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Mitochondria-dependent apoptosis induced by a novel amphipathic photochemotherapeutic agent $ZnPcS_2P_2$ in HL60 cells[1]

Hui-fang HUANG, Yuan-zhong CHEN[2], Yong WU

Fujian Institute of Hematology, Union Hospital, Fujian Medical University, Fuzhou 350001, China

Key words

photodynamic therapy; zinc phthalocyanine; apoptosis; cytochrome c; caspases; HL60 cells

[1] Project supported by a grant from the Fujian Provincial Science Foundation (No 2003D10, No 2004Y020).

[2] Correspondence to Dr Yuan-zhong CHEN.
Phn 86-591-8335-1966.
Fax 86-591-8332-4116.
E-mail chenyz@pub3.fz.fj.cn

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Abstract

Aim: To investigate the mechanism underlying the killing effects of a novel amphipathic photosensitizer, disulfonated diphthalimidomethyl phthalocyanine zinc ($ZnPcS_2P_2$), mediated photodynamic therapy (ZnPc-PDT) in human myelogenous leukemia HL60 cells. **Methods:** After incubation for 5 h with 0.5 μmol/L $ZnPcS_2P_2$, the HL60 cells were exposed to a light source of 670 nm wavelength. Thereafter, the cells were detected at different time intervals after PDT. The characteristics of apoptosis were detected by observation of ultrastructure assay, DNA fragmentation assay and terminal deoxynucleotidyl transferase deoxyuridine nick-end labeling method (TUNEL). Mitochondria-dependent apoptosis was determined by the detection of mitochondrial membrane potential ($\Delta\psi m$), activities of caspase family protease and of caspase-3, cytosol cytochrome c. Proteins Bcl-2 and Bax were detected by immunoblot analysis. **Results:** Evident characteristics of apoptosis were observed post-ZnPc-PDT with ultrastructure assay, DNA fragmentation assay and TUNEL staining. TUNEL assay showed that apoptotic rates in the cells collected from 6 h, 12 h and 24 h after PDT were 9.6%, 24.4%, and 33.0%, respectively. HL60 cells underwent mitochondria-dependent apoptosis as a result of cytochrome c release from mitochondria into cytosol accompanied by a reduction of $\Delta\psi m$. The activities of caspase family protease and of caspase-3 were elevated. Furthermore, ZnPc-PDT could remarkably down-regulate the Bcl-2 pro-apoptotic protein and up-regulate the anti-apoptotic Bax protein. **Conclusion:** ZnPc-PDT could induce mitochondria-dependent apoptosis in HL60 cells.

Introduction

Photodynamic therapy (PDT), a promising cancer therapeutic approach, utilizes a photosensitizing agent followed by illumination at a specific "activating" wavelength of light. PDT produces singlet oxygen and other reactive oxygen species (ROS), leading to lipid peroxidation and damage to membranes, DNA, cytoskeleton, and other sites, and eventual cell death[1–3]. Individually, photosensitizers and light are nontoxic, but tumoricidal when combined. Electron microscopy, histological and biochemical studies have shown that PDT with different photosensitizers induces apoptotic or necrotic cell death in different cell types[4–8]. Photosensitizers are key factors for PDT. According to previous studies[9,10], $ZnPcS_2P_2$ presents 4 advantages over conventional photosensitizers such as hematoporphyrin derivatives (HpD) or other porphyrin derivatives. The first advantage is that the wavelength of suitable exicited light for $ZnPcS_2P_2$ is 670 nm, which is good for penetrating into tissues. The second advantage is that $ZnPcS_2P_2$ has no obvious absorption in the visible part as with HpD with the result of decreased skin phototoxicity from natural light to a larger extent. The third advantage is that the exicited triplet state of $ZnPcS_2P_2$ has a larger quantum yield and longer lifetime. The fourth advantage is that the amphipathic structure of $ZnPcS_2P_2$ results in the increase of selective uptake of sensitizers by tumor cells. Our previous investigation manifested that leukemic cell lines exhibited higher susceptibility to ZnPc-PDT than normal hematopoietic cells and ZnPc-PDT could eliminate the residual leukemia cells in normal bone marrow mononuclear cells[11]. However, the mechanism underlying the killing ef-

fects of ZnPc-PDT on leukemic cells is still unknown. In the present paper, we studied the death pathway of HL60 cells induced by ZnPc-PDT and the mechanism of its action.

