Golgi apparatus-targeted AIEgens for effective photodynamic therapy through the crosstalk of Golgi apparatus and mitochondria

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Abstract

Golgi apparatus (GA) oxidative stress induced by *in situ* reactive oxygen species (ROS) could severely damage the morphology and function of GA, which may open up a new avenue for effective photodynamic therapy (PDT). However, due to the lack of effective design strategy, photosensitizers (PSs) with specific GA targeting ability have not been reported. Herein, we report aggregation-induced emission luminogen (AIEgen) based PSs that can effectively target to GA with Pearson correlation coefficient (PCC) up to 0.98 and singlet oxygen generation rate up to 77.8%. GA fragmentation and cleavage of GA proteins (p115/GM130) were observed upon light irradiation. Meanwhile, the apoptotic pathway was activated through a crosstalk between GA oxidative stress and mitochondria in HeLa cells. Finally, TPE-PyT-CPS can effectively inhibited tumour growth *in vivo* with negligible adverse effect. This work provided a promising design strategy for the development of PSs with specific GA targeting ability, which is of great importance for precise and effective PDT.

Introduction

Photodynamic therapy (PDT) is an attractive tumor treatment which could be spatially and temporally controlled by light.^[1] It shows advantages such as minimal invasiveness, selective killing of tumor by light induced cytotoxic reactive oxygen species (ROS), particularly singlet oxygen ($^1\text{O}_2$), and repeated administration without drug resistance, which are problems related to the use of chemotherapeutic drugs (e.g., cisplatin).^[2] However, traditional photosensitizers (PSs) tend to show diminished $^1\text{O}_2$ quantum yield at aggregated states due to the enhanced non-radiative decay rate, which might decrease their PDT efficiency.^[3-5] In this regard, PSs with aggregation induced emission (AIE) characteristics are superior to traditional PSs since they show both increased $^1\text{O}_2$ and fluorescence quantum yield upon aggregation.^[6-7] In addition, AIE luminogens (AIEgens) with intramolecular charge transfer (ICT) effect could facilitate the intersystem crossing (ISC) by decreasing ΔE_{ST} (energy gap between the lowest singlet state S_1 and the lowest triplet state T_1), which could be adopted for developing heavy-atom-free PSs that show minimum dark toxicity.^[8-9] Thus, AIEgen based PSs could serve as appealing alternatives for traditional PSs and have attracted much attention for efficient PDT.^[10-12]

Studies have revealed that $^1\text{O}_2$ shows very short lifetime (half-life: 0.03 to 0.18 ms) and narrow diffusion distance in biological systems (a radius of $< 0.02 \mu\text{m}$).^[1] Hence, it is desirable to develop PSs that could target organelles and generate high dosage of $^1\text{O}_2$ *in situ* to direct cause dysfunction of subcellular organelles and activate specific cell death signals. Several PSs targeting mitochondria,^[13-15] endoplasmic reticulum (ER),^[16-18] lysosome^[19-20] and cell membrane^[21-22] have been reported and exhibited high PDT efficiency. However, due to the lack of effective Golgi apparatus (GA) targeting strategies, specific GA targeting AIEgen based PSs have seldomly been reported. Golgi apparatus is a central node at the intersection point between the exocytic and endocytic channels in intracellular membrane transporting, which plays a pivotal role in the classification of newly synthesized and recycled

proteins and lipids to their final destinations.^[23] Oxidative stress will induce significant damages on the structure and physiological function of GA.^[24] These changes in GA may trigger and propagate downstream stress signals, leading to GA disruption or even apoptosis.^[25-28] Therefore, GA targeting AIEgen based PSs could serve as good candidates for efficient PDT.

Herein, we report AIEgen based GA targeting PSs and their applications in efficient suppression of tumor cells by PDT induced GA oxidative stress (**Figure 1A**). Among the AIEgens, TPE-PyT-CPS showed a high $^1\text{O}_2$ quantum yield of 77.8 % and excellent GA targeting ability with a Pearson's correlation coefficient (PCC) of 0.98. Structure-property relationship studies suggested that the cyano-group played a key role for specific GA targeting, while the strong ICT process and pyrene group contributed for high ISC rate and $^1\text{O}_2$ generation quantum yield. GA suffered from severe oxidative stress and fragmentation after ROS was generated *in situ* upon PDT. As shown in **Figure 1B**, the structural protein p115 of GA was found to be cleaved into N-terminal and C-terminal fragments, the latter then translocated into nucleus, resulting up regulation of p53 and dysfunction of mitochondria and activation of apoptotic pathway. Finally, prominent inhibition of tumor cell growth was demonstrated without noticeable adverse effect. This study not only provided an efficient strategy for developing GA targeted PSs, but also offered new insights for efficient and precise treatment of cancers.

Results And Discussion

Design, synthesis, spectroscopic and ROS generation properties

Traditional PSs usually have to face problems such as aggregation caused quenching (ACQ) and potential dark toxicity due to heavy atom modification.^[29-30] AIEgen based PSs with a heavy atom free D- π -A structure can efficiently separate the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) distribution, which is helpful to promote the rate of intersystem crossing (k_{ISC}) process and thus beneficial to increase $^1\text{O}_2$ generation.^[30] TPE-PyT-CPS was designed with an AIE active tetraphenylethene (TPE) derivative as the electron donating group (EDG) and a cyano-pyridinium salt moiety act as the electron withdrawing group (EWG). To improve the ISC efficiency, a pyrene ring and thiophene group was introduced as π spacer for better separation of HOMO-LUMO and decreasing ΔE_{ST} . The synthesis procedures of TPE-PyT-CPS and its control compounds were presented in Supporting Information. All the intermediates and target compounds were fully characterized by ^1H and ^{13}C NMR spectroscopy and HRMS.

