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Mitochondria-anchoring and AIE-active photosensitizer for self-monitored cholangiocarcinoma therapy†

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The morbidity of extrahepatic cholangiocarcinoma (CCA) has increased markedly in the past few decades. However, most patients with extrahepatic CCA are diagnosed at an advanced stage and thus cannot receive curative resection in time. To increase the long-term survival probability of nonresectable extrahepatic CCA patients, it is highly desirable to develop alternative treatment strategies. Photodynamic therapy is a highly efficient method for the ablation of cancer cells, but the conventional photosensitizers (PSs) suffer from self-quenching after local accumulation in organelles. To tackle this challenge, we herein develop a mitochondria-anchoring photosensitizer, **TTVPHE**, with aggregation-induced emission (AIE) characteristics for efficient ablation of extrahepatic CCA cells through the mitochondrial injury pathway. The **TTVPHE** probe shows significant advantages in terms of fast cell penetration ability, selective mitochondria-targeting ability, and excellent ROS generation ability under white light irradiation. Moreover, the probe can *in situ* monitor the mitochondrial injury process by translocation from mitochondria to the nuclear membrane. The ultrastructural changes in the morphology of mitochondria under PDT were also characterized using high resolution TEM. This AIE-active probe is promising for the treatment of advanced stage CCA.

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Introduction

Cholangiocarcinoma (CCA) is a malignancy originating in the biliary tree and the morbidity has increased markedly in the past few decades.¹ Surgical resection is the only curative therapy for all subtypes of CCA in those who are good surgical candidates.² However, approximately 80% of CCA patients belong to the extrahepatic subtype and easily lose the opportunity for surgical excision due to the aggressiveness and late

diagnosis of the extrahepatic subtype.^{3,4} Moreover, chemotherapy or radiotherapy in the clinic cannot increase the long-term survival of patients with nonresectable extrahepatic CCA.⁵ Therefore, the development of new therapy methods to improve the survival rate and life quality of extrahepatic CCA patients is an imperative task.

Photodynamic therapy (PDT) is a palliative and locoregional ablative treatment for extrahepatic CCA, and the first case of successful PDT for nonresectable CCA was reported by McCaughan's group in 1991.⁶ To date, many clinic trials have demonstrated that PDT combined with biliary decompression can improve stent patency, survival, and quality of life for patients with nonresectable CCA.^{7–11} The short life time of reactive oxygen species (ROS) requires the photosensitizers (PSSs) to be accumulated at desired sites.^{12,13} However, the conventional PSSs, such as porphyrins and phthalocyanines, feature discotic structures and suffer from poor ROS generation efficiency at a high local concentration.^{14–16} In contrast with the traditional PSSs, the aggregation-induced emission fluorogens (AIEgens) with rotor structures have significant advantages in terms of high emission efficiency and photodynamic activity in the aggregate state.^{17–25}

Mitochondria are the cell's energy sources, and mitochondria-targeted PSSs could greatly improve the efficiency of photodynamic

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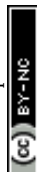
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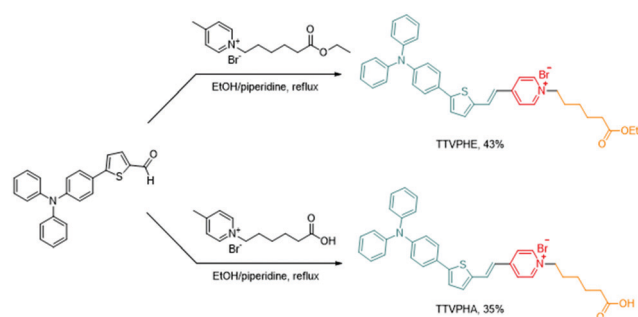


