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Studies on photodynamic mechanism of a novel chlorine derivative (TDPC) and its antitumor effect for photodynamic therapy *in vitro* and *in vivo*

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Photodynamic therapy (PDT) represents a promising method for treatment of cancerous tumors. The chemical and physical properties of used photosensitizer (PS) play key roles in the treatment efficacy. In this study, a novel PS, 5,10,15,20-tetrakis((5-dipropylamino)pentyl)-chlorin (TDPC) which displayed a characteristic long wavelength absorption peak at 650 nm were synthesized. It also shows a singlet oxygen generation rate of 4.257 min^{-1}. Generally, TDPC is localized in mitochondria and nucleus of cell. After light irradiation with 650 nm laser, it can kill many types of cell, in addition, TDPC–PDT can destroy ECA-109 tumor in nude mice and a necrotic scab was formed eventually. The expression levels of many genes which regulated cell growth and apoptosis were determined by RT-PCR following TDPC–PDT. The results showed that it either increased or decreased, among which, the expression level of TNFSF13, a member of tumor necrosis factor superfamily, increased significantly. In general, TDPC is an effective antitumor PS *in vitro* and *in vivo* and is worthy of further study as a new drug candidate. TNFSF13 will be an important molecular target for the discovery of new PSs.

Keywords: Photosensitizer; photodynamic therapy; tumor; chlorin; TDPC.

1. Introduction

Photodynamic therapy (PDT) has emerged as a promising new approach for treating and curing cancerous tumors.[1,2] It takes effect through localization of photosensitizer (PS) in the target tissues prior to radiation with an appropriate wavelength.[3,4]

PDT has potential advantage over surgery and radiotherapy due to its tissue-sparing properties. However, most of PSs also display only a slight preference for malignant cells, resulting in significant skin photosensitivity and high uptake by healthy cells and tissues. To enhance the treatment of cancer, new PS with absorption spectra at long wavelengths (650–800 nm) which can improve tumor selectivity and a quicker clearance are urgently needed to be developed.[5]

Chlorin-based PSs have been found to have applications as phototoxic drugs for PDT.[6–12] They are often selected because of their characteristic absorption spectra with a Soret band at approximately 415 nm and a usually narrow but very strong Q-band around 650 nm.[13] Such an inherent long wavelength characteristic provides more efficient light penetration in tumor tissues as compared to those PSs absorbing light at short wavelength.[14,15] Therefore, efforts in our laboratory were directed toward synthesis of chemically pure new longer wavelength absorbing PSs related to it.

In the present study, a novel chlorin-based compound 5,10,15,20-tetrakis[(5-dipropylamino) pentyl]-chlorin (TDPC) (see Fig. 1) was studied and its photophysical and photochemical properties have been evaluated. The localization pattern of TDPC was determined. We further explored the changes in gene expression that occurred in response to TDPC–PDT. The photosensitizing antitumor activities *in vitro* and *in vivo* were evaluated.

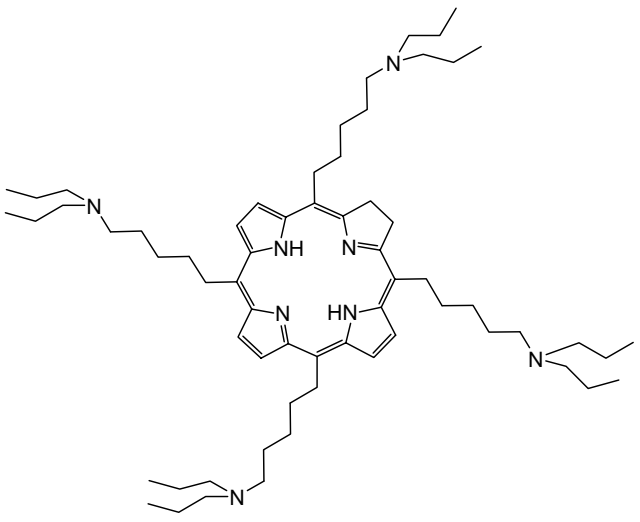


Fig. 1. Chemical structure of TDPC.

2. Material and Methods

2.1. *Materials*

TDPC was synthesized in our laboratory and Patent has been applied. All other chemicals and reagents were of analytical grade and used without any purification.

