Original Article
Inhibition of breast cancer cell proliferation by a newly developed photosensitizer chrolophyll derivative CPD4

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Abstract: To investigate the mechanism of inhibition of breast cancer cell proliferation by a newly developed photosensitizer chrolophyll derivative CPD4. MCF-7 cells were treated with CPD4 in different concentrations for 2 h, followed by irradiation (670 nm, 10 J/cm^2) using a semiconductor laser. Apoptotic mode and ratio of treated cells were analyzed. Subcellular localization of CPD4 were observed. Mitochondrial membrane potential was measured, and expression of Bcl-2 and Bax was detected. Four groups were set in this study: (1) no photosensitizer or light irradiation treatment, (2) photosensitizer (2.5 µg/mL CPD4) alone, (3) light irradiation alone, and (4) combined photosensitizer and light irradiation treatment (2.5 µg/mL CPD4-PDT). Flow cytometry assays revealed that apoptotic ratio of CDP4-PDT treated cells increased significantly in a CDP4 dose-dependent manner whereas apoptotic and necrotic ratio in the control group was only 4.9%-6.2% and 1.3%-2.9%, respectively. CPD4 was primarily distributed in mitochondria. While no significant difference in mitochondrial membrane potential was observed among three control groups (BG, PG and IG group) (*P*<0.05), mitochondrial membrane potential in CDP4-PDT group decreased significantly after irradiation treatment (*P*>0.05). A simutanous downregulation of Bcl-2 protein and overexpression of Bax protein in CDP4-PDT treated cells led to a reduction in the Bcl-2/Bax ratio. CPD4 photodynamic treatment induced decreased mitochondrial membrane potential, and might thereby trigger apoptosis through mitochondrial pathway in MCF-7 cells. The treatment stimulated Bcl-2 expression but down-regulated Bax expression, which might suggest an alternative mechanism of apoptotic effect of CPD4.

Keywords: Chrolophyll derivative CPD4, photodynamic therapy, breast cancer, apoptosis, mitochondrial membrane potential

Introduction

Breast cancer is one of the most common cancers in women worldwide, and its incidence rate of each year trends to increases gradually. Traditional treatments for breast cancer include surgery, chemotherapy, radiotherapy and endocrine therapy. Currently, the recurrence rate after treatment of breast cancer is 5%-19% [1, 2]. The primary treatment objectives for patients with recurrent or metastatic breast cancer are to alleviate their anguish, improve the quality of life, and extend survival duration. Photodynamic therapy (PDT) is a therapeutic modality based on photosensitization reaction in the presence of molecular oxygen which consequently generates a series of biological effects. During PDT, photochemical reactions of photosensitizers enriched in tumor tissues are triggered with a certain wavelength of light

to generate reactive oxygen species (ROS) such as excited states of singlet oxygen and superoxide anion free radical, leading to damage of biomacromolecules in tissues and cells, which eventually achieve therapeutic effects through killing tumor cells. In a recent clinical report, PDT has been shown to be highly effective for recurrent breast cancer, with an efficiency of 89%-92% [3, 4].

Photosensitizer is one of three key components in PDT. With the development of PDT technology, first generation of photosensitizers, represented by hematoporphyrin derivatives, have been gradually replaced by second generation photosensitizers such as cyanine and degradation derivatives of chlorophyll, due to their drawbacks including low tumoricidal effects, strong phototoxic reaction in normal tissues, unknown effective components, strict requirement for

light protection, etc.. The photosensitizer CPD4 [2-devinyl-2-(1-methoxyl-ethyl) chlorin f] used in this study is a dihydrogen porphyrin derivative, and is a monomer compound made from silkworm faeces chlorophyll. The photodynamic effects of CPD4 on human breast cancer cell line MCF-7 and possible mechanisms of its tumor killing activity were investigated.

Materials and methods

Materials

The photosensitizer CPD4 was provided by the College of Pharmacy in the Second Military Medical University (Shanghai, China). CPD4 was dissolved in 0.1 N NaOH and then diluted to final concentrations in RPMI-1640 medium purchased from Beijing Tianrun Cassidy Biological Technology Co., Ltd. (Beijing, China). Fetal bovine serum (FBS) was obtained from Beijing Yuan Heng Jinma Biological Technology Co., Ltd. (Beijing, China). Human breast cancer cell line MCF-7 was obtained from the Immunology Department in Cancer Hospital of Tianjin Medical University. Rhodamine-123 was purchased from Sigma (St. Luis, MO, USA). Annexin V FITC apoptosis detection kit was purchased from BD Biosciences (San Diego, CA, USA). The laser light source of 670 nm was generated by a semiconductor photodynamic therapy apparatus (manufactured by the Institute of Modern Optics in Nankai University, Tianjin, China) with a maximum output power of 2 W. The electrophoresis system was purchased from Bio-Rad Laboratories (Richmond, CA, USA) and gel imaging system was manufactured by Alpha Innotech (San Leandro, CA, USA).

