### Original Article Chlorin A-mediated photodynamic therapy induced apoptosis in human cholangiocarcinoma cells via impaired autophagy flux

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Abstract: Background: Photodynamic therapy (PDT) is a promising strategy for multiple cancers. Chlorin e6 and its derivative 13<sup>1</sup>-[2'-(2-pyridyl)ethylamine] Chlorin e6 (Chlorin A) are effective photosensitizers, although their cytotoxic mechanisms have not yet been fully characterized. Methods: Cell viability and apoptosis were evaluated by CCK8 assay, TUNEL assay, and Annexin V/PI staining. The expression levels of different proteins were analyzed by Western blot analysis and immunofluorescence. The crosstalk between autophagy, endoplasmic reticulum stress (ERS), and mitochondrial dysfunction was investigated using reactive oxygen species (ROS) scavenger N-acetyl cysteine (NAC), PERK inhibitor GSK2606414, autophagy inhibitor 3-MA, and mitochondrial stabilizer elamipretide. Furthermore, the extent of ROS production, lysosomal damage, autophagy flux, and mitochondrial membrane potential (MMP) were tracked using established probes. An in vivo xenograft model of cholangiocarcinoma (CCA) was established in BALB/c-nude mice by inoculation with EGI-1 cells, and Chlorin A was administered topically or intravenously, followed by light irradiation. Results: Chlorin A-PDT decreased the viability of CCA cells and induced apoptosis. Intriguingly, Chlorin A-PDT promoted autophagy via activation of ROS-induced ERS-related PERK/p-eif2α/CHOP axis, and blocked the ensuing autophagy flux by lysosomal damage. The PERK inhibitor GSK2606414 and NAC alleviated apoptosis and autophagy induced by Chlorin A-PDT. Furthermore, mitochondrial dysfunction aggravated ERS, and stabilizing the mitochondria reduced both apoptosis and autophagy. Finally, Chlorin A-PDT significantly reduced tumor growth in vivo. Conclusions: Chlorin A-PDT induced apoptosis in CCA cells by initiating autophagy and impaired the autophagy flux via ROS-mediated ERS and lysosomal damage.

Keywords: Chlorin, PDT, autophagy, endoplasmic reticulum stress, ROS

#### Introduction

Cholangiocarcinoma (CCA) is the second most common type of liver tumor, which originates from the bile duct epithelium [1]. It accounts for about 3% of all gastrointestinal cancers diagnosed worldwide [1, 2]. However, due to an asymptomatic early stage, CCA is usually diagnosed at an advanced stage when the 5-year survival rate is a dismal 10% [2]. In general, surgery is the best treatment option for early stage CCA and liver transplantation for unresectable CCA [3-5]. Traditional chemotherapy involving gemcitabine and cisplatin can be employed for patients unqualified for either surgical option, but is associated with serious side effects. Therefore, novel strategies are currently being explored to destroy cancer cells with minimal side effects. Photodynamic therapy (PDT) can improve the quality of life of cancer patients and prolong their survival [6, 7]. It involves light, oxygen, and a photosensitizer, which are non-toxic when used alone, but generate the highly toxic singlet oxygen free radical when used as a combined treatment modality, eventually lead-ing to cancer cell death [8-11].

Type II programmed cell death or autophagy is a self-catabolic process in which a cell degrades its own organelles and proteins [12]. Its

function in cancer is uncertain, and it can promote or inhibit tumor cell proliferation depending on the progression stage and stress conditions. Studies show that PDT can trigger both autophagy and apoptosis, although the role of the former during PDT depends on the photosensitizer [13, 14]. Therefore, a better understanding of the role of autophagy in cancer will enable the development of more efficient therapies to treat cancer. Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) lumen activates the unfolded protein response (UPR), which in turn leads to ER stress (ERS) and apoptosis. ERS is driven by the proapoptotic transcription factor CCAAT/enhancer binding mediated protein homologous protein (CHOP) via PERK/eIF-2α or IRE-1α/JNK, and aggravated ERS can induce autophagy and cell death or promote cell survival [15]. Therefore, a link between ERS and autophagy may also be relevant in anti-cancer therapy.