ablation of cancer cells.²⁶ Recently, mitochondria-targeted and AIE-active PSs with high ROS generation efficiency have shown great potential to kill cancer cells.^{27–30} However, the ultrastructural changes in the morphology of mitochondria caused by PDT of the AIE-active PSs have been rarely investigated, which is critical for revealing of the therapeutic mechanism. Meanwhile, the uncontrollable cell death induced by ROS generation can lead to tumor lysis syndrome and metabolic derangements, including severe renal impairment, blood electrolyte disturbance, cardiac arrhythmias, and ultimately death.^{31,32} Therefore, the development of mitochondria-targeted PSs with self-monitoring ability, which can precisely control the light treatment dose to avoid the excessive generation of ROS, is highly desirable. Recently, two AIE-active PSs were developed with self-monitoring ability, which could translocate from mitochondria to the nucleus and bind with nucleic acids during PDT treatment.^{33,34} However, their ultimate binding with nucleic acids may lead to genotoxic, carcinogenic, and teratogenic risks.³⁵ To endow the AIE-active PSs with a safe self-monitoring ability, we herein develop an AIE-active PS, **TTVPHE**, which could not only efficiently destroy the mitochondria in cancer cells, but also *in situ* monitor the therapeutic process by translocating from the damaged mitochondria to the nuclear membrane. Moreover, the ultrastructural morphology of the damaged mitochondria was characterized using high resolution transmission electron microscopy (TEM). The AIE-active PS, **TTVPHE**, also has significant advantages of fast cell penetration ability in living cells, high intracellular retention ability after being hydrolysed by esterase, and excellent mitochondrial disruption ability through fast ROS generation. Based on these distinctive features, **TTVPHE** is promising for the treatment of advanced stage CCA.

Results and discussion

Synthesis of mitochondria-targeted AIE probes

The synthetic routes of **TTVPHE** and **TTVPHA** are shown in Scheme 1. The probe **TTVPHE** consists of a hydrophilic moiety of positively charged pyridinium (abbreviated as “P”, red colour) and hydrophobic moieties of the (*E*)-4-(2-(5-(4(diphenylamino)phenyl)thiophen-2-yl)vinyl) group (abbreviated as “TTV”, green colour), and 6-ethoxy-6-oxohexyl group (abbreviated as “HE”, yellow colour). The positive charge and delocalized electrons of the lipophilic **TTVPHE** can facilitate its penetration into cells and



Scheme 1 The synthetic routes of **TTVPHE** and **TTVPHA**.

selectively stain mitochondria with a negative membrane potential of ~ -180 to 220 mV.³⁶ The compound **TTVPHA** with a hydrophilic carboxylic acid group was prepared as a control. The structures of **TTVPHE** and **TTVPHA** are well characterized and verified using NMR measurements (Fig. S1 and S2, ESI†).

The investigation of photophysical properties

We then investigated the photophysical properties of **TTVPHE** and **TTVPHA** (Fig. 1A–F). Both **TTVPHE** and **TTVPHA** show typical electron-donating and -accepting (D–A) structural features, with the “TTV” moiety as the electron-donating group and the “P” moiety as the electron-accepting group, which will facilitate the red-shift emission based on a twisted intramolecular charge transfer (TICT) process. Moreover, the TTV moiety can freely rotate in solution with non-radiative relaxation as the predominant pathway, while the restriction of intramolecular motion in the aggregated state will facilitate the radiative relaxation associated with a strong emission. In DMSO solution, **TTVPHE** showed a maximum absorption wavelength at 482 nm and a very weak emission with a low quantum yield ($\Phi_F = 0.7\%$) due to the free intramolecular rotation and TICT effect. In DMSO/toluene mixture with increased toluene fractions (f_{Tol}), a gradually increased and blue-shifted emission was observed. When the f_{Tol} increased to 99% , a maximum emission wavelength at 596 nm was observed with an 80.24 -fold increase in emission intensity and the Φ_F increased to 16.3% . These experiments verified the typical AIE properties of **TTVPHE**, which can be ascribed to the restriction of intramolecular motion and suppression of the TICT effect in the aggregate state. The compound **TTVPHA** also showed a typical AIE behaviour in DMSO/toluene mixture with increasing f_{Tol} from 0% to 90% with a 17.7 -fold increase in emission intensity. However, the further increase of f_{Tol} from