Methods

Cell culture: Human breast cancer cell line MCF-7 was cultured in RPMI-1640 medium containing 10% FBS, 1% penicillin/streptomycin at 37°C in an incubator with 5% CO_2, 95% humidity.

Photodynamic treatment: The following four groups (4 wells for each group) were set to study the photodynamic effects of CPD4 on MCF-7 cells: (1) no photosensitizer or light irradiation treatment (blank control group, BG), (2) photosensitizer (2.5 μg/mL CPD4) alone (PG) (3) light irradiation alone (IG), and (4) combined photosensitizer (2.5 μg/mL CPD4) and light

irradiation treatment (CPD4-PDT). Cells in exponential growth phase were inoculated into 96-well plates (about $1×10^4$ cells per well) and cultured for 24 h. 20 μL of normal saline containing different concentrations of CPD4 (to a final concentration of 0.5, 1.0, 1.5, 2.0, and 2.5 μg/mL, respectively) was added to the culture medium. Cells were incubated at 37°C for additional 2 h and then washed 3 times with PBS. Culture medium was replaced with fresh medium supplemented with 10% of FBS. Cells were irradiated at 670 nm using a semiconductor laser at a light dose of 10 J/cm², incubated for 24 h, and collected for subsequent experiments.

Apoptosis detection assay: Collected cells in each group were washed 2 times using prechilled PBS and a cell suspension in a density of $1×10^6$/mL was prepared. 5 μL of Annexin V-FITC and 5 μL of PI were added, and cells were incubated in dark for 15 min, followed by adding 400 μL of reaction buffer. Apoptosis detection assay was performed by a flow cytometry.

Confocal imaging of CPD4 and probes in MCF-7 cells: Cells at exponential growth phase were digested with 0.25% trypsin, and subcultured in 35 mm culture dishes with a density of $5×10^3$/mL. Normal saline containing CPD4 was added to a final concentration of 2.5 μg/mL once cells became completely adherent. After 2 h incubation, cells were incubated with mitochondrial probe Rhodamine-123 for additional 15 min, followed by washing the cells using PBS to remove the residual probe dye. Cells were observed under laser confocal microscopy. The green fluorescence represented locations of Rhodamine-123 and red for CPD4, whereas the common fields were yellow in the overlay of the green and red fluorescence.