2.2. *Absorption and emission spectra*

UV-Vis absorption spectrum was recorded on an ultraviolet visible spectrophotometer (Model V-530, Japan). Fluorescence spectra were measured on a Fluorescence Spectrometer (FluoroMax-4, France). 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 cuvettes with path length of 1 cm. TDPC was dissolved in N, N-dimethylformamide (DMF) as 5 μM.

2.3. *Singlet oxygen generation*

1,3-diphenylisobenzofuran (DPBF) was used as a $^1O_2$ trapping reagent in DMF solution. DPBF and TDPC were dissolved in DMF. Two groups were placed in sealed quartz cuvettes and tested in each experiment: (1) DPBF and TDPC (5×10^{-5}M, 5×10^{-7}M); (2) DPBF(5×10^{-5}M). They were all at a reagent grade and used without further purification. A 5 mW Nd: YAG laser (650 nm) was used as the light source. The absorbance of the solution at 410 nm was measured every 10 s for a 120 s period with an UV-Vis spectrophotometer. Ratio of Singlet oxygen was carried out according to the equation mentioned below: In $A_0/A = kt$, where A_0 is absorbance in t_0; A is absorbance in t; k is the reaction rate constant[s^{-1}] and t is the time [s].[16]

2.4. *In vitro photosensitizing efficacy*

2.4.1. *Cell lines and cell culture*

Human esophageal carcinoma Eca-109 cell line was purchased from Chinese Academy of Sciences. The cells were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum and incubated at 37° in a humidified atmosphere containing 5% CO_2. Cell viability was detected via 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and expressed as the percentage of control cells.

All cells were incubated at 37° in 5% CO_2 in a humidified incubator.

2.4.2. *Phototoxicity studies*

The MTT assay was used to monitor the cytotoxicity mediated by TDPC–PDT. Briefly, Eca-109 cells, which were incubated in media containing 10, 1, 0.1, 0.01, 0.001 μM TDPC for 24 h, were irradiated at 650 nm by a Nd:YAG laser and the MTT assay was then used to analyze cellular sensitivity. All cells were seeded at 2000 cells per well in 96-well plates, each group in triplicate at least, and then incubated in a cell culture incubator with 5% CO_2 at 37°C. Five microliter of MTT (5 mg/mL) reagent (Sigma, MO, USA) was added to each well and incubated for 4 h at 37°C. At the end of the incubation period, the medium was removed and the formazan complex was solubilized with 100 μL DMSO. Absorbance of the complex was measured with a micro-plate reader (Bio-Rad, California, USA) at a wavelength of 570 nm.[17]

2.4.3. *Intracellular localization*

Eca-109 cells were grown in 8-wells plates on poly-L-lysine coated coverslips, incubated in the dark at 37°C with 4 μM TDPC for 24 h, then rinsed in the medium and incubated with 0 nm MitoTracker Green (Invitrogen, Carlsbad, CA) for 30 min at 37°C. After washing with PBS, cells were re-incubated with 1 mg/mL Hoechst 33258 (Beyotime, Nanjing, China) for 10 min at 37°C. Double-stained cells were imaged under a confocal microscope (LSM 510 META, Carl Zeiss, Göttingen, Germany). Images were analyzed with the LSM Image Browser Version 2.8.

2.4.4. *Real-time PCR*

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNAs were synthesized using M-MLV reverse transcriptase (Promega) for real-time PCR analysis. Real-time PCR was performed using SYBR Master Mixture (TAKARA, Japan) to measure mRNA levels of selected genes. Data were analyzed using the standard curve analysis method with Light-Cycler 480 Software (Roche Applied Science).

2.5. *In vivo photosensitizing efficacy*

A total of 18 nude BALB/C mice (9 males and 9 females, weighing 15–20 g) were used for the study. The mouse tumor models were set up by subcutaneous injection of 1×10^6 Eca-109 cells in the flank. Experiments were initiated when tumors reached 1 cm in diameter (between 21 and 28 days after tumor injection). The tumor volume (TV) $= 1/2 \times$ length $\times$ width.^{2} The mice were randomly divided into three groups: laser radiation group (6 mice), treatment group (6 mice) receiving 8 μM TDPC without laser application, and TDPC+ laser radiation group (6 mice) which was given 8 μM TDPC followed by laser radiation after 24 h. The mice were restrained in rat holders and exposed to laser ($\lambda = 650$ nm) with a light dose of 78 J/cm^2. Tumor regression was evaluated every 5 days for 20 days.

