV-vis spectrum (a) and fluorescence spectrum (b) of Ppa in DMSO; the highest peak (Sorat band) is at 416 nm and second highest peak (Q-band) is at 669 nm, while the highest fluorescence emission is at 685 nm

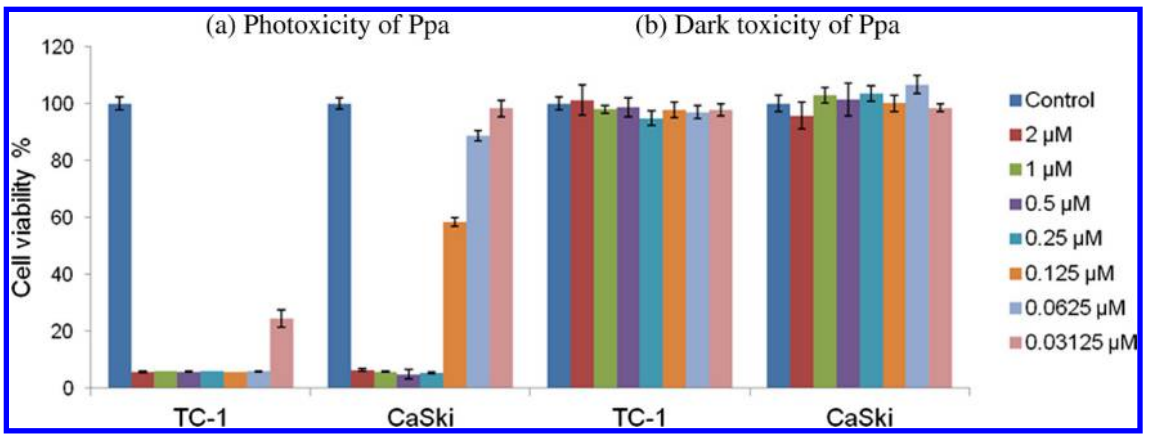

Fig. 3. Phototoxicity and dark toxicity results of Ppa at various concentration on TC-1 and CaSki cell lines. MTT assay was performed under dark and cells were incubated at 37 °C with 5% CO_2 for 24 h. Error bars represent standard deviation

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay on two cell lines was performed in the presence of light and in the absence of light. In both cases, after seeding in 96 well plates the cells were incubated at 37 °C under 5% CO_2 for 24 h, cells were treated with Ppa in DMSO and medium solution at concentrations 0.03, 0.06, 0.125, 0.25, 0.5, 1 and 2 μM. The light at 665 nm with 6.25 J/cm^2 was given to the cell-containing plate at 24 h after treatment of Ppa under darkness. Further, the cells were incubated for 24 h and 20 μL of MTT reagent was added into each well. Following additional 4 h incubation, absorption of the cells at 570 nm was recorded by ELISA reader. Figure 3 shows the PDT and dark toxicity effect on the two cell lines.

Phototoxicity of Ppa on the two cell lines was observed to be different as shown in Fig. 3. In case of TC-1 cell line, around 100% efficiency was observed at the concentration range of 0.06–2 μM. At 0.03 μM concentration, Ppa revealed around 75% efficiency. In case of CaSki cell line, 100% efficiency was observed at 0.25–2.0 μM while at 0.125 μM it exhibited only around 30% efficiency. It was noted that at concentration of 0.06 and 0.03 μM, Ppa revealed negligible phototoxicity. In addition, there was no dark toxicity of Ppa in both cell lines as shown in Fig. 3. Thus, Ppa on TC-1 cell line was over 10 fold more phototoxic as compared to CaSki cell line. This photosensitizing activity may be attributed to the localization difference of Ppa in each cell or the over-expression of ABCG2 protein in CaSki cells that can efflux Ppa derivatives. To evaluate whether ABCG2 protein expression can lead to photosensitizing resistance, we performed western blot analyses as shown in Fig. 4.

