ZnPcS$_2$P$_2$-based Photodynamic Therapy Induces Mitochondria-dependent Apoptosis in K562 Cells

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Abstract Mitochondria play a key role in the regulation of apoptosis induced by numerous antitumor chemotherapeutic and other toxic agents. Photodynamic therapy (PDT) exerts significant cellular killing efficacy through either an apoptotic or necrotic cell death pathway. This study investigated the mechanism underlying the killing effects of a novel amphipathic photosensitizer [di-sulfonated di-phthalimidomethyl phthalocyanine zinc (ZnPcS$_2$P$_2$)]-mediated photodynamic therapy (ZnPcS$_2$P$_2$-PDT) on K562 cells. Apoptosis was evident in the post-PDT cells through the TdT-mediated dUTP nick end labeling (TUNEL) method and DNA fragmentation assay. After ZnPcS$_2$P$_2$-PDT, K562 cells underwent mitochondria-dependent apoptosis as evidenced by the release of cytochrome c from mitochondria into cytosol, accompanied by mitochondrial membrane potential ($\Delta\psi_m$) reduction, indicating the opening of the mitochondrial permeability transition pore (PTP). The activities of protease from the caspase family and caspase-3 were also significantly elevated. Furthermore, ZnPcS$_2$P$_2$-PDT down-regulated the expression of chimaeric Bcr-Abl oncoprotein, which is the molecular hallmark of chronic myelogenous leukemia (CML).

Key words photodynamic therapy; zinc phthalocyanine; apoptosis; mitochondria; K562 cell

Photodynamic therapy (PDT), a promising novel therapeutic method for the treatment of many tumors, utilizes a photosensitizer, visible light and oxygen to produce singlet oxygen and other reactive oxygen species (ROS), leading to lipid peroxidation, photo-oxidation of DNA guanine and damage to membranes, cytoskeletons and other sites, and eventual cell death [1–4]. Low-frequency electromagnetic field treatment changes the membrane permeation of photosensitizers and amplifies the photodynamic effect in the killing of human cancer cells [4–6]. Photosensitizers and light are, on their own, non-toxic, but are tumoricidal when combined. PDT may induce apoptotic or necrotic cell death in cancer cells in culture [7]. However, cell response to PDT varies according to the physical properties of the photosensitizer employed, PDT dose and the cell type [7–11]. A different subcellular localization of the photosensitizer results in a different response [9,10]. Even the same photosensitizer-mediated PDT can lead to a different outcome. At low doses of PDT, the cellular machinery for apoptosis is activated; however, higher doses lead to inhibition of apoptosis, with cell death taking place via a necrotic process [7,11].

The photosensitizer is a key element in PDT. According to previous studies [12,13], ZnPcS$_2$P$_2$ presents four advantages over conventional photosensitizers, such as hematoporphyrin derivatives (HpD) or other porphyrin derivatives. The first advantage is that the wavelength of suitable excited light for ZnPcS$_2$P$_2$ is 670 nm, which is good for tissue penetration. The second advantage is that ZnPcS$_2$P$_2$ has no obvious absorption in the visible part of the light spectrum compared with HpD, which leads to a greater extent of decreased skin phototoxicity from natural light. The third advantage is that the excited triplet state of ZnPcS$_2$P$_2$ has a larger quantum yield and a longer...
lifetime. The fourth advantage is that the amphipathic structure of ZnPcS₂P₂ results in the increase in the selective uptake of sensitizers by tumor cells. Our previous investigation has shown that leukemic cell lines exhibit a higher susceptibility to ZnPcS₂P₂-PDT than normal hematopoietic cells, and ZnPcS₂P₂-PDT can eliminate the residual K562 cells in normal bone marrow mononuclear cells [14]. However, the mechanism underlying the killing effects of ZnPcS₂P₂-PDT on leukemic cells is still unclear. In the present study, we investigated the K562 cell death pathway induced by ZnPcS₂P₂-PDT and the mechanism of its action.

Mitochondria play a central role in the control of apoptosis induced by PDT. It is universally accepted that PDT induces the mitochondrial permeability transition pore (PTP) to 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. When released from 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 and nuclear chromatin condensation, and cause cell death [15–25]. Furthermore, it is well known that the chimaeric gene bcr-abl is the molecular hallmark of chronic myelogenous leukemia (CML). The product of the gene is the 210 kDa protein, tyrosine kinase, which is expressed in more than 95% of haematopoietic cells of CML patients [26]. The chimaeric oncoprotein is associated with resistance to apoptosis [27,28].