2.6. *Statistical analysis*

One-way ANOVA and the Student's t-test were used to measure differences using the SPSS16.0 software package. Data were reported as mean $\pm$ SD and p values <0.05 were considered significant.

3. Results

3.1. *UV-Vis absorption spectrum*

The UV-Vis absorption spectrum of TDPC (see Fig. 1) was determined in DMF at 0.5 μM. As can be seen, the TDPC had an elevated absorption peak at 650 nm (see Fig. 2), suggesting that TDPC might be an effective PS.

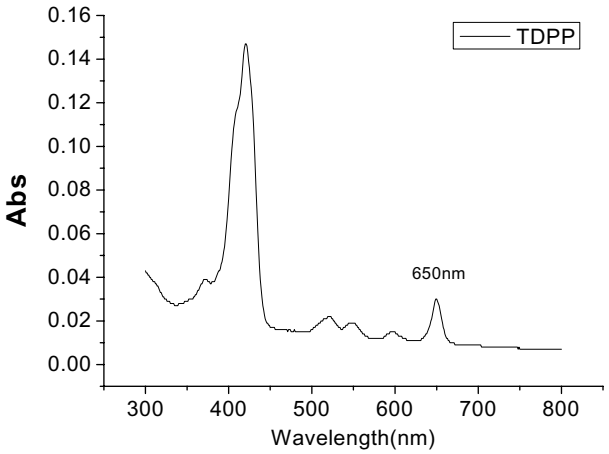


Fig. 2. UV-Vis absorption spectrum of TDPC in DMF.

3.2. *Fluorescence spectrum*

Fluorescence spectra were measured on a Fluorescence Spectrometer. TDPC can be excited at 418 nm, and its emission was monitored at wavelengths 651 and 722 nm (see Fig. 3).

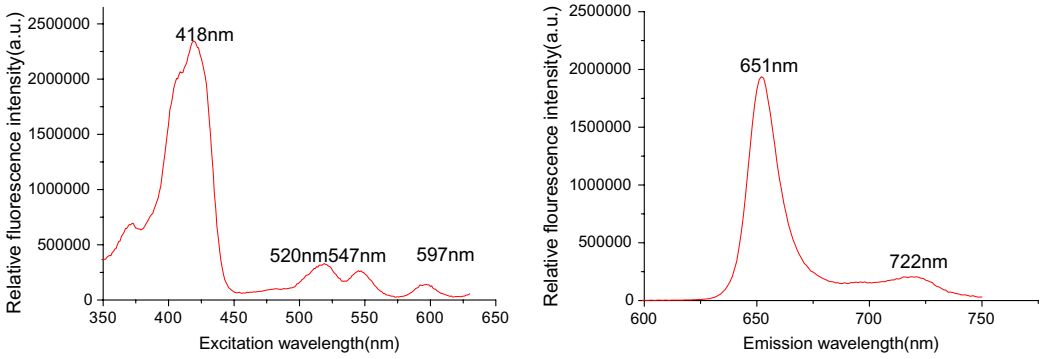
3.3. *Singlet oxygen generation*

Diphenylisobenzofurane (DPBF) as an effective singlet oxygen quencher has been widely used in many areas. Its conjugated structure is destroyed after photo-oxidation reaction with singlet oxygen which can be measured by UV-Vis spectrophotometer. Solution of DPBF and TDPC has a strong absorption peak at 413 nm. Monitoring the changes in intensity of the Q band can prove singlet oxygen production generation. As shown in Fig. 4, singlet oxygen generation rates of TDPC in DMF is $4.257\,\text{min}^{-1}$.