The mitochondrion plays a central role in the control of apoptosis by releasing the apoptogenic proteins apoptosis-inducing factor (AIF) and cytochrome c into the cytosol. The released proteins activate pathways essential for carrying out the morphological and biochemical changes. For example, cytochrome c, normally residing between the mitochondrial outer and inner membranes and serving as a diffusible electon carrier in the intermembrane space, has been demonstrated to be a co-activator of caspase-9, which in turn activates caspase-3. The active caspase-3 then activates caspase-activated DNA (CAD). Active CAD initiates chromatin and DNA fragmentation[12–22]. Therefore, in the present study, we investigate the relationship between the death mechanism induced by ZnPc-PDT and the mitochondria-dependent apoptosis. Furthermore, it is well known that Bcl-2 serves to inhibit apoptosis by antagonizing the release of mitochondrial cytochrome c, whereas Bax promotes apoptosis[24,25]. The Bcl-2 protein was reported to be a damage target of PDT and contributes to PDT-efficient induction of apoptosis[25]. Therefore, we also detect the kinetic change of Bcl-2 and Bax expression following ZnPc-PDT.

Materials and methods

Cell line Human myelogenous leukemia HL60 cells provided by the Shanghai Institute of Cytobiology (Institute of Chinese Academy of Sciences, China) were maintained in continuous culture in RPMI-1640 (GIBCO BRL), supplemented with 10% (*v*/*v*) heat-inactivated fetal calf serum (FCS), penicillin 100 U/mL, 100 μg/mL streptomycin, and 0.25 mmol/L *L*-glutamine at 37 °C under 5% CO_2 humidified air. The viability of cells was determined by Trypan blue dye exclusion. Cells were maintained in log phase with viability greater than 95%.

Photosensitizer $ZnPcS_2P_2$ was a gift from the Department of Chemistry, Institute of Research on Functional Materials, Fuzhou University, China. It was dissolved in a solution comprised of Cremophor EL 2%(*v*/*v*), propylene glycol 20% (*v*/*v*), NaCl 0.9%(*w*/*w*) and stored in the dark at 4 °C.

Photodynamic treatment HL60 cell suspensions (5×10^6/mL in RPMI-1640) were incubated with 0.5 μmol/L of $ZnPcS_2P_2$ for 5 h. The cell suspensions were then photoirradiated using a LD-670 semi-conductor laser (Laboratory of Laser Medicine, Tianjin Medical University, China) emitting red light at a 670 nm wavelength. The power density at the illumination area was 53 mW/cm^2 and total light dose was 2.1 J/ cm^2. A control group (cells incubated with same volume of saline for 5 h) was established. Thereafter, cells were harvested at 6 h, 12 h, and 24 h, respectively.

Apoptosis assays Ultrastructure was analyzed under a HU-12A electron microscope. DNA fragmentation assay was followed as described[26]. TUNEL stains were carried out according to the manufacture's instructions (Promega) and the apoptotic cells characterized with brown nuclei were counted under microscope and photographed.