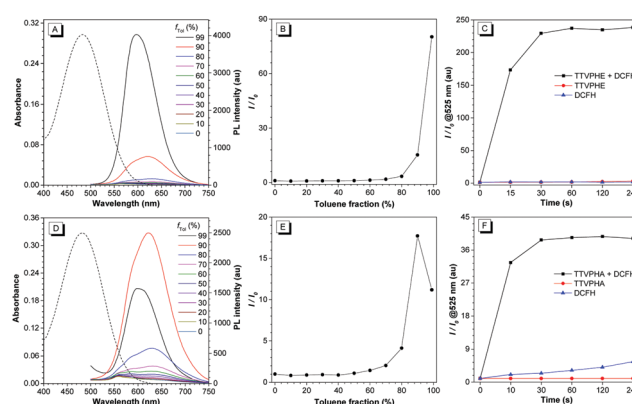


Fig. 1 (A) and (D) The normalized UV-vis absorption spectra (dashed line) of **TTVPHE** and **TTVPHA** in DMSO solution and their PL spectra in DMSO/toluene mixture with different toluene fractions (f_{Tol}); for **TTVPHE**, $\lambda_{\text{ex}} = 482$ nm; for **TTVPHA**, $\lambda_{\text{ex}} = 478$ nm. (B) and (E) The plots of the relative maximum emission intensity (I/I_0) of **TTVPHE** and **TTVPHA** versus the solvent composition of DMSO/toluene mixture. (C) and (F) The ROS generation of **TTVPHE** and **TTVPHA** in aqueous solution measured using DCFH under white light irradiation (10 mW cm^{-2}). [**TTVPHE**] = [**TTVPHA**] = $10 \mu\text{M}$; [DCFH] = $10 \mu\text{M}$; $\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 525$ nm.

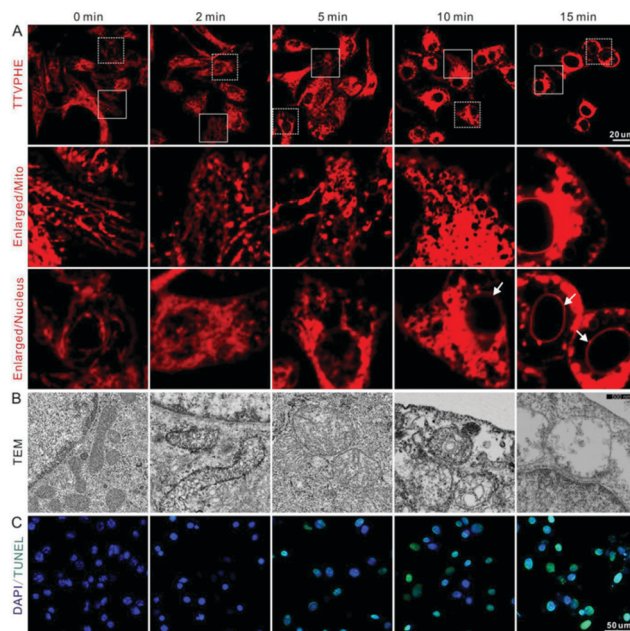


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low cytotoxicity was observed for **TTVPHE** in the dark (Fig. S8, ESI[†]), which is critical to guarantee the selective killing of cancer cells with a high spatiotemporal resolution *via* light irradiation. The cytotoxicity of **TTVPHE** under light irradiation is closely related with the light dose and the incubation concentration. By prolonging the irradiation time to 15 min or increasing the concentration of **TTVPHE** to 7 μM , the cell viability decreased to almost zero, which clearly verified the photodynamic killing ability of **TTVPHE** through mitochondrial damage (Fig. 3B and C). Moreover, the changes in the cell morphology of QBC939 cells after PDT treatment with **TTVPHE** further verified the efficient inhibition of cancer cell growth (Fig. 4).