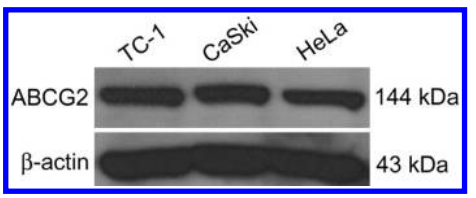

Fig. 4. *In vitro* expression of ABCG2 protein using western blotting. After 24 h of incubation, the cells were collected and proteins were extracted from TC-1, CaSki, and HeLa cells

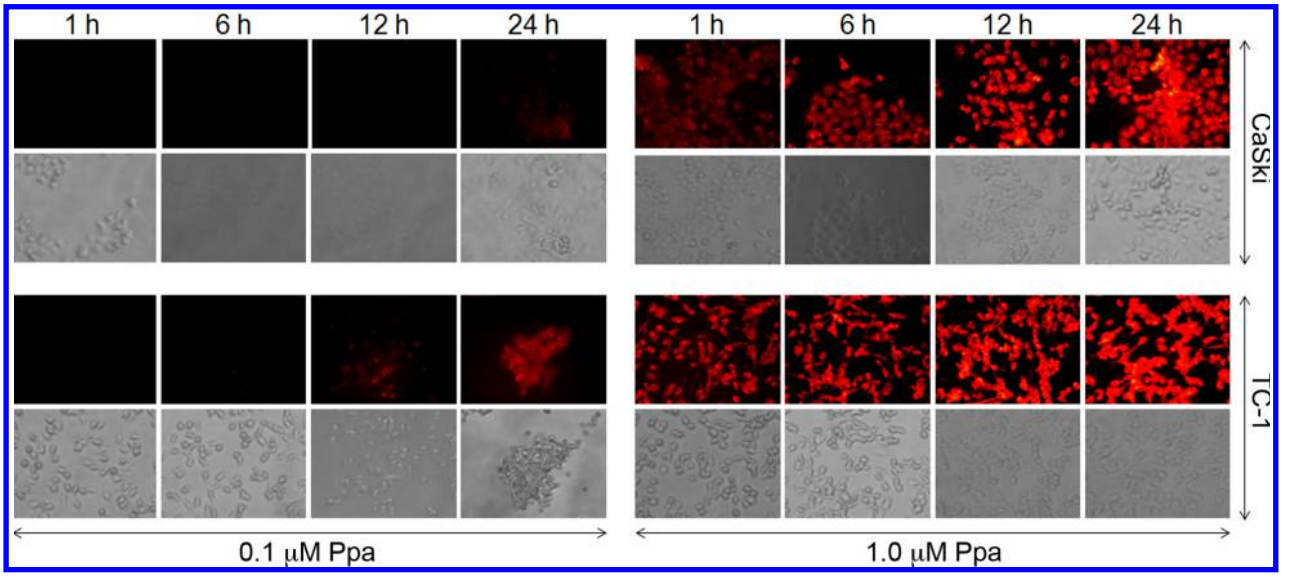

Fig. 5. Cellular uptake of Ppa in TC-1 and CaSki cells. The cells were incubated with Ppa in medium containing 10% FBS at 37 °C, and their concentrations in the cells after incubation for 1–24 h were measured by fluorescence microscopy

In CaSki, HeLA, and TC-1 cells, the levels of ABCG2 protein expressions were similar as compared with β-actin. These data suggest that ABCG2 protein expression did not regulate the phototoxicity in the cervical cancer cells.

We examined the uptake of Ppa (0.0, 0.01, 0.1 and 1.0 μM) into these cells by fluorescence microscopy as shown in Fig. 5. Only 1.0 μM of Ppa began to penetrate into the cells after incubation for 1 h. 0.1 μM of Ppa started showing higher uptake into TC-1 cells than CaSki cells after incubation for 12 h. And 0.1 μM of Ppa exhibited much higher accumulation in TC-1 cells compared to CaSki cells at 24 h. High accumulation of 1.0 μM of Ppa in these cells was observed at all time points, while 0.01 μM of Ppa did not show any accumulation for 24 h (Supplementary material). We observed that 0.1 μM of Ppa facilitated the enhanced accumulation in TC-1 cells, which is consistent with the phototoxicity of TC-1 cells at 0.125 μM. This increased accumulation and penetration may be attributed to the efficient internalization of Ppa by the endocytic cell uptake mechanisms of TC-1 cells.