Detection of mitochondrial membrane potential: MCF-7 cells were seeded into 24-well plates ($1×10^5$ per well) and cultured overnight. Cells were washed 3 times using PBS, and either 500 μL of 2.5 μg/mL CPD4 or RPMI-1640 medium was added to each well. Plates were incubated in dark for 2 h, and medium of each well was replaced with 1 ml of fresh RPMI-1640 medium. Each well was irradiated at 670 nm with a 10 J/cm² dose for 5 min by a semiconductor laser instrument. 10 μg/mL of Rhodamine-123 was added to each well at 2 h
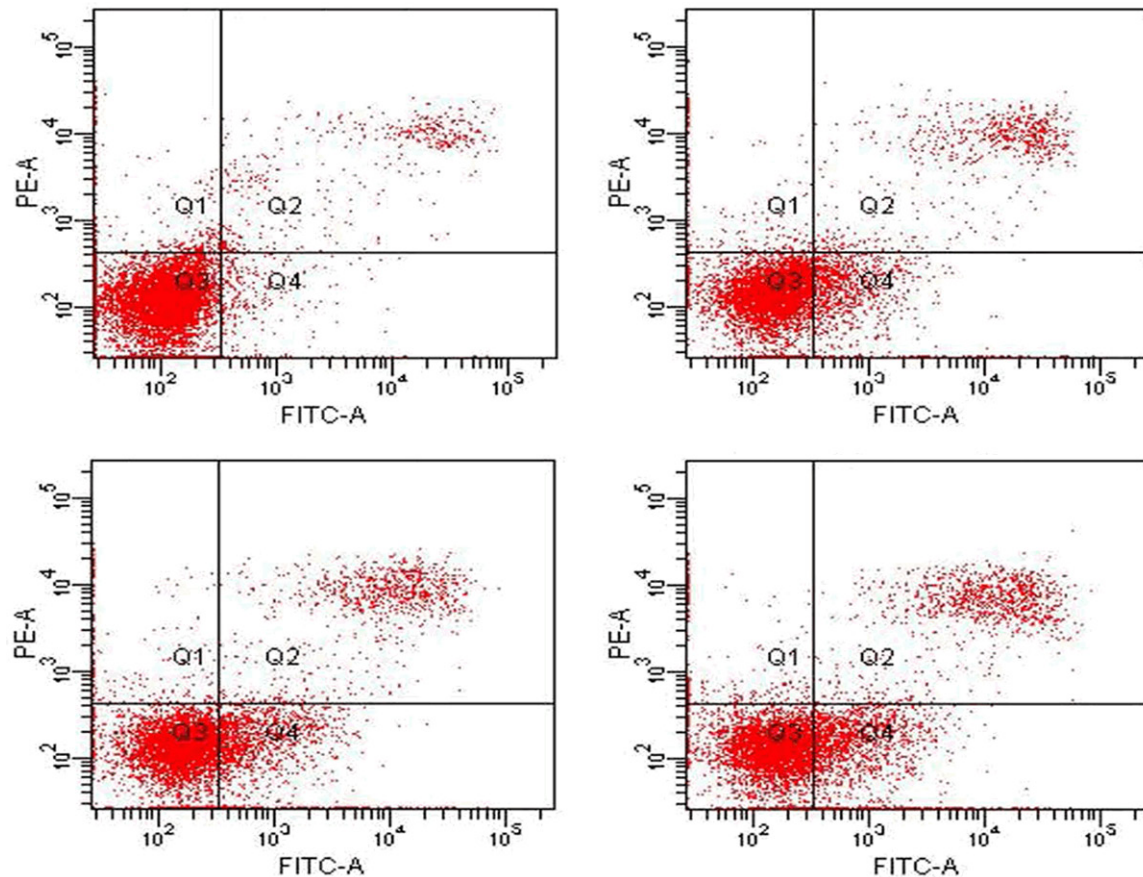
Figure 1. Apoptotic ratio of MCF-7 cells after CPD4-PDT treatment for 24 h.

**Table 1.** Apoptotic ratio of MCF-7 cells after CPD4-PDT treatment (%) ($\overline{x}$±S.D.)

Group	Apoptotic Ratio (%)
BG	2.2±1.27*
IG (10 J/cm²)	4.0±1.52*
PG (2.5 µg/mL)	2.9±2.16*
CPD4-PDT 1 (1.5 µg/mL)	31.25±0.21#,®
CPD4-PDT 2 (2.0 µg/mL)	34.1±3.11#,®
CPD4-PDT 3 (2.5 µg/mL)	41.2±1.41#,®

Apoptosis detection assay was performed on cells treated with CPD4 for 2 h, followed by 24 h of irradiation (670 nm, 10 J/cm²) using a semiconductor laser. #$P<0.01$, CPD4-PDT group was compared with BG, PG and IG groups, respectively. *$P>0.05$, BG group was compared with PG and IG groups, respectively. ®, $P<0.01$, Pairwise comparison between different CPD4-PDT groups.

after irradiation, and the plates were incubated in the dark for 5 min. Medium were removed and cells were washed 3 times with PBS, digested by 200 µL of trypsin, and collected by centrifugation. Cells were then washed 3 times using PBS, and subject to flow cytometry assay.

Expression of Bcl-2 and Bax by Western blotting analysis: Cells in both photodynamic treatment and control groups were harvested at 2 and 6 h after treatment. 100 µg of Total proteins were extracted and the concentration was determined by Mircro BCA protein Assay kit (Pierce, IL, USA) according to manufacturer's instruction. 10% SDS-PAGE gel was prepared and 40 µg of protein was loaded onto each lane for electrophoresis.