In this study, we investigated the relationship between the cell death mechanism induced by ZnPcS₂P₂-PDT in K562 cells and the mitochondria-dependent apoptosis. We also detected the kinetic change in p210 expression post-ZnPcS₂P₂-PDT.

Materials and Methods

Cell line

Leukemia K562 cells obtained from the Shanghai Institute of Cytobiology (Institute of Chinese Academic Medical Science, Shanghai, China) were maintained in continuous culture in RPMI 1640 (Invitrogen, California, USA), supplemented with 10% (V/V) heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 mM L-glutamine, at 37 °C in 5% CO₂ 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

ZnPcS₂P₂, a gift from the Research Center of Functional Materials (Department of Chemistry, Fuzhou University, Fuzhou, China) was dissolved in a solution comprising 2% (V/V) Cremophor EL, 20% (V/V) propylene glycol and 0.9% (W/W) NaCl, and stored in the dark at 4 °C.

Photodynamic treatment

K562 cell suspensions (5×10⁶ cells/ml in RPMI 1640) were incubated with 0.25 µM ZnPcS₂P₂ for 5 h. Then the cell suspensions were photo-irradiated using an LD-670 semi-conductor laser (Laboratory of Laser Medicine, Tianjin Medical University, Tianjin, China) emitting red light at a wavelength of 670 nm. The power density at the illumination area was 53 mW/cm² and the total light dose was 2.1 J/cm². A solution control group (cells incubated with the same volume of solution for 5 h), a light control group (cells incubated with the same volume of solution for 5 h and then irradiated with a laser) and a photosensitizer control group (cells incubated with ZnPcS₂P₂ for 5 h without irradiation) were established. Thereafter, cells were harvested at 6, 12 and 24 h.

Apoptosis assay

The apoptosis was detected by observing the DNA fragmentation assay and TdT-mediated dUTP nick end labeling (TUNEL) assay. The DNA fragmentation assay was carried out as described by Wu et al. [29]. TUNEL staining was carried out according to the manufacturer’s instructions (Promega, Madison, USA). The procedure is carried out as follows. Biotinylated nucleotide is incorporated at the 3’-OH DNA ends using the Terminal deoxynucleotidyl transferase (rTdT) recombinant enzyme. Horseradish peroxidase-labeled streptavidin (streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown. The apoptotic cells were counted under the 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 manufacturer’s instructions (Biovision, Mountain View, USA). The results were then analyzed by flow cytometry.
The mitochondrial membrane potential (Δψm) detection kit utilized MitoCapture™, a cationic dye that fluoresces differently depending on whether cells are healthy or apoptotic. In healthy cells, MitoCapture accumulates and aggregates in the mitochondria, giving off a bright red fluorescence. In apoptotic cells, MitoCapture cannot aggregate in the mitochondria because of the altered Δψm, and thus remains in the cytoplasm in its monomer form, emitting green fluorescence. The fluorescent signal can be analyzed by the flow cytometry FITC (fluorescein) channel for green monomers and the propidium iodide (PI) channel for red aggregates. The assay for caspase activity is based on the cleavage of (aspartyl)-2-Rhodamine 110 (D2R), a reported substrate for members of the caspase family of proteases. D2R is non-fluorescent; however, upon cleavage of the substrate by cellular caspses, the released Rhodamine 110 gives rise to 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 is cell permeable, non-toxic and binds irreversibly to activated caspase-3 in apoptotic cells.

**Assay for cytochrome c**

Western blotting was performed according to previously published methods [30], with minor modification. Briefly, cell pellets were washed once with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (20 mM HEPES-KOH, 250 mM sucrose, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM dithiothreitol and 0.1 mM 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 12,000 g for 15 min at 4 °C, and the resulting supernatants were cytosol without mitochondria. The protein concentration of cell extracts was determined using a Bradford protein assay (Coomassie brilliant blue G-250 obtained from BBI (Toronto, Canada). 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 using a chemiluminescent detection system according to the manufacturer’s instructions (KPL, Gaithersburg, MD, USA). Essentially, the membranes were blocked in blocking buffer for 1 h at room temperature, and then incubated with the anti-human cytochrome C mouse monoclonal antibody (Neomarkers, Fremont, USA) 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 the 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 level of cytochrome c was obtained after normalization with the level of β-actin in the same lane.