Inspired by the excellent photodynamic activity of **TTVPHE**, we further investigated its mitochondria-targeted injury process. The morphology of mitochondria under PDT could be *in situ* monitored with an excellent contrast based on the high emission efficiency of **TTVPHE** in the aggregate state. As shown in Fig. 5A, the morphology of mitochondria changed from rod to swelling circles by prolonging the irradiation time from 0 to 15 min. Meanwhile, the nucleus membrane showed a gradual turn-on fluorescence, which suggests that the **TTVPHE** probe could translocate from damaged mitochondria to the nuclear membrane. Meanwhile, mitochondria in cells without light irradiation kept rod or sphere-shape and well-organized cristae. Furthermore, the changes in the ultrastructural morphology of mitochondria under PDT were characterized using high resolution TEM (Fig. 5B), which showed disorganized cristae and loss of matrix under light irradiation. An increased DNA fragmentation was also observed using the TUNEL assay under PDT by prolonging the irradiation time (Fig. 5C), which suggests that the ROS generation can efficiently induce cancer cell apoptosis.

To further verify the cancer cell apoptosis caused by ROS generation in mitochondria, the ROS quencher NAC was added as a control. After incubation with both **TTVPHE** and NAC, the mitochondrial morphology, characterized using CLSM and TEM, kept normal under light irradiation, which suggests that



the generation of ROS is the predominant pathway for mitochondrial damage (Fig. 6A and C). Meanwhile, the mitochondrial membrane potential ($\Delta\psi_m$) measured by JC-1 showed that NAC could efficiently inhibit the decrease of $\Delta\psi_m$ under light irradiation (Fig. 6B). All these results suggest that the **TTVPHE** probe could efficiently destroy the mitochondrial structure and membrane potential. To determine whether the mitochondria-dependent apoptotic pathway was activated, Western blotting was conducted for the measurement of cytosolic cytochrome-*c* (Cyto *c*), and cleaved caspase-9 and caspase-3. As shown in Fig. 6D, their expression levels up-regulated significantly under light irradiation, and this tendency could be alleviated by the addition of NAC. These results verified that **TTVPHE** could efficiently activate the mitochondria-dependent apoptotic pathway.

Interestingly, the translocation of **TTVPHE** to the nuclear membrane at the late stage of PDT could also improve the nuclear membrane permeability *via* ROS generation under light irradiation. As shown in Fig. 6E, the live QBC939 cells were stained with a low dose of DAPI ($1.0 \mu\text{g mL}^{-1}$) for 2 h, but the DAPI dye distributed in the cytoplasm around the nucleus and could not penetrate through the nuclear membrane owing to its barrier function. On the contrary, DAPI could rapidly enter into the nucleus after the nucleus membrane was stained by **TTVPHE**, at the late stage of PDT treatment. Moreover, the TEM images of the nucleus before and after PDT treatment further verified the damaged nucleus structure (Fig. S9, ESI[†]). Therefore, the translocation of **TTVPHE** from damaged mitochondria to the nuclear membrane could further improve the PDT efficacy for efficient killing of cancer cells which is illustrated in Fig. 6F.