Assays for mitochondrial membrane potential (Δψm), caspase-family protease activity and caspase-3 activity The procedure was carried out according to the manufacture's instructions (Biovision). The results were then analyzed by flow cytometry (BD). The Δψm detection kit utilized MitoCapture^{TM} (BioVision), a cationic dye that fluoresces differently in healthy cells and apoptotic cells. In healthy cells, MitoCapture accumulated and aggregated in the mitochondria, giving off a bright red fluorescence. In apoptotic cells, MitoCapture could not aggregate in the mitochondria due to the altered Δψm, and thus it remained in the cytoplasm in its monomer form, emitting green fluorescence. The fluorescent signal could be analyzed by flow cytometry FITC channel for green monomers and PI channel for red aggregates. The assay for caspase activity was based on the cleavage of $(aspartyl)_2$-Rhodamine 110 (D_2R), a reported substrate for members of caspase family proteases. D_2R was non-fluorescent, however, upon cleavage of the substrate by cellular caspases, the released rhodamine 110 resulted in a green fluorescence. The caspase-3 activity detection kit utilized the caspase-3 inhibitor DEVD-FMK, conjugated to FITC as the fluorescent *in situ* marker. FITC-DEVD-FMK was cell-permeable, nontoxic, and irreversibly binds to activated caspase-3 in apoptotic cells.

Assay for cytochrome c Western blotting was performed according to previously published methods[27], with minor modification. In brief, cell pellets were washed once with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (in mmol/L: 20 Hepes-KOH, 250 sucrose, pH 7.5, 10 KCl, 1.5 $MgCl_2$, 1 sodium ethylene diaminetetraacetic acid (EDTA), 1 dithiothreitol, and 0.1 phenylmethylsulfonyl fluoride). The cells were homogenized on ice for 15 min and the homogenates were centrifuged twice at 1000×*g* for 10 min. The supernatants were centrifuged at 12000×*g* for 15 min at 4 °C, and the supernatants were cytosol without mitochondria. Protein concentration of cell extracts was determined with Bradford protein assay (Coomassie Brilliant Blue G-250 obtained from BBI). Cytosol protein was loaded and separated on 12% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to a nitrocellulose membrane

by standard electric transfer protocol. The chemiluminescent detection of membrane-bound proteins was performed according to the manufacture's instructions (KPL, Kirkegaard & Perry Laboratories). In brief, the membranes were blocked in blocking buffer for 1 h at room temperature and then incubated with anti-human cytochrome c mouse monoclonal antibody (Neomarkers) in blocking buffer overnight at 4 °C. After washing, the membranes were incubated with anti-mouse secondary antibody horseradish peroxidase conjugate and then detected by enhanced chemiluminescent detection system. Finally, the bands were visualized by autoradiography using X-ray film and scanned and quantified by Imager Quant (Alpha Innotech Corporation, San Leandro, USA). The relative levels of cytochrome c were obtained after normalization with β-actin values in the same lane. At the same time, the distribution of cytochrome c was detected by immunohistochemistry staining and the signals were visualized with an alkaline phosphatase-linked assay system (DAKO LSAB labeled streptavidin-biotin, DakoCytomation Inc). The proteins were detected under microscope and photographed.

Assay for Bcl-2 protein and Bax protein To prepare lysates, cells were initially washed once with ice-cold PBS. Cell pellets were treated with lysis buffer [1% Nonidet P-40 detergent (NP-40), 50 mmol/L Tris-HCl buffer, pH 8, 0.1% SDS, 0.1 mmol/L phenylmethylsulphonyl fluoride (PMSF), 0.02% sodium azide] for 15 min on ice. Lysates were centrifuged at 12 000×*g* 4 °C for 15 min. Protein concentration of cell extracts was determined with Bradford protein assay. The detergent-soluble proteins were detected using the same procedure as cytochrome c. The intensities of the bands were determined with the utilization of the ratios of Bax (21 kDa) to Bcl-2 (26 kDa). The anti-human monoclonal antibodies were purchased from NeoMarkers.

Statistical analysis All data were presented as mean±SD of 3 independent experiments. Statistical significance was determined with Student's unpaired two-tailed *t*-test (SPSS 10.0 software, SPSS Inc, Chicago, USA). $P<0.05$ was considered statistically significant.