3.4. *In vitro studies*

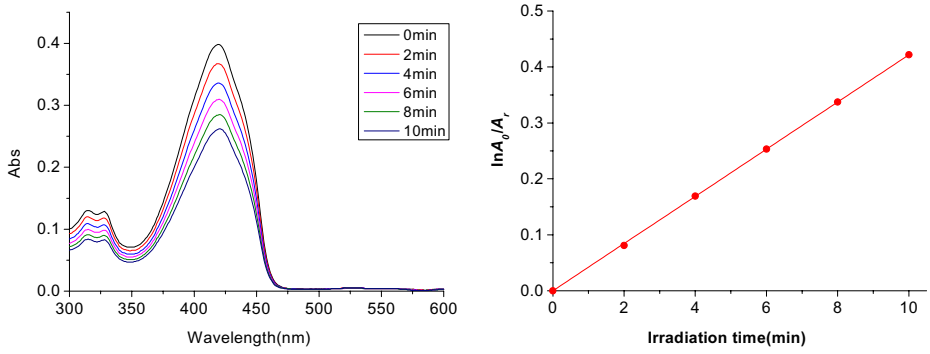
3.4.1. *Phototoxicity studies*

Eca-109 cells were incubated with TDPC for 24 h and exposed to light as described above. After PDT, cells were incubated in the dark for 8 h. Then cell viability was measured by MTT assay. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), was reduced to purple formazan in living cells. DMSO was added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution could be quantified by measuring at a certain wavelength by a spectrophotometer. The results were compiled by origin system. As shown in Fig. 5. It could be seen that at examined concentrations no apparent cytotoxic effects of the drugs were observed without light. The survival fraction still reached $75.20 \pm 0.093\%$ when concentration of drug was $10\,\mu$M. In the light/no drug



(a) (b)

Fig. 3. Excitation and emission spectrum of TDPC in DMF. (a) Excitation spectrum of TDPC; (b) Emission spectrum of TDPC, which was excited at 418 nm, and the peak was at 651 and 722 nm.



(a) (b)

Fig. 4. (a) Absorption spectra for the photo-oxidation of DPBF photosensitized by TDPC; (b) First-order plots for the photo-oxidation of DPBF photosensitized by TDPC($k = 4.257\,\text{min}^{-1}$).

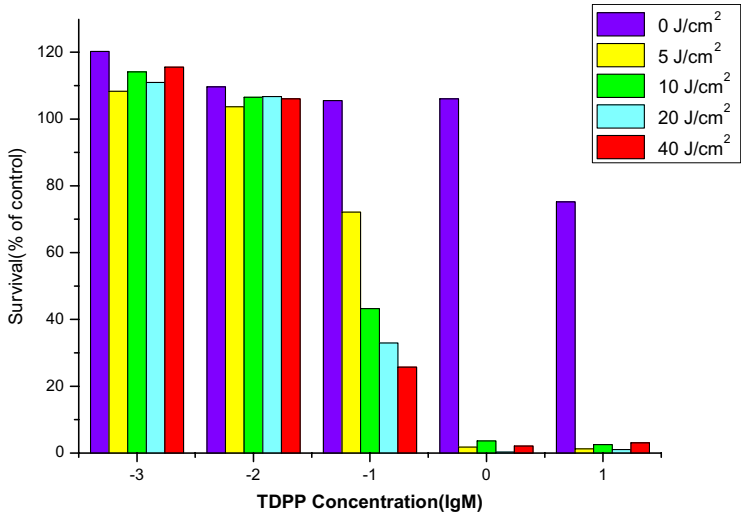


Fig. 5. Cytotoxicity to Eca-109 cells with different concentrations of TDPC and different laser dose.

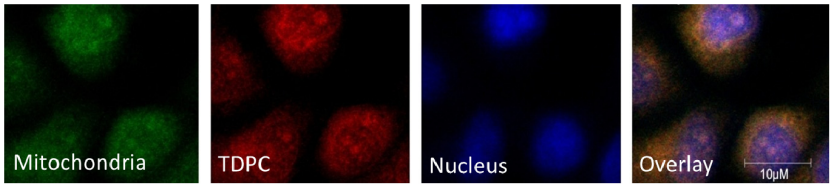


Fig. 6. The intracellular localization of TDPC in Eca-109 cells with MitoTracker Green and Hoechst 33258, respectively.

groups, decreased cell viability was not noticed ($p > 0.05$). Survival of cells dropped only by a combination of the PS and light. Following PDT, when the concentration of TDPC was increased, the viability of cells was declined. However, the PDT effect was not strong at 0.01 and 0.001 μM concentration but opposite at the concentration above 0.1 μM. Besides that, there was no significant difference between light doses. The viability of cells appeared gradient relation only at 0.1 μM concentration of TDPC. It was indicated that TDPC may be an excellent PS and pro-oxidant in biological applications.