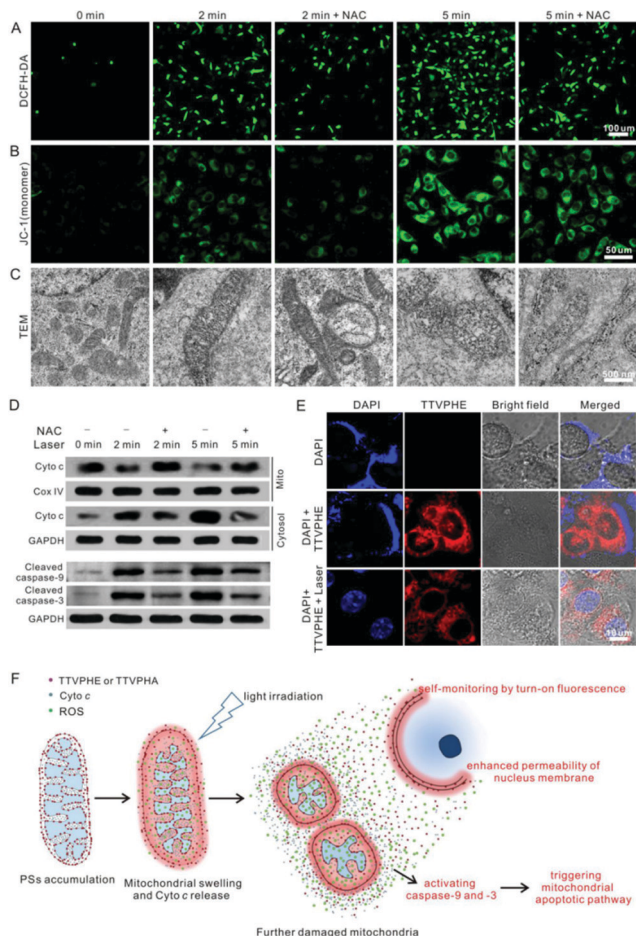


Fig. 6 (A) The intracellular ROS generation, (B) mitochondrial membrane potential ($\Delta\psi_m$), and (C) mitochondrial morphology were respectively measured using DCFH-DA, JC-1, and TEM in the absence and presence of NAC. (D) The western blot analysis of Cyto c, cleaved caspase-9 and caspase-3. (E) The CLSM images of QBC939 cells stained with DAPI, "DAPI + TTPPHE" without light irradiation, and "DAPI + TTPPHE" with light irradiation (180 mW cm⁻², 5 min). [DAPI] = 1 μ g mL⁻¹; TTPPHE = 5 μ M. (F) The schematic illustration of the mitochondrial apoptotic pathway, which can be self-monitored by the translocation of TTPPHE from damaged mitochondria to the nuclear membrane.

Conclusions

In conclusion, we have developed a mitochondria-anchoring and AIE-active PS, TTPPHE, which can not only selectively anchor in the mitochondria of CCA cells in a fast manner, but also *in situ* monitor the photodynamic process. At an early stage of PDT, changes in the morphology of mitochondria, which could be ascribed to the local accumulation of TTPPHE in the mitochondria at a high concentration, can be observed using high contrast fluorescence imaging. At a late stage of PDT, TTPPHE could translocate from the mitochondria to the nuclear membrane and further improve the PDT efficacy. It is noteworthy that the changes in the ultrastructural morphology of mitochondria under PDT were also characterized using high resolution TEM. We believe that this AIE-active probe will be promising for the photodynamic ablation of cancer cells with a

high spatiotemporal resolution and excellent self-monitoring capability.

Experimental section

Materials and equipment

Ethyl 6-bromohexanoate and 4-methylpyridine were purchased from Aladdin (Shanghai, China), 6-bromohexanoic acid was purchased from Energy Chemical (Shanghai, China), 5-(4-(diphenylamino)phenyl)thiophene-2-carbaldehyde was purchased from Psai Chemical Products Co., Ltd (Henan, China), and piperidine was purchased from Kai Xin Logistics Co., Ltd (Guangdong, China). The commercial chemicals were directly used without further purification. QBC939 human extrahepatic cholangiocarcinoma cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). RPMI 1640 medium, fetal bovine serum (FBS) and JC-1 mitochondrial membrane potential probe were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Penicillin-streptomycin mixture, trypsin and *N*-acetyl-L-cysteine (NAC) were bought from Sigma-Aldrich (Saint Louis, USA). Cell counting kit-8 (CCK8) was purchased from Dojindo Laboratories (Kumamoto, Japan). 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) and MitoTracker Green (MTG) were purchased from Yeasen Biotech Co., Ltd (Shanghai, China). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) and the TUNEL apoptosis assay kit were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Cleaved caspase-9, cleaved caspase-3, cytochrome-*c* and Cox-IV antibodies were bought from Cell Signaling Technology (Danvers, MA, USA). RIPA, ECL substrate, GAPDH antibody, HRP-labeled anti-mouse secondary antibody, the BCA protein assay kit, and the mitochondria and nuclear protein extraction kits were purchased from Wuhan Servicebio Technology Co. Ltd (Wuhan, China).