The hydroporphyrin photosensitizer Chlorin A localizes to the mitochondria, which is also the main site of ROS production during PDT [16]. The mitochondrial dysfunction resulting from high levels of ROS also activates the intrinsic apoptosis pathway, aggravates ERS, and may also trigger autophagy in cancer cells. Apart from the mitochondria, Chlorin A has also been detected in the lysosomes and endoplasmic reticulum, which indicates multiple possible mechanisms of Chlorin A-mediated PDT. Our previous study showed that Chlorin A-PDT effectively killed esophageal cancer cells both in vitro and in vivo [16]. In this study, we showed for the first time that Chlorin A-PDT not only induced cell death by initiating autophagy via ROS-mediated ERS and mitochondria dysfunction, but also blocked the autophagy flux via lysosome damage. Thus, our findings provide novel insight into anti-cancer mechanisms of PDT.

### Materials and methods

### Reagents

The stock solution of  $13^{1}$ -[2'-(2-pyridyl) ethylamine] Chlorin e6 (Chlorin A) was prepared in DMSO and sterilized by filtering through a 0.22-µm membrane. Temoporfin, GSK26064-14, N-acetylcysteine (NAC), 3-methyladenine (3-MA) and Elamipretide were purchased from MedchemExpress (Monmouth Junction, NJ, USA), and 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was from Sigma-Aldrich (St. Louis, MO, USA). The maximum DMSO concentration used was less than 1%. The Annexin V-FITC/PI Apoptosis detection kit was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Antibodies against cleaved-Caspase-3, BIP, CHOP, actin and Beclin-1 were purchased from Protein tech (Chicago, IL, USA), and those targeting LC3B-I/II, C-PARP, mTOR, P-mTOR, AKT, P-AKT, EIF2 $\alpha$ , P-EIF2 $\alpha$ , PERK, and P-PERK from Cell Signaling Technology (Beverly, MA, USA).

### Cell lines

The human liver bile duct carcinoma cell lines HuCCt1 and EGI-1 were respectively purchased from Japanese Collection of Research Bioresources Cell Bank, and the German Collection of Microorganisms and Cell Cultures. HuCCt1 cells were cultured in RPMI-1640 (Hyclone, Logan, UT, USA) and the EGI-1 cells in DMEM (Hyclone, Logan, UT, USA), each supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Both lines were incubated in a humidified atmosphere containing 5%  $CO_2$  at 37°C.

### Cell viability assay

Hucct1 and EGI-1 Cells were seeded in a 96well plate at the density of  $1 \times 10^4$  cells/well and incubated for 12 h. Following incubation with different drug concentrations (0.125, 0.25, 0.5, 1, and 2 µM) and treated with PDT after certain time points (3, 6, 12, and 24 h), the proportion of viable cells were evaluated using the Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan) according to the manufacturer's instructions. The absorbance of the media was measured at 450 nm on a microplate reader, and cell viability (%) was calculated as  $OD_{treatment}/OD_{control} \times 100\%$ .

### Photodynamic therapy

The cells were divided into the untreated control, drug-treated (only Chlorin A), light-treated (only light without Chlorin A), and PDT (Chlorin A with light) groups. Temoporfin was used as the control to assess the efficacy of Chlorin A. Then, the cells were incubated with Chlorin A and treated with PDT. Semiconductor lasers (664 nm and 652 nm) were used as the light source for PDT at 9 mW/cm<sup>2</sup>. The total dose (J/  $cm^2$ ) was calculated as the fluence rate (mW/  $cm^2$ ) × treatment duration (s).

### TUNEL staining

TUNEL staining was performed using the One Step TUNEL Apoptosis Assay kit according to the manufacturer's instructions (Beyotime, Shanghai, China). Briefly, the HuCCt1 cells were seeded in 24-well plates, allowed to adhere overnight, and treated as described above. The drug and energy doses were determined according to their respective IC50 values calculated from the cell viability experiment. After washing once with PBS, the cells were fixed with 4% paraformaldehyde and washed twice. Next, the cells were permeabilized with Enhanced Immunostaining Permeabilization Buffer (Beyotime, Shanghai, China) provided in the kit for 5 minutes at room temperature, and washed twice with PBS. After incubating with the TUNEL reagent for 1 h at room temperature, cells were washed twice with PBS, counterstained with DAPI, and observed under a fluorescence microscope.