**Assay for p210**

The positive expression rate of p210 was analyzed by flow cytometry. Briefly, cells were incubated first with the primary antibody against p210 (Coulter, California, USA) for 30 min and then incubated with the FITC-conjugated rabbit anti-mouse antibody for 30 min at 37 °C in the dark. The cells were then measured by a flow cytometer (BD) and the percentage of positive cells was determined. The contents of p210 were analyzed by Western blotting. To prepare lysates, cells were initially washed once with ice-cold PBS. Cell pellets were treated with lysis buffer [1% Nonidet P-40 (NP-40), 50 mM Tris-HCl, pH 8, 0.1% SDS, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.02% sodium azide] for 15 min on ice. The lysates were centrifuged at 12,000 g and 4 °C for 15 min. The protein concentration of cell extracts was determined by a Bradford protein assay, and then the detergent-soluble proteins were detected using the same procedure as that for cytochrome c. The intensities of the bands were determined using the mass ratio of p210 to the intrinsic control p120. The anti-human c-Ab1 monoclonal antibody was purchased from Santa Cruz (California, USA).

**Statistical analysis**

All data were presented as mean±SD of three independent experiments. Statistical significance was determined using Student’s unpaired two-tailed t-test (SPSS 10.0 software). P<0.05 was considered statistically significant.

**Results**

**Characteristics of apoptosis in K562 cells after ZnPcS₂P₂-PDT**

After ZnPcS₂P₂-PDT, K562 cells showed typical apoptotic features, such as cell shrinkage, chromatin condensation and nuclear fragmentation (data not shown). The ZnPcS₂P₂-PDT-induced apoptosis was monitored by oligonucleosomal DNA fragmentation, observable as DNA...
“ladders” upon agarose gel electrophoresis [Fig. 1(A)]. In contrast, cells not treated with ZnPcS2P2-PDT did not reveal any DNA ladders. Quantification of the number of K562 cells in apoptosis was accomplished by TUNEL staining analysis. At 6 h after PDT, 13.4% of the cells appeared to be in apoptosis. At 12 h and 24 h after PDT, the apoptosis rates were 28.8% and 42.6%, respectively [Fig. 1(C)], whereas no apoptotic cells were found in any of the cultures that were not exposed to PDT. Similar results were obtained when the apoptosis was monitored by cell cycle analysis. ZnPcS2P2-PDT can induce a progressive increase in the sub-G1 cell fraction (data not shown). The results indicate that ZnPcS2P2-PDT induces apoptosis in K562 cells in a time-dependent manner.

**Characteristics of mitochondria-dependent apoptosis in K562 cells after ZnPcS2P2-PDT**

At different time intervals after treatment, the cells were analyzed for $\Delta\psi_m$ and activities of caspase-family proteases and caspase-3. Less than 1% of the cells not treated with PDT were found to be positive for these three indexes. However, compared with the control groups, the positive levels of the three indexes in cells subjected to PDT increased remarkably ($P<0.01$). The changes in these three indexes are shown in Fig. 2. PDT caused a marked reduction of $\Delta\psi_m$ in 40.5% of the cells at 6 h. Thereafter, the fraction of cells with low $\Delta\psi_m$ increased gradually to about 62.4%. Caspase-3 was rapidly activated. At 6 h post-PDT, 100% of the cells displayed protease activities. With the increase of time, the mean fluorescence intensity also changed insignificantly (not shown). However, caspase-family proteases were activated comparatively slightly and slowly after PDT. After PDT, 30.6% of the cells showed total caspase activity. Thereafter, the fraction of cells with caspase activity increased to 48.6%. The Western blot analysis indicated the release of cytochrome c from the mitochondria into cytosol after PDT (Fig. 3). Increased levels of cytochrome c were notably detected in the cytosol after 6 h. Its release peak occurred between 6 h and 12 h post-PDT.

Putting together the results from different observations mentioned above, it can be concluded that ZnPcS2P2-PDT induces K562 cell apoptosis through mitochondria-dependent processes.

**Effects of ZnPcS2P2-PDT on the expression of p210**

As shown in Fig. 4(A), the expression rate of p210 was reduced after ZnPcS2P2-PDT treatment. The rates

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**Fig. 1** Results of DNA fragmentation assay and TUNEL staining

(A) Results of DNA fragmentation assay. 1–3, results of the 24 h treatment of the solution control, light control and photosensitizer control, respectively, where no DNA ladders can be seen; 4–6, results after 6 h, 12 h and 24 h of ZnPcS2P2-PDT, respectively, where characteristic DNA ladders appeared. (B) Results of TUNEL. Many apoptotic cells characterized with brown nuclei can be seen in the ZnPcS2P2-PDT groups. Magnification, 400×.