UV-vis absorption spectra were measured using a Shimadzu UV-2600 spectrophotometer, at medium scanning rate, with quartz cuvettes of 1 cm path length. Photoluminescence spectra were recorded using a Horiba Fluoromax-4 spectrofluorometer. The absolute fluorescence quantum yield was measured using a Hamamatsu quantum yield spectrometer C11347 Quantaury_QY. ¹H and ¹³C NMR spectra were measured using a Bruker AV 500 NMR spectrometer. High resolution mass spectra (HRMS) were recorded using a Bruker maxis impact mass spectrometer operated in MALDI-TOF model.

Synthesis of TTPPHE and TTPPHA

1-(6-Ethoxy-6-oxohexyl)-4-methylpyridin-1-ium bromide (63.2 mg, 0.2 mmol) and 5-(4-(diphenylamino)phenyl)thiophene-2-carbaldehyde (71 mg, 0.2 mmol) were refluxed in ethanol (6 mL) under nitrogen and further added with piperidine (200 μ L). The mixture was stirred at reflux under nitrogen for 1 h and then cooled to room temperature. The solvent was removed by rotary evaporation. The crude product was purified using silica-gel chromatography with CH₂Cl₂/CH₃OH = 10/1 as the eluent to afford TTPPHE as a red powder with 43% yield.

1-(5-Carboxypentyl)-4-methylpyridin-1-ium bromide (57.6 mg, 0.2 mmol) and 5-(4-(diphenylamino)phenyl)thiophene-2-carbaldehyde



4% paraformaldehyde for 10 min and permeabilized with 0.3% Triton X-100 in PBS for 5 min at room temperature. After rinsing with PBS three times, the cells were incubated with TUNEL reaction mixture for 1 h at 37 °C in the dark. Finally, the nuclei were labelled with DAPI for 15 min and then the fluorescence was observed using CLSM.

Mitochondrial membrane potential ($\Delta\psi_m$) analysis

The $\Delta\psi_m$ was analyzed using JC-1 according to the manufacturer's protocol. The cells were incubated with 5 μ M **TTVPHE** for 30 min and then 2 μ g mL⁻¹ of JC-1 for 20 min at 37 °C. To alleviate the mitochondrial injury, 10 μ M NAC was co-incubated with **TTVPHE** for 30 min to quench the generated ROS from the PDT effect. After rinsing with PBS three times, the cell images were taken using CLSM. For JC-1(monomer): λ_{ex} = 488 nm and band-pass filter λ = 500–530 nm.

Western blotting analysis

The cells were incubated with 5 μ M **TTVPHE** for 30 min. After PDT treatment, the cytosolic and mitochondrial proteins were extracted using mitochondria and nuclear protein extraction kits, respectively. The total protein was extracted using a RIPA lysis buffer. The protein concentration was measured using a BCA protein assay kit. Then equivalent amounts of protein were loaded in the wells of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and separated by gel electrophoresis. After being transferred onto PVDF membranes and blocked using 5% non-fat milk for 1 h, the proteins were incubated with primary antibodies overnight at 4 °C. Primary antibodies included anti-Cyto c (1:200), anti-Cox IV (1:500), anti-cleaved caspase-9 (1:500), anti-cleaved caspase-3 (1:500) and anti-GAPDH antibody (1:1000). Then, they were incubated with HRP-conjugated secondary antibodies, and the immunoreactivity was detected using an ECL substrate and recorded using the ChemiDoc imaging system (Bio-Rad, Hercules, USA).

Conflicts of interest

There are no conflicts to declare.

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