Results

Characteristics of apoptosis in HL60 cells after ZnPc-PDT Electron microscopy studies showed that in ZnPc-PDT group, the features of HL60 cells appeared obviously as following: the cell size decreasing, cell membrane blebbing,

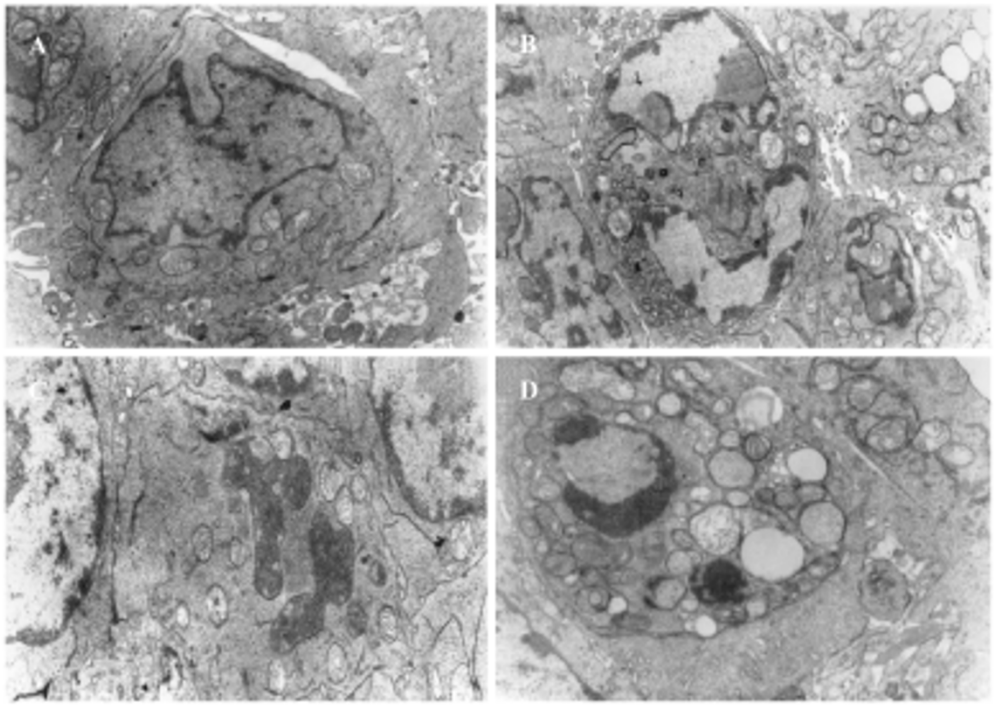

Figure 1. ZnPc-PDT induced apoptosis in HL60 cells. Electron micrographs of HL60 cells in control group (A, ×6000) or in ZnPc-PDT group(B–D, incubated with 0.5 μmol/L of $ZnPcS_2P_2$ for 5 h and then exposed to 670 nm light source of total light dose of 2.1 J/cm^2). B: nuclear chromatin condensation (×4800, 6 h post-PDT); C: nuclear chromatin cleavage (×7000, 24 h post-PDT); D: formation of apoptotic bodies (×7000, 24 h post-PDT).

cytoplasm shrinkage, nuclear chromatin condensation or cleavage and formation of apoptotic bodies (Figure 1). And characteristic chromosomal DNA fragmentation appeared in the cells after ZnPc-PDT. By contrast, no DNA ladder existed in the control groups (Figure 2). TUNEL staining analysis showed many apoptotic cells which nuclei stained brown existed in ZnPc-PDT groups and the average apoptosis rates significantly increased in a time-dependent manner. At the 3 different time points after PDT, the mean TUNEL-positive rates were 9.6%, 24.4%, and 33.0%, respectively (Figure 3). However, few apoptotic cell was detected in the control group. The results indicated that ZnPc-PDT could induce evident apoptosis in HL60 cells.