### Annexin V/PI staining

HuCCt1 and EGI-1 cells were seeded in a 6-well plate at the density of  $3 \times 10^5$  cells/well, cultured overnight, and treated appropriately. The harvested cells were then stained with the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay kit (Becton Dickinson) and analyzed by flow cytometry.

### Transmission electron microscopy

After treatment with Chlorin A-PDT for 12 h, HuCCt1 cells were harvested and fixed overnight at 4°C with 2.5% glutaraldehyde. Then, cells were treated with 1% buffered osmium tetroxide for 1 h at 4°C. Dehydrated cells were embedded and stained both with uranylacetate. Representative areas were chosen for ultrathin sectioning and cells were visualized by transmission electron microscopy (TEM).

# Mitochondrial membrane potential assessment

HuCCt1 cells were seeded in a 6-well plate at the density of 3  $\times$  10<sup>5</sup> cells/well and treated using 0.25  $\mu M$  Chlorin A and/or 0.48 J/cm²

light after 12 h of incubation. The cells were harvested and washed twice with PBS, mixed with 500  $\mu$ I JC-1 working solution, and vortexed. After incubating at 37°C for 15 min, cells were washed twice with 1 × assay buffer, resuspended in the buffer, and analyzed by flow cytometry and the FlowJo VX software (Becton Dickinson, Franklin Lakes, NJ, USA).

### Western blot analysis

Proteins were extracted from the suitably-treated cells using the RIPA lysis buffer (Beyotime, Shanghai, China), and resolved by SDS-PAGE. The protein bands were transferred to polyvinylidene fluoride (PVDF) membranes that were then blocked for 1 h at room temperature with 5% non-fat milk. After incubating overnight with the primary antibodies at 4°C, the blots were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibody. The positive bands were visualized using an ECL detection kit (Millipore, Burlington, MA, USA), and gray values were analyzed using Image J software. Experiments were performed in triplicate.

### Measurement of ROS levels

HuCCt1 cells were seeded in 6-well plates at a density of  $3 \times 10^5$  cells/well, cultured overnight, and treated with 0.25 uM Chlorin A and/or 0.48 J/cm<sup>2</sup> light. Cells were incubated with 25  $\mu$ M DCFH-DA staining solution (Sigma-Aldrich, St. Louis, MO, USA) for 20 min at 37 °C in the dark. The medium was discarded and the cells were washed twice with cold PBS. ROS levels were analyzed by fluorescence microscope and flow cytometry, and fluorescence intensities were quantified using FlowJo VX software.

### Immunofluorescence

Cells grown on coverslips in a glass-bottomed culture dish were exposed to Chlorin A-PDT for 12 h at the aforementioned doses. Cells were then fixed with 4% paraformaldehyde for 15 min, washed three times with PBS, permeabilized with 0.5% Triton 100 for 20 minutes, and washed again. After blocking with 3% BSA for 30 minutes at 37°C, cells were incubated with primary antibodies according to the recommended concentrations by the manufacturers for 12 h at 4°C, followed by the secondary antibodies for 1 h at 37°C. To track the autophagy flux, cells were transfected with GFP-RFP-LC3 for 24 h, then subjected to Chlorin A-PDT for another 12 h. The nuclei were counterstained with DAPI for 5 min, and images of the stained cells were captured using a fluorescence microscope and confocal microscope (Leica, Wetzlar, Germany).

### Cell cycle analysis

HuCCt1 cells were subjected to Chlorin A-PDT for 12 h, harvested, and fixed overnight with 75% ice-cold alcohol at 4°C. After centrifuging and washing twice with PBS, the cells were incubated with PI/Rnase staining solution for 30 min at 4°C in the dark. The cells were acquired on a flow cytometer (BD, C6) and the cell cycle phases were analyzed using the Modifit Lt 5.0 software.