Tumors treated with Ppa (1.25 or 2.5 mg/kg) showed rapid shrinkage after irradiation, while tumors treated with the same amount of Ppa-only (no irradiation) showed a slow growth (Fig. 6). In case of mice treated with PBS and PBS-irradiation, the tumor growth continuously increased up to 19 days. After 3 days, tumor growth in mice treated with Ppa (2.5 mg/kg) significantly decreased and was comparable to that treated with Ppa (1.25 mg/kg). Until 10 days we observed shrinkage in tumor size by as much as half the initial tumor size. After 13 days, tumor recurrence was observed in mice treated with Ppa (2.5 mg/kg) compared to 5 days in mice treated with Ppa (1.25 mg/kg). Although *in vivo* the PDT effect of Ppa (1.25 or 2.5 mg/kg) on the tumor shrinkage in the mice bearing TC-1 cells did not show a significant

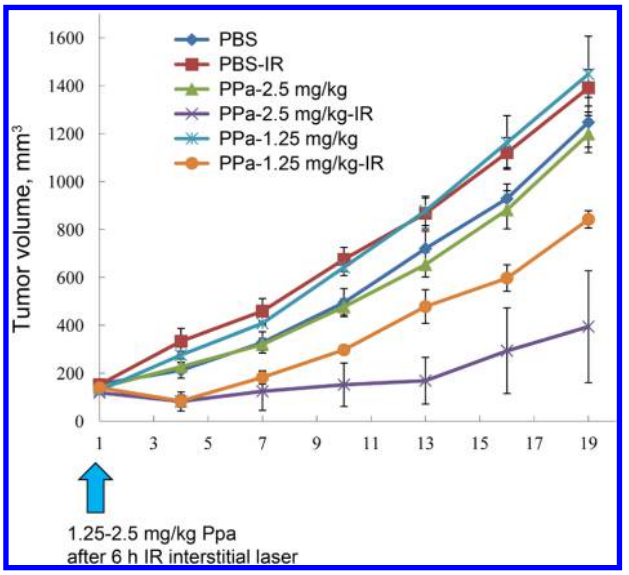

Fig. 6. PDT effect of Ppa on tumor growth in C57BL/6 mice bearing TC-1 cells. Groups of five mice were given intravenous injection of Ppa (1.25 or 2.5 mg/kg) or PBS as a control. At 6 h after administration, the tumors were irradiated by laser with a 670 ± 10 nm wavelengths and interstitial total impact energy of 150 J/cm^2

difference from each other, they exhibited greater effects relatively on the tumor recurrence after their application. These data were consistent with the results of *in vitro* experiments.

CONCLUSION

We tested the phototoxicity and dark toxicity of Ppa on two cell lines; TC-1 and CaSki. It was evident that Ppa at

concentration of 0.06 and 0.125 μM exhibit much higher (70–90% higher) growth inhibition effect on TC-1 cells compared to CaSki cells. However, Ppa at concentrations >0.25 μM displayed similar effects on both cell lines. These results were consistent with the cellular uptake assay. Taken together, Ppa at lower concentration is more toxic to TC-1 cells than CaSki cells. Our future work will be focused on determining whether it is cell line-dependent phototoxicity or cell-specific localization of Ppa [26].

Acknowledgements

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Supporting information

Cellular uptake of Ppa in TC-1 and CaSki cells was observed by fluorescence microscope (Figures S1–S6) after Ppa treatment (0.0, 0.01, 0.1 and 1.0 μM). Fluorescence was recorded at 1, 3, 6, 12, 24 and 48 h. This material is available free of charge *via* the internet at http://www.worldscinet.com/jpp/jpp.shtml.

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