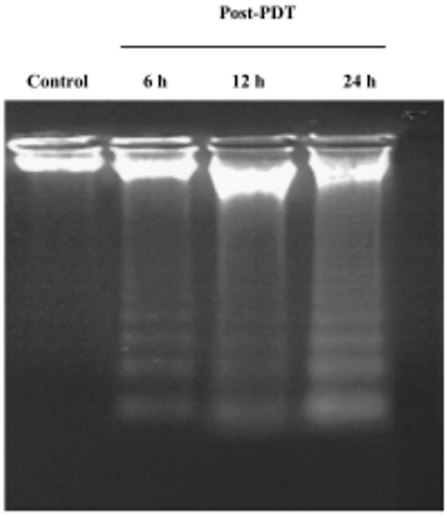

Figure 2. DNA electrophoresis of HL60 cell extracts. Control group: No DNA ladder existed. Post-PDT: HL60 cells were incubated with 0.5 μmol/L of $ZnPcS_2P_2$ for 5 h and then exposed to 670 nm light source of total light dose of 2.1 J/cm^2. The cells were harvested at 6 h, 12 h and 24 h post-PDT. Characteristic DNA ladder appeared. (Original magnification×1.5)

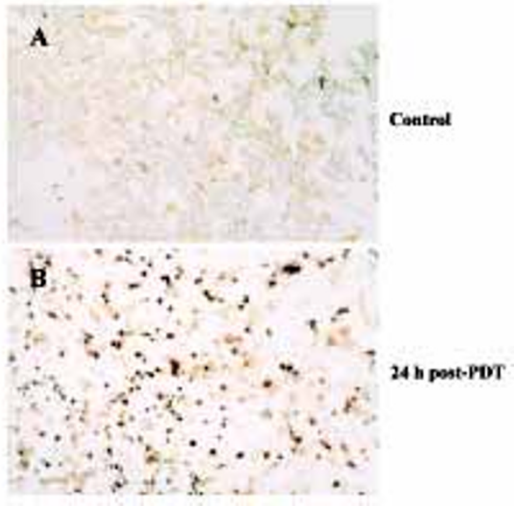

Figure 3. TUNEL staining analysis. A: Control group. B: 24 h post-PDT (HL60 cells were incubated with 0.5 μmol/L of $ZnPcS_2P_2$ for 5 h and then exposed to 670 nm light source of total light dose of 2.1 J/cm^2). (Original magnification×400.)

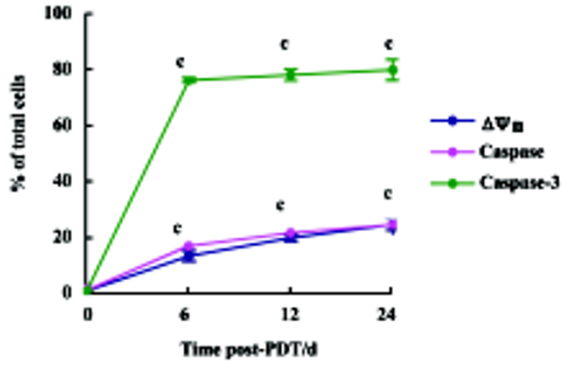

Figure 4. Time course of changes in Δψm, activities of caspase and caspase-3 post-PDT (HL60 cells were incubated with 0.5 μmol/L of $ZnPcS_2P_2$ for 5 h and then exposed to 670 nm light source of total light dose of 2.1 J/cm^2). n=3. Mean±SD. ^cP<0.01 *vs* control.

Characteristics of mitochondria-dependent apoptosis in HL60 cells after ZnPc-PDT In the control group, the positive rates of assays for Δψm, activities of caspase family proteases and caspase-3 were all less than 1%. In the ZnPc-PDT groups, compared with the control group, the positive rates of the 3 indexes increased remarkably (P<0.01) as shown in Figure 4. In the ZnPc-PDT groups, Δψm altered significantly and the positive rates increased in a time-dependent manner. When detecting caspase-3 activity, approximately 75.4% cells presented proteases activities at 6 h post-PDT. Caspase family proteases were activated comparatively slightly and slowly after PDT.