### Establishment of xenograft model

The in vivo anti-tumor effects of Chlorin A were analyzed in 4-6 weeks old male nude mice (Shanghai Laboratory Animal Center of the Chinese Academy of Sciences, Shanghai, China). Experiments were approved by the Ethics Committee of the Shanghai General Hospital, HuCCt1 cells were subcutaneously injected into the right flanks of the mice, which were then divided into the untreated control, intravenous and topical groups (6 mice each). Once the tumor volume reached 50-100 mm<sup>3</sup>, the mice were either injected or locally smeared with Chlorin A (2 mg/kg), followed by PDT. The treatment was repeated one week later. The tumor dimensions were measured every two days, and the volume (TV) was calculated as  $1/2 \times (\text{length} \times \text{width} \times \text{width}).$ 

### Histopathological analysis

The mice were sacrificed 12 Days after the treatment, and the tumors and major organs were dissected and fixed in 4% paraformaldehyde. After dehydration in an automated apparatus, the fixed tissues were embedded with paraffin and cut into 4  $\mu$ m-thick sections. The organs were stained with hematoxylin and eosin as per standard protocols, and the tumor tissues were subjected to TUNEL assay. Briefly, the sections were dewaxed, hydrated through an ethanol gradient, and incubated with 20  $\mu$ g/ml proteinase K for 30 minutes. After washing twice with PBS, the TUNEL reagent was added and the sections were incu-

bated for 1 hour in the dark. The stained sections were washed again with PBS and observed under a fluorescence microscope (Leica, Wetzlar, Germany).

### Statistical analysis

Data were presented as the mean  $\pm$  standard deviation (SD), and analyzed using GraphPad Prism 6 software (GraphPad Software Inc, La Jolla, CA, USA). Student's t-test, Fisher's Exact test, and one-way ANOVA were used for comparing different groups as appropriate. *P*<0.05 was considered statistically significant.

### Results

# Chlorin A-PDT decreased CCA cell viability via apoptosis and cell cycle arrest

The viability of human CCA cell lines exposed to Chlorin A and light decreased in a dose dependent manner. The half-maximum inhibitory concentration of Chlorin A in the HuCCt1 and EGI-1 cells were respectively 0.25 µM and 0.5 µM in the presence of 0.48 J/cm<sup>2</sup> light, and were therefore used for the subsequent experiments (Figure 1A). Furthermore, Chlorin A was more potent compared to the classical photosensitizer temoporfin (Figure 1B). Chlorin A-PDT significantly increased the levels of apoptosisrelated proteins such as BAX, C-caspase 3 and C-PARP (Figure 1C), as well as the percentage of apoptotic cells (Figure 1D and 1F) compared to the untreated control (P<0.05), whereas no obvious changes were observed the cells treated with either Chlorin A or light. Furthermore, the proportion of cells in the GO/G1 phase increased significantly following Chlorin A-PDT, resulting in a concomitant decrease in the cells in G2/M phase (Figure 2A and 2B), while the cell cycle was not altered in the untreated control or light/Chlorin A-treated groups. Consistent with this, the key GO/G1 checkpoint factors Cyclin D1 and CDK2 were significantly downregulated in cells receiving Chlorin A-PDT in a time-dependent manner, while p21 was upregulated (Figure 2C). Taken together, Chlorin A-PDT inhibited proliferation of human CCA cells by inducing apoptosis and cell cycle arrest.

# The apoptotic effect of Chlorin A-PDT was dependent on autophagy

To determine whether Chlorin A-PDT also resulted in autophagy, we next analyzed the expres-



**Figure 1.** Chlorin A-PDT inhibited proliferation and induced apoptosis in human CCA cells. A. The cell viability of HuCCt1 cells and EGI-1 cells was decreased following incubation with 0-2  $\mu$ M Chlorin A for 12 h and irradiation with 0-2 J/cm<sup>2</sup> light in a dose dependent manner. B. The viability of HuCCt1 cells treated with Chlorin A-PDT was significantly lower compared to cells treated with temoporfin-PDT (0-2  $\mu$ M). C. Chlorin A-PDT increased the protein expression of C-PARP and C-caspase 3 in HuCCt1 cells. D. The percentage of TUNEL+ cells was significantly increased after treatment with Chlorin A-PDT. E, F. The percentage of apoptotic cells that were positive for Annexin V/PI was significantly increased after treatment with Chlorin A-PDT. \*P<0.05 compared to untreated controls.