The separated proteins were semi-dry transferred to PVDF membranes, washed with TBST buffer, blocked with 5% defatted milk for 2 h at room temperature (RT), and rinsed with TBST buffer. The treated PVDF membranes were then incubated overnight with the solution containing primary monoclonal antibodies (rabbit anti-β-actin at 1:1000 dilution, anti-Bcl-2 at 1:500 and anti-Bax at 1:500, respectively). The PVDF membranes were washed with TBST buffer and incubated with HRP-labeled secondary antibodies (Beijing ZSGB-Bio, Inc. Beijing, China) for 1 h at RT. The membranes were
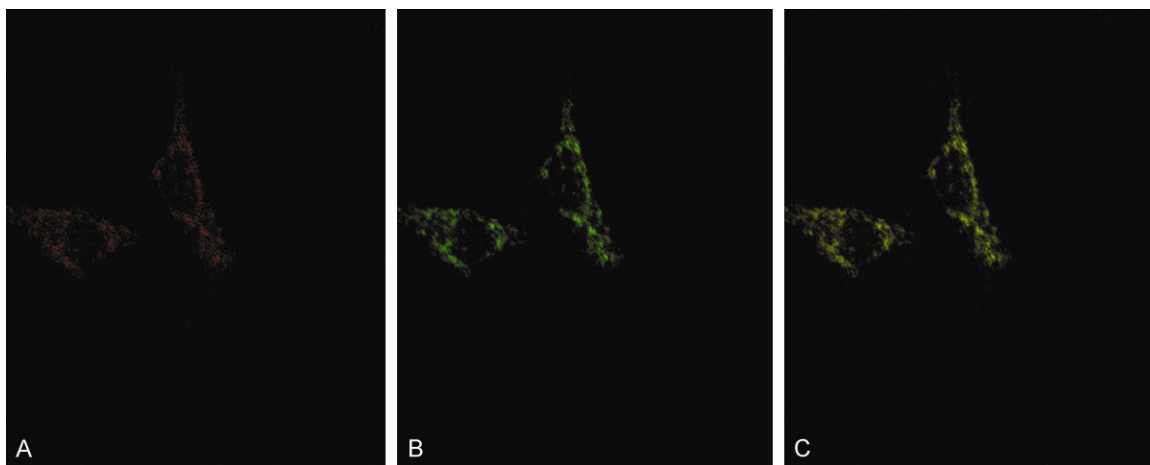
Figure 2. Fluorescence images of photodynamically treated MCF-7 cells. A. Fluorescence image of CPD4. B. Fluorescence image of probe-labeled mitochondrial and C. the overlay of two images.
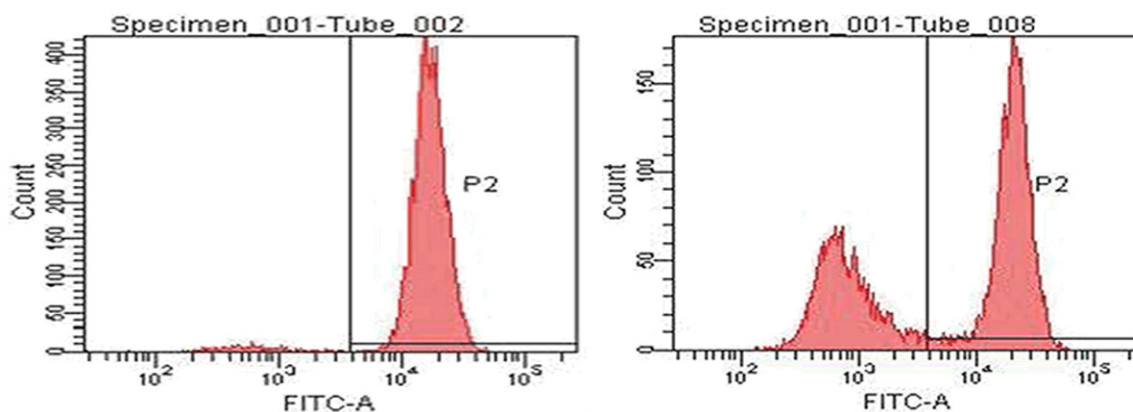
Figure 3. Mitochondrial membrane potential detected by flow cytometry assay.

rinsed with TBST buffer and developed using Supersignal West Pico kit. β-actin was used as an internal control.

Statistical analysis

In this study, all procedures were performed independently in triplicate. Data were expressed as mean ± S.D. and differences among the four test groups were compared by one-way ANOVA (SPSS, version 13.0; SPSS Inc., Armonk, New York, USA). *P* values less than 0.05 were considered statistically significant.

Results

Photodynamic effects of CPD4 on apoptosis of human breast cancer cell line MCF-7

Apoptosis and necrosis was detected by flow cytometry analysis in 4.9%-6.2% and 1.3%-

2.9% of cells in BG group, respectively, which was consistent with normal growth characteristics of passage cell lines. However, the apoptotic ratio in photodynamic groups increased significantly in a CPD4 dose-dependent manner. 41.2%±1.41 of cells underwent apoptosis but necrotic ratio remained apparently unchanged in CPD4-PDT group (2.5 μg/mL CPD4-PDT), indicating that cytocidal effects of CPD4 was predominantly mediated by apoptosis induction (**Figure 1**; **Table 1**).