Compared with the control groups, in the ZnPc-PDT groups the ratio of cytosol cytochrome c (15 kDa) to β-actin (43 kDa) assessed by Western blot analysis increased significantly (P<0.01). Significant difference existed between 6 h and 12 h post-PDT (P<0.01). However the difference between 12 h and 24 h points was minor (P>0.05) (Figure 5A). Immunochemistry assay showed that after ZnPc-PDT, cytochrome c translocated from its original location adjacent to nucleus and dispersed into cytosol and the positive particles were stained obviously darker (Figure 5B). The 2 indexes showed that ZnPc-PDT induced evident release of cytochrome c from the mitochondria into the cytosol.

Summing up the results from different observations as

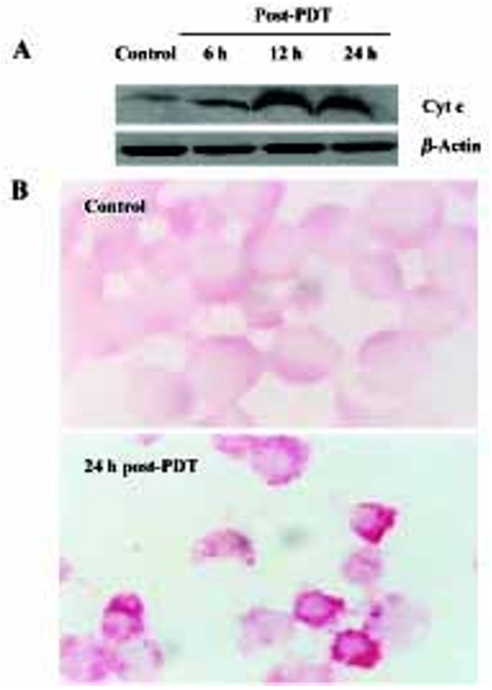

Figure 5. Results of the detection of cytosol cytochrome c. A: Western blotting showed that the intensity of cytochrome c bands strengthen remarkably after ZnPc-PDT (HL60 cells were incubated with 0.5 μmol/L of $ZnPcS_2P_2$ for 5 h and then exposed to 670 nm light source of total light dose of 2.1 J/cm^2). (Original magnification ×2) B: Immunohistochemistry assay showed that after PDT, cytochrome c translocated from its original location adjacent to nucleus to cytosol (×1000).

previously discussed, we concluded that ZnPc-PDT could induce HL60 cells apoptosis through mitochondria-dependent ways.

Effects of ZnPc-PDT on the expression of Bcl-2 and Bax

Western blot assays showed that ZnPc-PDT could down-regulate Bcl-2 expression and up-regulate Bax expression. After PDT, the ratio of Bax to Bcl-2 increased significantly in a time-dependent manner (Figure 6).

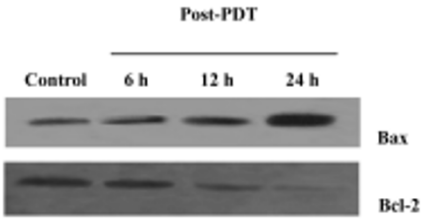

Figure 6. Western blot analysis of Bax and Bcl-2. Bcl-2 expression decreased and Bax expression increased after ZnPc-PDT (HL60 cells were incubated with 0.5 μmol/L of $ZnPcS_2P_2$ for 5 h and then exposed to 670 nm light source of total light dose of 2.1 J/cm^2). (Original magnification ×2)