sion levels of autophagy-related proteins, and observed significant upregulation of LC3B-II/I

and Beclin 1 following Chlorin A-PDT compared to the control groups. Furthermore, Chlorin



**Figure 2.** Chlorin A-PDT induced GO/G1 cell cycle arrest in CCA cells. A. In HuCCt1 cells, Chlorin A-PDT treatment increased the DNA content the in GO/G1 phrase. B. Histogram showing the percentage of cells in the different cell cycle phases. C. Immunoblot showing the expression levels of cell cycle-related proteins p21, CDK2, CDK4, and Cyclin D1. Data are presented as the mean  $\pm$  SD (n=3). \*P<0.05 vs the control group, #P<0.05 vs the light-treated group.

A-PDT also increased the phosphorylation of AKT and mTOR in a time dependent manner. indicating initiation of autophagy (Figure 3A and 3B). In addition, TEM analysis showed that significantly more autophagosomes were present in the Chlorin A-PDT-treated group compared to the Control group (Figure 3C). Since autophagy can be both pro- and anti-survival, we examined the effect of inhibiting autophagy in the cells exposed to Chlorin A-PDT. Pretreatment with 3-MA abrogated the anti-proliferative and apoptotic effects of Chlorin A-PDT (Figure 3D and 3E), and decreased the proportion of LC3B-II/I as well as Beclin-1 and C-caspase 3 levels (Figure 3F). In conclusion, Chlorin A-PDT induced autophagy in CCA cells via the AKT/mTOR pathway, which was essential for apoptosis.

# Chlorin A-PDT impaired the formation of autophagolysosomes

To further explore the effect of Chlorin A-PDT on autophagy, we analyzed the levels of the ubiquitin-binding scaffold protein p62 and the

lysosomal protein LAMP1 in the suitably treated cells. Chlorin A-PDT significantly increased endogenous p62 protein levels in a timedependent manner, and markedly decreased that of LAMP1 (Figure 4A), indicating a decrease in autophagy flux. To validate these findings, we directly monitored the autophagy flux in the Chlorin A-PDT exposed cells using RFP-GFP-tagged LC3, and observed numerous autophagosomes (yellow dots) but very few autophagolysosomes (red dots) (Figure 4B). Consistent with these data, Lysotracker Red indicated significantly fewer lysosomes in the Chlorin A-PDT compared to the untreated group (Figure 4C). Taken together, Chlorin A-PDT decreased the autophagy flux in CCA cells by inhibiting the formation of autophagolysosomes and lysosomal damage.

### Chlorin A-PDT triggered the ERS-related PERK/ EIF2α/CHOP pathway

To elucidate the underlying mechanisms of the cytotoxic effects of Chlorin A-PDT in CCA cells, we analyzed the expression levels of ESR-re-



**Figure 3.** Chlorin A-PDT-induced apoptosis is dependent on autophagy. A. Immunoblot showing levels of LC3B, Beclin 1, p-AKT, AKT, p-mTOR and mTOR in CCA cells subjected to Chlorin A-PDT. B. Representative fluorescence images showing significant accumulation of LC3 in HuCCt1 cells treated with Chlorin A-PDT. C. Representative TEM micrographs showing accumulation of autophagosomes in CCA cells after Chlorin A-PDT. Arrows indicate autophagosomes, and arrowheads indicate ER. D. Pre-treatment with 5 mM 3-MA alleviated the decreased cell viability in HuCCt1 cells that underwent treatment with Chlorin A-PDT. E. Pre-treatment with 5 mM 3-MA alleviated the increase in cell apoptosis in HuCCt1 cells that were treated with Chlorin A-PDT. Data was derived from three independent experiments. F. Immunoblot showing levels of C-caspase 3, LC3B and Beclin-1 in HuCCt1 cells pre-treated with 5 mM 3-MA followed by Chlorin A-PDT. \*P<0.05 vs the control group, #P<0.05 vs the PDT-treated group.



Figure 4. Chlorin A inhibited autophagy flux in CCA cells. A, B. Immunoblot showing P62 and Lamp1 levels in HuCCt1 and EGI-1 cells after Chlorin A-PDT. C. Representative fluorescence images of HuCCt1 cells transiently transfected with GFP-tagged LC3 followed by Chlorin A-PDT. D. Representative fluorescence images of decreased lysosomes in Hucct1 cells after treatment with Chlorin A-PDT.



