Journal of Photochemistry & Photobiology, B: Biology 176 (2017) 62–68

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

# The interaction between the light source dose and caspase-dependent and -independent apoptosis in human SK-MEL-3 skin cancer cells following photodynamic therapy with zinc phthalocyanine: A comparative study

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ARTICLE INFO

*Keywords:*
Photodynamic therapy
Zinc phthalocyanine
SK-MEL-3
Caspase-dependent apoptosis
Caspase-independent apoptosis
Laser diode

ABSTRACT

The aim of this study is to determine the behavior of relative expression of Bcl-2, caspase-8, caspase-9, and caspase-3 genes of/in SK-MEL-3 cancer cells and explore molecular mechanisms responsible for the apoptosis response during an in vitro photodynamic therapy (PDT) with Zinc Phthalocyanine (ZnPc) using different doses of the light source. In this study, firstly the cytotoxic effects of ZnPc-PDT on SK-MEL-3 cells were evaluated. By irradiating the laser, ZnPc induced a significant amount of apoptosis on SK-MEL-3 cells in three $IC_{50}$s including 0.064 ± 0.01, 0.043 ± 0.01, and 0.036 ± 0.01 μg/mL at the doses of 8, 16, and 24 J/cm$^2$, respectively. Moreover, flow cytometry and QRT-PCR experiments were done. The high percentage of apoptotic cells was seen in the early apoptosis stage. The expression of Bcl-2 and caspase-8 genes at all doses of laser experienced an obvious reduction in comparison to the control group. On the other hand, although the expression of caspase-9 and caspase-3 genes remains almost constant at 8 J/cm$^2$, but they faced an increment at 16 and 24 J/cm$^2$ doses. These data reveal caspase-dependent apoptosis in high and caspase-independent apoptosis in low doses of laser. Based on the results of present work, it can be suggested that the dose of the light source is a key factor in induction of caspase-dependent and caspase-independent apoptosis pathways following PDT.

## 1. Introduction

Photodynamic therapy (PDT) is a new approach for cancer treatment with the minimum invasive effect on non-cancer tissues, which functions by exciting a non-toxic light-sensitive compound (photosensitizer) using a laser light [1,2]. This therapy has been used to treat cancers including head and neck [3], esophageal [4], bladder [5], liver [6], lung [7], breast [8], brain tumors [9], Kaposi's sarcoma, oral cavity [10,11], skin [12], prostate [13], the cervical [14], and gastrointestinal cancers [15] with promising results in comparison to other cancer therapies.

The main problems of conventional cancer therapies including chemotherapy, radiation therapy, hormone therapy, and surgery are high toxicity, serious side effects, and damage to the healthy tissues [16]. Meanwhile, in PDT, photosensitizer delivery to the target tissues and focused light source precisely on them, can limit cell damage in the treated area and consequently reduce the damage inflicted to normal tissue [17].

PDT itself consists of three main elements including photosensitizer, harmless visible light, and reactive oxygen species (ROS) [18,19]. Among the factors influencing the efficiency of PDT, the photosensitizer has a key role [20]. Owing to some predominant characteristics of metal phthalocyanine (the second generation of photosensitizers) such as high stability, high optical toxicity, low dark toxicity, strong absorption in phototherapeutic window (a wavelength range of 600 to 900 nm), high ROS production, and selective absorption by malignant tissues, zinc phthalocyanine is considered among the most promising photosensitizers used in PDT [21–24].

The photosensitizer can be placed within the organelles such as mitochondria, lysosome, endoplasmic reticulum (ER), Golgi, and the plasma membrane [25]. It has to be noted that the type of cell death after PDT is highly dependent on replacement of photosensitizer inside the cells due to the instability of reactive oxygen species (half-life from 1 to 4 microseconds and the radius migration of < 30 nm [26]) [27]. In addition, the type of cell death also depends on photosensitizer, the

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http://dx.doi.org/10.1016/j.jphotobiol.2017.09.020
Received 15 June 2017; Received in revised form 3 September 2017; Accepted 21 September 2017
Available online 22 September 2017
1011-1344/ 

total delivered energy, the type of cells, and the applied protocol [28–31].

Mechanisms of tumor destruction in the PDT include direct effects (direct damage to tumor cells via apoptosis/necrosis) and indirect effects (damage to the blood vessels supplying the tumor tissue and host immune system activation against the tumor) [32]. PDT can induce different cell death mechanisms such as apoptosis/necrosis [33]. Apoptotic cell death mechanism is an ATP-dependent process [34], activated by external stimuli such as TNF-α, Fas, and TRAIL or through internal stimuli and mitochondrial signaling pathway [35,36].

In terms of morphology, features of apoptosis include mitochondrial membrane permeability, condensation chromatin, fragmentation of DNA, cell shrinkage, bubbled plasma membrane, the formation of apoptotic bodies and exposed phosphatidylserine to the outer layer of the plasma membrane. At the biochemical level, apoptosis is a caspase-dependent phenomenon and various morphological changes in cells are caused by caspases activity [37,38]. However, according to available studies, caspase-independent apoptosis is also possible to occur. The main mediator in the caspase-independent apoptosis is an apoptosis-inducing factor (AIF) released from mitochondria. In caspase-independent apoptosis, morphology of the nucleus such as a part of chromatin in nucleus is condensation without DNA fragmentation. This feature is distinct from apoptosis, while many other features of apoptosis have maintained [39–42].