3.4.2. *Subcellular localization of TDPC*

Photodynamic efficacy is principally determined by the subcellular localization of a PS. The localization of TDPC ($\lambda_{max} = 650$ nm) in cancer cells was analyzed by MitoTracker Green and Hoechst 33258 counterstain. It was found that TDPC initially localized in mitochondria with some accumulation in the nucleus in Eca-109 cells (see Fig. 6).

3.4.3. *The changes of gene expression in ECA-109 cells treated by TDPC–PDT*

To explore the changes of gene expression after TDPC–PDT, total cellular RNA was harvested

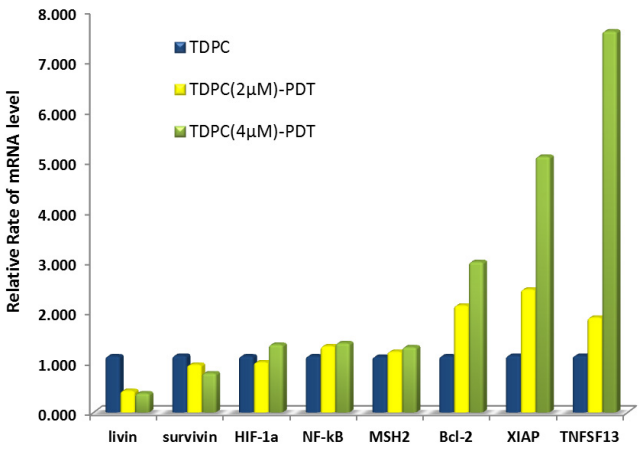


Fig. 7. The Changes of gene expression in Eca-109 cells after TDPC–PDT. RT-PCR results showed the expression changes of 8 genes in Eca-109 cells treated by PDT with 2 or 4 μM TDPC, or with 4 μM TDPC with no photo excitation after 24 h.

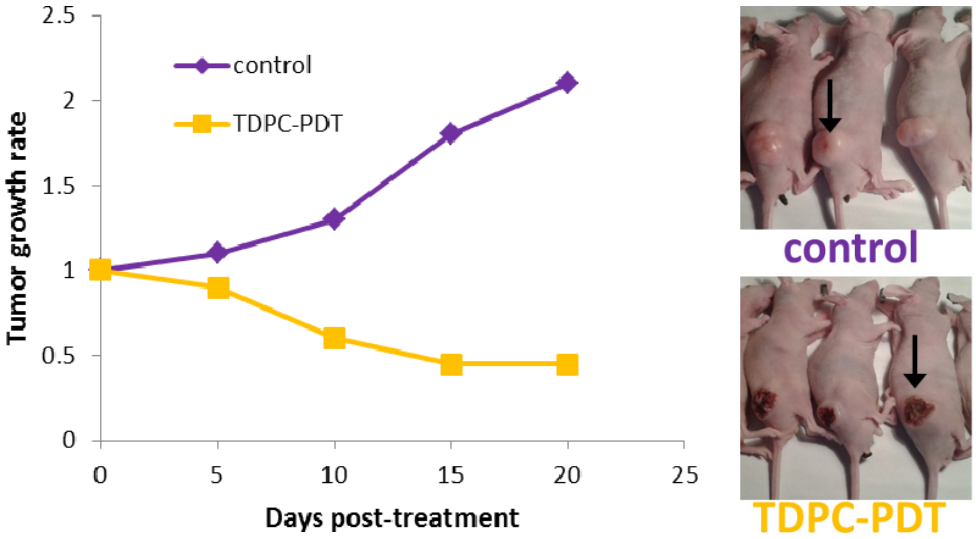


Fig. 8. *In vivo* photosensitizing efficacy of TDPC against lung cancer in nude mice bearing ECA-109 tumors (6 mice/group). The mice in PDT group were treated with laser light (698 nm, 78 J/cm^2) at 24 h post injection of the drug. The mice in PS group were treated with 8 μM TDPC, but not exposed to light. The control mice were not subjected to any PS or light.

from ECA-109 cells after 24 h incubation in medium containing 2 or 4 μM TDPC followed by irradiation with a dose of 0.18 J/cm^2 light ($\lambda = 650$ nm). The expression levels of genes involved in apoptosis and cell growth were then detected by real-time PCR assay (see Fig. 7).