Figure 5. Chlorin A-mediated autophagy and apoptosis was dependent on endoplasmic reticulum stress. A. Immunoblot showing levels of endoplasmic reticulum stress (ERS)-related proteins including PERK, p-PERK, BIP, EIF2 $\alpha$ , p-EIF2 $\alpha$ , and CHOP in HuCCt1 cells after treatment with Chlorin A-PDT. B. Pre-treatment with 2  $\mu$ M GSK2606414 alleviated the cell viability in HuCCt1 cells that was decreased after treatment with Chlorin A-PDT. C, D. Pre-treatment with 2  $\mu$ M GSK2606414 alleviated the cell apoptosis in HuCCt1 cells that was increased after treatment with Chlor rin A-PDT. Data was obtained from three independent experiments. E. Immunoblot showing the levels of apoptosis, autophagy and ER stress-related proteins, including C-caspase-3, LC3B, Beclin-1, p-PERK, BIP, p-EIF2 $\alpha$ , and CHOP in HuCCt1 cells that were treated as indicated above. \*P<0.05 vs the control group, #P<0.05 vs the PDT-treated group.

lated proteins, including p-PERK, p-EIF2 $\alpha$ , CH-OP, and BIP. In the cells subjected to Chlorin A-PDT, the expression levels of these proteins increased significantly compared to the untreated controls in a time-dependent manner (**Figure 5A**). Furthermore, blocking the PERK/ EIF2 $\alpha$ /CHOP pathway using the PERK inhibitor GSK2606414 significantly attenuated the inhibitory effect of Chlorin A-PDT on HuCCt1 cells and reduced the apoptosis rate (Figure 5B-D). Consistent with this, inhibition of the PERK/EIF2 $\alpha$ /CHOP pathway also downregulated C-caspase 3, BAX, LC3B-II and Beclin-1 levels in the combination treatment group (Figure 5E). Therefore, Chlorin A-PDT activated the ER stress-related PERK/p-EIF2 $\alpha$ /CHOP pathway to induce apoptosis and autophagy in the human CCA cells.



Figure 6. Chlorin A-PDT triggered the production of reactive oxygen species in CCA cells. A. Representative fluorescent images showing HuCCt1 cells expressing the oxidized DCFH-DA probe. B. Bar graph showing the mean fluorescence intensity of DCFH-DA. Data are presented as the mean  $\pm$  SD from three independent experiments. C. Pre-treatment with 5  $\mu$ M NAC alleviated cell viability in HuCCt1 cells that was decreased after treatment with Chlorin A-PDT. D. Pre-treatment with 5  $\mu$ M NAC alleviated the cell apoptosis in HuCCt1 and EGI-1 cells that was increased by treatment with Chlorin A-PDT. E. Immunoblots showing the levels of C-caspase 3, LC3B, Beclin-1, p-PERK, p-EIF2 $\alpha$ , and CHOP in HuCCt1 cells that were treated as described above. \**P*<0.05 vs the control group, #*P*<0.05 vs the PDT-treated group.

### Chlorin A-PDT induces ROS production in CCA cells

Several studies have implicated ROS production as the mechanistic basis of PDT. In agreement with previous findings, we demonstrated that Chlorin A-PDT significantly increased the fluorescence intensity of the oxidized ROS probe DCFH-DA compared to the untreated control (**Figure 6A** and **6B**), indicating significant ROS production. To further explore the causative role of ROS in PDT-induced apoptosis, autophagy and ER stress, we treated cells with the ROS scavenger NAC prior to Chlorin A-PDT. NAC not only restored cell viability but also alleviated apoptosis (**Figure 6C** and **6D**) induced by Chlorin A-PDT. Furthermore, pretreatment with NAC significantly inhibited the p-PERK/p-EIF2/CHOP axis as well as C-caspase-3 and Bax expression levels in Hucct1 cells (**Figure 6E**). Therefore, Chlorin A-PDT increased ROS production which, in agreement with previous studies, induced the UPR/ERS pathway eventually leading to apoptosis and autophagy [13].