**Fig. 2** Changes in $\Delta\psi_m$ and activities of caspase and caspase-3 over time
decreased from approximately 99% to 53.7%. Western blot results also showed that ZnPcS2P2-PDT can down-regulate the expression of p210. At 24 h after PDT, the ratio of p210 to p120 was reduced from about 12.7 (control groups) to 2.5 [Fig. 4(B)].

Discussion

The cytotoxic efficacy of PDT relies on a bimodal protocol comprised of a chemical photosensitizer and light irradiation. Neither the photosensitizer nor the light alone is toxic [1,14]. The present study shows that no apoptotic phenomena were observed in K562 cells treated either with a photosensitizer only or light only. However, these two elements in combination induced apoptosis in K562 cells, which are particularly resistant to cell death via apoptosis, irrespective of the stimuli [31]. The results are consistent with the conclusion mentioned above.

For the first time, the present study provides direct evidence of the role of mitochondria in the apoptosis in K562 cells induced by ZnPcS2P2-PDT. Cytochrome c appeared in the cytosol 6 h following light activation of
ZnPcS\textsubscript{2}P\textsubscript{2}. The main cytochrome c release occurred later, between 6 h and 12 h after PDT. However, caspase-3 can be quickly activated by ZnPcS\textsubscript{2}P\textsubscript{2}-PDT. A remarkable level of caspase-3 activity was observed 6 h post-PDT in almost all cells. According to recent studies [32], the release of cytochrome c from mitochondria occurs in two distinct stages during genotoxic stress-induced apoptosis. The early release of low levels of cytochrome c into the cytosol precedes the activation of caspase-9 and caspase-3. The late-stage cytochrome c release results in a drastic loss of mitochondrial cytochrome c and the activities of the caspases contribute to the late cytochrome c release mentioned previously. In the present study, PDT induced the early release of low levels of cytochrome c into the cytosol in the first 6 h. Then the cytochrome c combined with procaspase-9 to form the "apoptosome". The apoptosome then activated the caspase-3 that induced the late cytochrome c release. Therefore, the main cytochrome c release occurred between 6 h and 12 h post-PDT. However, at the time of release of cytochrome c and caspase-3 activation (6 h after PDT), the disruption of \( \Delta \psi_m \) was detected only in 40.5\% of the cells. The data are in agreement with the previous conclusion [24] that dissipation of \( \Delta \psi_m \) is not required for the release of cytochrome c from mitochondria during the apoptosis in K562 cells induced by ZnPcS\textsubscript{2}P\textsubscript{2}-PDT.

It is well known that caspase-3 contributes to the overall caspase activity. However, in this study, the results show that when caspase-3 was highly activated at 6 h after irradiation, only 30\% of cells showed caspase activity. The reason for the inconsistency may be that the assay for caspase activity is based on the cleavage of a caspase substrate D\textsubscript{2}R, which is non-fluorescent, and the released Rhodamine 110 gives rise to fluorescence that can be measured. In contrast, the assay for active caspase-3 staining utilizes the caspase-3 inhibitor DEVD-FMK, which irreversibly binds to activated caspase-3, conjugated to FITC as the fluorescent in situ marker. Therefore, the method used for measuring caspase-3 activity is more sensitive.

The study also indicates that ZnPcS\textsubscript{2}P\textsubscript{2}-PDT can down-regulate the expression of p210 Bcr-Abl fusion protein, which inhibits apoptosis and plays an important role in the pathogenesis of CML. The data provide evidence that there may exist an inter-relationship between the apoptosis in K562 cells induced by ZnPcS\textsubscript{2}P\textsubscript{2}-PDT and the down-regulation of p210. It has been reported that Bcr-Abl tyrosine kinase is the target for caspase cleavage [33–35]. The role of caspase in the down-regulation of Bcr-Abl by ZnPcS\textsubscript{2}P\textsubscript{2}-PDT should be investigated.

In conclusion, the evidence provided by our data shows that mitochondria play a central role in the control of apoptosis induced by ZnPcS\textsubscript{2}P\textsubscript{2}-PDT by releasing cytochrome c into the cytosol and activating caspase-3. Our findings provide a fundamental basis for the clinical application of ZnPcS\textsubscript{2}P\textsubscript{2}-PDT.

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