Cellular and molecular mechanisms of apoptosis induced by PDT are highly complex and not fully known. Therefore, characterizing these mechanisms leads to design better PDT protocols that widely enhance its effectiveness [25,43].

In this study, the effectiveness of PDT on SK-MEL-3 cell line using different concentrations of Zinc-Phthalocyanine as a photosensitizer and a diode laser with a wavelength of 675 nm is evaluated. The purpose of this study is to compare the effects of different laser doses on the type of induced cell death and, finally, the analysis of cellular and molecular mechanisms involved in this process.

## 2. Materials and Methods

### 2.1. Cell Culture

The SK-MEL-3 cell line was purchased from the Pasteur Institute (Tehran, Iran). Firstly, the cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS, Gibco, USA) and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin) (Gibco, USA). Consequently, cells were incubated at 37 °C in 5% $CO_2$ and 95% humidity until they reached a 70% confluency. The passage number of cells were 3 for all experiments.

### 2.2. Photosensitizer

Zinc phthalocyanine (ZnPc) was purchased from Sigma-Aldrich (USA). Since ZnPc is strongly hydrophobic, dimethylsulfoxide (DMSO) was used to make ZnPc dissolve in the culture medium [44]. The stock solution of ZnPc (5 μg/mL) was prepared using DMSO and RPMI1640 (Gibco, USA) and then sonicated in a sonicator (bath type, Elma transonic T420, Germany). The total concentration of DMSO in the stock solution was reached 2% (v/v) in RPMI1640. In other words, ZnPc was firstly dissolved in DMSO by sonication, and then gradually added to RPMI-1640 medium until the final concentration of ZnPc reached 5 μg/mL including 2% (v/v) DMSO. All the concentrations (0.00001–5 μg/mL) tried in the experiments were diluted using RPMI1640. It is worth mentioning that ZnPc has a weak absorption peak at 345 nm and a strong one at 675 nm [1,45].

### 2.3. Light Source

As stated above, the strong absorption peak of ZnPc is indexed at 675 nm; therefore, to match the wavelength light source with the maximum absorption of ZnPc, a continuous wave diode laser (Shenzhen Taiyong Technology, China) with a wavelength of 675 nm and 80 mW output power at different times including 20, 40, and 60 s (i.e., 8, 16, and 24 J/cm$^2$ laser doses, respectively) was utilized.

### 2.4. PDT Treatment

For in vitro PDT, after reaching almost a 70% confluency of cells in the flask, they were centrifuged, counted, and seeded in 96-well plates with the number of 10,000 cells per well. Then, they were incubated overnight (at 37 °C, 95% humidity, and 5% $CO_2$). The examined cells in this study were categorized into four different groups. The first group, specified as the control group, received neither ZnPc nor laser. The second group was treated only with different concentrations of ZnPc without laser irradiation. The third group, defined as laser control group, was exposed to different doses of the laser (8, 16, and 24 J/cm$^2$) in the absence of ZnPc. The final group received both ZnPc and laser exposure.

To carry out the examinations, firstly, the second and fourth groups were incubated with different concentrations of ZnPc in the total dark condition. After 24 h, all the wells were rinsed with PBS. Next, the cells in the groups three and four were exposed to the laser with three different doses in a dark room and eventually incubated for 24 h at 37 °C, 95% humidity, and 5% $CO_2$.

### 2.5. MTT Assay

According to our previously reported article [1] and with slight changes, cytotoxic effects of PDT treatment with ZnPc were investigated using methylthiazole tetrazolium kit (MTT assay, Sigma). In brief, 24 h after laser irradiation, 50 μL of MTT solution (2 mg/mL in PBS) was added to each well and incubated at 37 °C for 4 h. This process led to metabolize of the MTT to formazan crystals by the succinate-tetrazolium reductase system active only in viable cells. To dissolve the insoluble formazan crystals, 200 μL DMSO was added to each well and incubated for 20 min. Eventually, optical density (OD) of each well was measured at the wavelength of 570 nm using an ELISA reader (Sunrise ELISA Plate Reader, Tecan, Salzberg, Austria). All the tests were done in dark condition and repeated three times. To obtain the percentage of viable cells in each well, the following formula was used.

$$\text{Cell viability (\%)} = (\text{OD of sample}/\text{OD of control}) \times 100$$

### 2.6. DAPI

The changes in the cell nucleus morphology characteristic of apoptosis were examined by citation 5 system (Biotek, USA) of DAPI-stained cells. The SK-MEL-3 cells were seeded 5000 cells per well in a 96 well culture plate. After 24 h, PDT treatment with ZnPc was performed on three previously introduced groups. The cells were fixed with 4% paraformaldehyde for 10 min. The plate was washed twice for 3 min with PBS. Next, the cells were permeable by 0.1% Triton-X-100 for 10 min. Then, after washing three times, the cells were stained with 0.15% DAPI in PBS for 10 min at room temperature (18–20 °C) in the dark. Triplicate random fields were chosen for the experiment. Subsequently, a ratio of the cells showed some evidence of apoptosis.

### 2.7. Detection of Apoptosis/Necrosis by Annexin-V-FLUOS Staining

The apoptosis/necrotic percentages of cells after PDT treatment in three obtained $IC_{50}$s were examined using Annexin-V-FLUOS staining Kit (Roche, Germany). Shortly, 24 h after the treatment of SK-MEL-3 cells with PDT, cells were washed twice with PBS and centrifuged. Afterward, the suspended cells were stained with 1 μL Annexin and 1 μL PI and incubated again in the dark for more 15 min. At last, cell