Figure 7. Chlorin A-PDT decreased the mitochondrial membrane potentialin CCA cells. A. Representative fluorescence images showing JC-1 stained HuCCt1 cells after Chlorin A-PDT. B. Flow cytometry plots indicating mitochondrial membrane potential (MMP) regarding JC-1 intensity. C. Immunoblot showing levels of C-caspase-3, LC3B, Beclin-1, P-EIF2 $\alpha$ , and CHOP in HuCCt1 cells that were treated with 2  $\mu$ M Ela before Chlorin A-PDT. D. Pre-treatment with 2  $\mu$ M Ela alleviated the cell viability in HuCCt1 cells that was decreased after treatment with Chlorin A-PDT. Data are presented as the mean ± SD from three independent experiments. \**P*<0.05 vs the control group, #*P*<0.05 vs PDT-treated group.

# Chlorin A-PDT activated the mitochondrial apoptosis pathway

ROS is primarily produced in the mitochondria and Chlorin A also localizes to this organelle, therefore, we next analyzed the indices of mitochondrial dysfunction. The JC-1 fluorescent probe showed a significant reduction in the red/green fluorescence ratio in the Chlorin A-PDT group compared with the control groups (P<0.05, **Figure 7A** and **7B**), indicating loss of MMP. Furthermore, the mitochondrial stabilizer elamipretide decreased the expression levels of C-caspase 3, LC3bII, Beclin 1, CHOP, and BIP in the combination treated cells (**Figure 7C**), and restored cell viability (**Figure 7D**). Taken together, Chlorin A-PDT disrupted the MMP and triggered the intrinsic apoptosis pathway.

### Chlorin A-PDT inhibited tumor growth in vivo

To evaluate the effect of Chlorin A-PDT *in vivo*, we established a tumor xenograft model and administered Chlorin A either intravenously or topically. Both delivery methods significantly inhibited the growth of CCA, as indicated by



Figure 8. PDT inhibited growth of CCA cells *in vivo*. A. Tumor volume in the PDT group was significantly lower compared with that in the control group and showed a time-dependent change in a xenograft tumor model. B. Representative images of TUNEL-stained tumor tissues showing apoptotic cells. C. Immunoblot showing levels of Beclin 1, C-caspase 3, LC3B and BIP in xenograft tumor tissues from the different groups. D. Representative HE-stained images of the heart, liver, spleen, lung and kidney tissues (200 × magnification).



Figure 9. Proposed mechanism of Chlorin A-PDT-induced apoptosis and autophagy in cholangiocarcinoma cells.

the time-dependent decrease in tumor volume (Figure 8A). Furthermore, TUNEL staining showed significant apoptosis in tumor tissues of the Chlorin A-PDT-treated group compared to the control group (Figure 8B), which was consistent with the increased expression levels of pro-apoptotic proteins in the Chlorin A-PDT treated mice (Figure 8C). Finally, HE staining of major organs showed that no obvious pathological changes induced by the Chlorin A (Figure 8D). Taken together, Chlorin A-PDT effectively inhibited CCA growth in vivo without any systemic adverse effects. Figure 9 showed Chlorin A-mediated photodynamic therapy induced apoptosis in human cholangiocarcinoma cells via impaired autophagy flux.

### Discussion

PDT is an effective alternative for patients who are ineligible for the standard therapeutic modalities against CCA, such as surgery, liver transplantation, and chemotherapy. The photosensitizer Chlorin e6 and its novel derivative Chlorin A have shown encouraging results against CCA [17]. In this study, we explored the effect of Chlorin A-PDT on CCA cells and related mechanisms. We demonstrated that Chlorin A was more potent than temoporfin, a photosensitizer routinely used to treat head and neck cancer, and also proved to be effective against CCA [18-20], regarding decreasing cancer cell viability. Furthermore, Chlorin A-PDT increased the rate of both early and late apoptosis in human CCA cell lines.