During TDPC–PDT, some genes involved in apoptosis such as Bcl-2 and X-linked inhibitor of apoptosis (XIAP) were significantly up-regulated, and the tumor necrosis factor superfamily member 13 (TNFSF13) also greatly increased after PDT with the higher dose of TDPC (4 μM). TDPC–PDT at the dose of 2 μM caused hypoxia-inducible factor-1a (HIF-1a) to be down-regulated, however increasing the dose of TDPC furthermore could promote HIF-1a expression.

3.5. *The efficacy of TDPC–PDT against esophagus cancer in vivo*

To determine the effects of TDPC–PDT on esophagus cancer *in vivo*, 1×10^6 ECA-109 cells were injected into the axilla of nude mice to form solid tumors (1 cm^3 in size). 100 μL of 8 μM TDPC was then injected into the tail vein and 24 h later the tumor was irradiated with 130 mW/cm laser light for 10 min (78 J/cm^2, $\lambda = 650$ nm). As shown in Fig. 8, PDT-treated tumors began to shrink within five days and became dark, hardened and dried over the course of the next 10 days, eventually formed a scab by 20 days after treatment. The normal skin phototoxicity subsided within 4 days. In contrast, the tumors in PS or control group continued to grow and were significantly larger than in PDT-treated group after 10 days post-treatment (see Fig. 8).

4. Discussion

An ideal PS should have absorption spectra at long wavelengths, which allows deeper tissue penetration and decreases nonspecific lesions.[18] For instance, 630 nm light penetrates less than 0.5 cm whereas 700 nm light reaches a depth of nearly 0.8 cm.[19] TDPC can be excited by 650 nm light. Both *in vitro* and *in vivo* data supported that TDPC might be an ideal PS.

Not only many PSs are bright fluorophores, but also they tend to emit in the nearinfrared (NIR) portion of the spectra that is useful for *in vivo* imaging. Excited by 418 nm light, TDPC can emit red fluorescence at 651 and 722 nm, which means that it has the prospects to become a new reagent for targeting fluorescence imaging in tumor diagnosis.

In numerous PDT studies, it has been demonstrated that generation of $^1O_2$ is responsible for the initiation of cell death.[20–24] The singlet oxygen was identified as ROS involved in cell photosensitization by TDPC.

TDPC showed significant concentration and light dose dependence and induced no dark toxicity

in the range of concentrations used in the present photodynamic studies via MTT assay.

The chemical properties of a PS determine its subcellular localization, which in turn affect the cytotoxicity of photoactivation. Some PSs show a broad distribution, while some may localize more specifically. In general, the PSs that localize in mitochondria show more effectiveness. We found that TDPC localized primarily in mitochondria and, to a lesser degree, the nucleus. Therefore, TDPC–PDT-mediated apoptosis may be triggered via mitochondrial pathway.

It was found that the expression of some apoptosis factors including survivin and livin/KIAP were up-regulated significantly during TDPC–PDT, and it potentially contributed to cell apoptosis. Nuclear factor kB (NF-kB) is a nuclear transcription factor that regulates the expression of a large number of important genes for apoptosis.[25] The activation of NF-kB is regarded as a stress response and has been proved to be activated by TDPC–PDT. MSH2 functioned in DNA repair is increased after TDPC–PDT. Thus, the apoptosis induced by TDPC–PDT is likely the result of an integrative response based on the activation and deactivation of different genes that are critical for growth, apoptosis and DNA repair. The expression level of TNFSF13, a member of tumor necrosis factor superfamily, increased significantly. It will be an important molecular target for the discovery of new photosensitizers.

5. Conclusions

In general, TDPC with an absorption peak at 650 nm endowed with high singlet oxygen generation rates of $4.257\,\mathrm{min}^{-1}$. It localized primarily in mitochondria in cancer cells. TDPC–PDT caused a rapid and robust apoptosis response in Eca-109 cells when exposed to 650 nm laser light irradiation. Eca-109 tumors in nude mice were destroyed and a small scab was formed eventually after TDPC–PDT. So, TDPC is an effective PS which is worth further study as a new drug candidate and TNFSF13 may be an important molecular target in the discovery of new PSs for PDT.

Acknowledgments

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