Cellular distribution and subcellular localization of CPD4 in MCF-7 cellls

Figure 2A and **2B** showed the fluorescence image of photosensitizer CPD4 and probe-labeled mitochondrial, receptively, and **Figure 2C** demonstrated the overlay of both images, suggesting significant overlaps of signals of

Table 2. Changes in mitochondrial membrane potential ($\overline{x}$±S.D.)

Group	Decrease of mitochondrial membrane potential (%)
BG	2.2±1.27*
IG (10 J/cm^2)	4.0±1.52*
PG (2.5 µg/mL)	2.9±2.16*
CPD4-PDT (2.5 µg/mL)	41.2±1.41*,#

*,#$P<0.01$, CPD4-PDT group was compared with BG, IG and PG group after treatment, respectively. *$P>0.05$, BGwas compared with IG and PG group, respectively.

CPD4 and Rhodamine-123 in photodynamically treated cells. CDP4 was shown to be primarily distributed between nuclear membrane and cell membrane, where mitochondrial membrane were located as confirmed by fluorescence signal of mitochondrial-specific probes. In addition, considerable amounts of CDP4 were also found in both cell membrane and nuclear membrane (**Figure 2A**), whereas only a small amount of CDP4 was dispersed in cytoplasm.

Photodynamic effects of CPD4 on mitochondrial membrane potential of MCF-7 cells

As shown in **Figure 3** and **Table 2**, no significant difference in mitochondrial membrane potential as indicated by mean fluorescence intensity of rhodamine-123 existed between all three control groups (BG, PG and IG group) ($P<0.05$), whereas mean fluorescence intensity of CDP4-PDT group decreased significantly after irradiation treatment compared with the control groups ($P>0.05$).

Photodynamic effects of CPD4 on expression of Bcl-2 and Bax in MCF-7 cells

Western blotting analysis revealed that photodynamic effects of CDP4 induced a significant reduction in Bcl-2 expression, but a dramatic increase in Bax expression (**Figure 4**), leading to a decreased Bcl-2/Bax ratio, which suggested that both Bcl-2 and Bax might be involved in CPD4-PDT-associated apoptosis.

Discussion

PDT is a novel therapeutic modality aimed at treating cancers without surgery or chemotherapy, during which photosensitization is triggered to induce cell death when the cells are
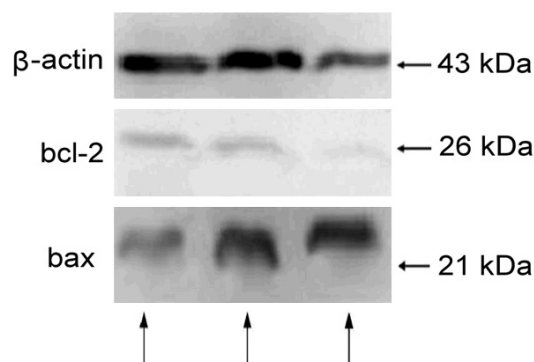
Figure 4. Western blotting analysis of expression of Bcl-2 and Bax proteins.

exposed to a particular type of light. In this study, the therapeutic effect of PDT using a newly developed photosensitizer chrolophyll derivative CPD4 was examined in human breast cancer cell line MCF-7. Intracellular concentration and distribution of drugs are two major factors that affect the photodynamic effect. Therefore, the present study focuses on cell-killing effect of CPD4 in different doses and its intracellular localization.

The main mechanism of PDT involves generation of singlet oxygen and other oxygen radicals, which destruct intracellular biomacromolecules and induced cell death in a variety of cancers. However, it has been reported that the molecular mechanisms behind each death event may differ [5-7], although apoptosis induction is considered to be the predominant mechanism involving tumor-killing of PDT [8, 9]. Rapozzi et al. found out that a high dose of pheophorbide a-PDT induced apoptosis in HeLa cells, but necrosis in HepG2 cells [10]. We herein investigated the cell death mechanism by CPD4-PDT. We previously showed that MCF-7 cells displayed typical apoptotic morphological changes including cell shrinkage, extensive plasma membrane blebbing and nuclear fragmentation after CPD4-PDT treatment, indicating that photodynamic effects of CPD4 on MCF-7 cells may be mainly mediated by apoptosis induction. In this study, photodynamic effects of CPD4 on apoptosis of MCF-7 cells were further investigated by Annexin V/PI double staining in flow cytometry assays. No apoptotic effect was observed in CPD4 or laser alone treated cells, but apoptotic ratios of MCF-7 cells in CPD4-PDT groups were signifi-