Increasing evidence has shown that multiple photosensitizers can initiate autophagy [21, 22]. Chlorin A-PDT significantly increased the levels of the phagophore membrane elongating protein LC3-II and the autophagosomal protein Beclin 1, clearly indicating the induction of autophagy. Furthermore, the autophagymediating pathway PI3K/AKT/ mTOR was also activated by Chlorin A-PDT. Autophagy has a dual role in cancer since it can promote both cell survi-

val and cell death depending on the stimulus. In our study, we found that blocking autophagy by 3-MA, an inhibitor of LC3BI to LC3BII conversion, alleviated apoptosis induced by Chlorin A-PDT. These findings strongly suggested that the CCA cell death induced by Chlorin A-PDT was dependent on autophagy. During the autophagy flux, the autophagosomes fuse with lysosomes for further degradation. Chlorin A-PDT blocked the autophagy flux as indicated by fewer autophagolysosomes and P62 accumulation, likely via lysosomal damage since the mature lysosome marker LAMP1 was also significantly decreased. The inhibition of autophagic flux eventually resulted in cell death [15]. Therefore, Chlorin A-PDT caused apoptosis of the CCA cells by initiating autophagy, followed by blocking the autophagy flux. Our findings are consistent with a previous study on the effects upconversion nanoparticle encapsulated chlorine e6 [14]. In contrast, two other studies using chlorine e6-PDT reported a pro-survival effect of the photosensitizer, which could be attributed to different target cells or mechanisms of action [22, 23]. Therefore, further research is needed to determine the role of autophagy in PDT mediated by different photosensitizers in order to achieve better clinical outcomes.

Since Chlorin A can localize to the ER, we hypothesized that Chlorin A-PDT damaged the ER structure and induced ERS. This process is accompanied by the activation of BIP, an early ERS marker that initiates the UPR pathway, and CHOP which is upregulated during severe ERS through the IRE1- and PERK signal pathways and triggers apoptosis. PERK is a type I transmembrane ER protein that inhibits mTORC1 activity and initiates autophagy under stress by activating the downstream eukaryotic initiation factor 2 alpha (eIF2α). Chlorin A-PDT induced ERS in a time-dependent manner, and inhibition of PERK with GSK2606414 blocked the UPR pathway and attenuated both apoptosis and autophagy. Thus, we concluded that the apoptosis and autophagy induced by Chlorin A-PDT were downstream of ERS.

ROS is the mechanistic basis of PDT, and intracellular ROS production was significantly increased in CCA cells following Chlorin A-PDT treatment. In a previous study, it was shown that the ROS scavenger NAC partly inhibited PDT-induced autophagy [15]. Consistent with this, NAC significantly decreased the levels of autophagy-related proteins and inhibited the PERK/EIF2α/CHOP axis in the CCA cells subjected to Chlorin A-PDT. These findings suggested that the ER stress induced by PDT is partially dependent on ROS. Since Chlorin A also localizes to the primary ROS producer mitochondria [24, 25], we next investigated whether Chlorin A-PDT induced the intrinsic apoptotic pathway through mitochondrial dysfunction. The MMP of the treated CCA cells decreased significantly as indicated by the JC-1 probe [26], and the cytochrome c and C-caspase 3 levels were upregulated. Mitochondrial depolarization released cytochrome c, which activated caspase-3 and eventually triggered the apoptotic cascade [27, 28]. The mitochondrial stabilizer elamipretide attenuated the effects of Chlorin A-PDT, indicating that the latter was dependent on mitochondrial dysfunction. Finally, Chlorin A-PDT effectively inhibited CCA growth in vivo, with more potent effects seen with topical as opposed to intravenous administration, without any adverse effects. Consistent with the in vitro findings, Chlorin A-PDT increased the number of apoptotic cells in the tumor tissues. Taken together, our findings indicated that Chlorin A was an effective and safe photosensitizing agent for PDT against CCA.

### Conclusions

Chlorin A-PDT inhibited CCA cell growth by inducing apoptosis and cell cycle arrest. Mechanistically, Chlorin A-PDT initiated autophagy and blocked the ensuing autophagy flux, resulting in apoptosis. Furthermore, it also induced ERS and mitochondrial dysfunction that further aggravated autophagy and apoptosis. Our findings provide novel insight into the biological effects of Chlorin A-PDT in cholangiocarcinoma.

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### Disclosure of conflict of interest

None.

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