Discussion

PDT has emerged as a promising therapeutic procedure for the treatment of many tumors. The cytotoxic efficacy relies on a bimodal protocol comprised of chemical-photosensitizer and light-irradiation. Separately, neither the photosensitizer nor the light is toxic[1,11]. The mechanism of the PDT-mediated cytotoxic effects is not well defined. However it is generally believed that the apoptotic response of PDT is critical for its therapeutic efficacy and the generation of ROS is essential for PDT effects[2,3,25]. Some results have demonstrated that PDT could induce the permeability transition pore open and the opening leads to the loss of integrity of the outer mitochondrial membrane, thus releasing the inter-membrane proteins, such as cytochrome c, into the cytosol[12–16]. When released from the mitochondria, cytochrome c combines with an inactive protease precursor, procaspase-9, to form the "apoptosome". The apoptosome attacks procaspase-3 and cleaves it to form active caspase-3, which can lead to DNA breakage, nuclear chromatin condensation, then cause cell death[20,21].

A number of studies have documented that cell death response to PDT varies due to the physical properties of the photosensitizer employed, PDT dose and the cell type[4–8]. Different subcellular localization of the photosensitizer results in different responses[6,7]. Even the same photosensitizer-mediated PDT could lead to different outcomes. At low doses, the cellular machinery for apoptosis is activated, however, higher doses lead to the inhibition of apoptosis with cell death via a necrotic process[4,8]. In the present study, a novel photosensitizer agent, $ZnPcS_2P_2$, developed in China, was employed. Our previous studies have demonstrated that ZnPc-PDT could exert significant photodamage to HL60 cells[11]. In the present study, 0.5 μmol/L drug level was selected as the experimental condition. Under the condition, ZnPc-PDT could inhibit the proliferation of HL60 cells at 51%.

It is well known that there are two major pathways that are involved in the initiation of apoptosis: the "extrinsic" death receptor pathway and the "intrinsic" mitochondrial pathway. The present study provides direct evidence for the role of mitochondria in HL60 cells apoptosis induced by ZnPc-PDT. Cytochrome c appeared in the cytosol 6 h following light activation of $ZnPcS_2P_2$. The main cytochrome c release occurred later, between 6 h and 12 h after PDT. However, caspase-3 could be quickly activated by ZnPc-PDT. Remarkable activity of caspase-3 was observed 6 h post-PDT in almost all cells. According to recent studies[28],

during genotoxic stress-induced apoptosis, the release of cytochrome c from mitochondria occurrs in 2 distinct stages. The early release of low levels of cytochrome c into the cytosol precedes the activation of caspase 9 and 3. The late stage cytochrome c release results in a drastic loss of mitochondrial cytochrome c and the activities of caspases contribute to the late cytochrome c release mentioned earlier. In the present study, PDT induced the early release of low levels of cytochrome c into the cytosol in the first 6 h. The cytochrome c then combined with procaspase-9 to form the "apoptosome". The apoptosome activated caspase-3 that induced the late cytochrome c release. Therefore, between 6 h and 12 h post-PDT, the main cytochrome c release occurred. At the time of cytochrome c release and remarkable caspase-3 activation (6 h after PDT), only a 12.6% fraction of the cells detected disruption of $\Delta\psi m$. The data are in agreement with the previous conclusion[19] that the dissipation of $\Delta\psi m$ is not required for the release of cytochrome c from the mitochondria. Also, the up-regulation of pro-apoptotic protein Bax, and the down-regulation of anti-apoptotic protein Bcl-2 by ZnPc-PDT appeared significantly and could result in activating mitochondria-mediated apoptosis. The results coincide with those of other previously published studies[25,29]. It is also reported that in many cell types, there is delicate coordination and cross talk between the extrinsic and intrinsic pathways that leads to the activation of the executioner caspase cascade[30,31]. Therefore, the study of the role of the death receptor pathway in the apoptosis induced by ZnPc-PDT should be pursued.

Thus, we conclude that the main mechanism for the apoptosis in HL60 cells induced by ZnPc-PDT appeared to be mitochondria-mediated pathway accompanied with cytochrome c release from the mitochondria into the cytosol followed by the activation of caspase-3. Our findings would provide a fundamental basis for the clinical application of ZnPc-PDT.

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