cantly higher than all control groups, indicating induction of apoptosis in MCF-7 cells by photodynamic effects of CPD4. It was further noticed that CPD4-PDT significantly inhibited cell proliferation in dose-dependent manner with the highest apoptotic ratio of 41.2±1.41% observed in the 2.5 µg/mL CPD4-PDT group. These results suggested that selecting the appropriate photosensitizer doses are really necessary for highly efficient PDT.

Subcellular localization of the photosensitizer determines the mode and degree of cell death after PDT due to the limited diffusion distance of singlet oxygen. It has been reported that photosensitizers located on cell membrane and lysosome mainly induce cell death through non-apoptotic pathways, whereas those distributed in cytoplasm primarily promotes apoptotic cell death. Furthermore, mitochondrial photosensitizers exhibit higher capacity to induce apoptosis [11]. In recent years, it has been found out that mitochondrial induced apoptosis is one of the major apoptotic signaling pathways. During apoptosis, mitochondria serve as a molecular relay station which transduces and amplifies an incoming apoptotic signal by releasing apoptosis-related substances [9, 12]. Therefore, it is essential to determine the distribution and localization of photosensitizers to clarify the mechanism underlining apoptotic induction of CPD4. In the present study, CPD4 was predominantly distributed in mitochondria as shown by confocal microscopy imaging although it was also found in cell membrane, nuclear membrane and cytoplasm, suggesting that mitochondria was the primary target for CPD4, and the photosensitizer might induce apoptosis through a mitochondrial pathway. It became evident that mitochondria are directly involved in the process of apoptosis by releasing a large amount of free radicals, nucleases and proteases. Depolarization of mitochondria is often the first measurable change during apoptosis [13]. We herein determined the mitochondrial membrane potential variations using the fluorescence probe Rhodamine-123, a widely application for detecting mitochondrial depolarization. The result demonstrated a rapid decrease in mitochondrial membrane potential in CPD4-PDT-treated MCF-7 cells thus indicating structural damages of mitochondrial membrane and thereby changes in membrane permeability.

It is currently believed that high permeability of mitochondrial permeability transition pore (PTP) is crucial for the occurrence of mitochondria-dependent apoptosis [14, 15]. Previous studies have found out that Bcl-2 family proteins play important regulatory roles for permeability of PTP [16]. Bcl-2 protein located in mitochondrial membrane is one of the key members of Bcl-2 family which inhibits the permeability transition of PTP through interaction with the adenine nucleotide translocator (ANT), a protein component of PTP, and thereby maintains membrane potential. Bax protein, another member of Bcl-2 family, is located in the cytoplasm and also regulates the permeability transition of PTP, but in an opposite manner [16, 17]. Therefore, the Bcl-2/Bax ratio is a key factor which determines if cell survival or apoptosis takes place [16]. In this study, we next examined the effect of CPD4-PDT on expression of Bcl-2 and Bax by Western blot analysis. Significant downregulation of Bcl-2 and overexpression of Bax was detected in CPD4-PDT group, resulting in a decreased Bcl-2/Bax ratio, which suggested that apoptotic induction of CPD4 photodynamic therapy might be associated with PTP opening in MCF-7 cells mediated by down-regulation of Bcl-2/Bax ratio. Furthermore, a reduction in Bcl-2 expression generally causes decreased antioxidant activity of mitochondria [18]. Therefore, singlet oxygen generated by CPD4 photochemical reaction might accumulate in the MCF-7 cells which further accelerate their apoptosis.

In conclusion, the potent antitumor activities of PDT using a newly developed photosensitizer CPD4 were confirmed in human breast cancer cell line MCF-7. CPD4 photodynamic treatment induced decreased mitochondrial membrane potential, and might thereby trigger apoptotic cell death by regulating mitochondrial integrity in human breast cancer cell line MCF-7. CPD4 photodynamic treatment up-regulated Bcl-2 expression but down-regulate Bax expression, leading to a reduction in the Bcl-2/Bax ratio, which might be an alternative mechanism behind apoptotic effects of CPD4 on MCF-7 cells. These findings suggest that CPD4-PDT could be a potent clinical therapeutic strategy for human breast cancers.

Disclosure of conflict of interest

None.

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