The spectroscopic properties of all compounds in water were studied with the UV-vis and photoluminescence (PL) spectrometer (**Figure S17**), in which the TPE-PyT-CPS shows a maximum absorption peak at 498 nm and an NIR emission at 693 nm, respectively. The Stokes shift was nearly 200 nm, which can reduce the interference of background signal and helps to improve the image-guided therapy. Moreover, compared with the TPE-PyT-PS and TPE-PyT-CP, the UV absorption spectra and fluorescence emission spectra of TPE-PyT-CPS and TPE-T-CPS show a significant red shift, which is due

to the stronger ICT effect caused by EDGs of cyano-group and pyridinium salt group. As depicted in **Figure 2A and B**, TPE-PyT-CPS in pure acetonitrile exhibited very weak emission, while with the water fraction increasing to 30%, the emission enhanced slightly. Further increasing the water fraction to 99%, lead to a distinct enhancement on PL intensity with a gradual red shift of the emission maximum to 680 nm, indicating typical AIE characteristics of TPE-PyT-CPS. The fluorescence quantum yields of TPE-PyT-CPS in acetonitrile and water (with 1% acetonitrile, v/v) were 2.74% and 17.1%, respectively. In addition, The AIE features of the other three AIEgens were also confirmed using a mixed solvent (acetonitrile and water) system with different water fractions (**Figure S18, Table S1**).

Next, dynamic light scattering (DLS) tests showed that the particle sizes of TPE-PyT-CPS in water and acetonitrile were around 410 nm and 1 nm, respectively (**Figure 2C**). The results suggested that the TPE-PyT-CPS is almost monodisperse in acetonitrile but form aggregates (rod-like according to TEM) in aqueous solution. The *in vitro* $^1\text{O}_2$ generation capability of TPE-PyT-CPS was evaluated under 532 nm laser irradiation with 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) as the $^1\text{O}_2$ indicator (**Figure 2D-F**). Upon light irradiation, the absorbance of ABDA in acetonitrile showed almost no change within 12 minutes in the presence of TPE-PyT-CPS, while in water (contains 1% acetonitrile) it decreased sharply within 150 seconds, confirming that TPE-PyT-CPS possessed a dramatically elevated ROS in aggregation state.

The $^1\text{O}_2$ quantum yield (Φ_Δ) of TPE-PyT-CPS in aqueous solution was determined to be 77.8%, while those of the control compounds were found to be 71.4%, 62.7% and 19.2% for TPE-T-CPS, TPE-PyT-PS and TPE-PyT-CP, respectively (**Figure S19, Table S1**). TPE-PyT-PS showed much higher Φ_Δ than TPE-PyT-CP, suggesting that the pyridinium group contributed more for ROS generation than the -CN group. The stronger electron withdrawing ability of pyridinium group than -CN group lead to stronger ICT effect and more efficient ISC of TPE-PyT-PS than TPE-PyT-CP. It is also reasonable that TPE-PyT-CP and TPE-PyT-PS showed lower ROS generation yields than TPE-PyT-CPS and TPE-T-CPS, since the former two showed weaker ICT effects due to the lack of EDGs of either pyridinium group or -CN group.

To understand the mechanism of higher ROS generation ability of TPE-PyT-CPS than that of TPE-T-CPS, which showed identical electron donor and acceptor but different π spacers, density functional theory (DFT) calculation was conducted. As shown in **Figure 3A**, although the HOMO and LUMO electronic distributions of TPE-T-CPS are well separated, there are some overlaps at the region of thiophene and the nearby phenyl ring of TPE-T-CPS. However, by introducing an additional large π spacer pyrene, the overlap between HOMO and LUMO of TPE-PyT-CPS was distinctly reduced. As a result, the ΔE_{ST} value of TPE-PyT-CPS was calculated to be 0.20 eV, smaller than that of TPE-T-CPS, which was calculated 0.39 eV (**Figure 3B, Table S2**). The DFT calculation was consistent with the experimental data that TPE-PyT-CPS showed higher $^1\text{O}_2$ quantum yield than TPE-T-CPS in water, which verified our design strategy that the introduction of pyrene spacer can help improve the HOMO-LUMO separation within the ICT system, thus elevate the $^1\text{O}_2$ generation ability.

Subcellular localization study

The intracellular localization of TPE-PyT-CPS was investigated by confocal laser scanning microscopy (CLSM). As shown in **Figure 4**, the Pearson's correlation coefficients (PCCs) between fluorescence images of marked commercial dyes and the TPE-PyT-CPS were determined to be 0.36 for Mito, 0.54 for ER, 0.98 for GA and 0.61 for lysosome, respectively. The results indicated that TPE-PyT-CPS localized preferentially in GA with high specificity. It was strange that TPE-PyT-CPS showed minimum localization in mitochondria, although it contained a cationic pyridinium group. Therefore, the structure-property relationships of the four compounds were investigated to figure out which moiety was responsible for the GA targeting ability (**Figure 1A**). The PCC values between the fluorescence images of GA marker and those of each compound were determined to be 0.98, 0.90, 0.89 and 0.68 for TPE-PyT-CPS, TPE-T-CPS, TPE-PyT-CP and TPE-PyT-PS, respectively (**Figure 4, Figure S21-S23**). We also tested the lipophilicity of all compounds (**Figure S20, Table S2**) because lipophilicity was reported to play an important role in the cell uptake and distribution of compounds in subcellular organelles.^[31] The $\log P_{O/W}$ values of each compound were determined to be 1.764, 1.582, 2.707 and 2.215 for TPE-PyT-CPS, TPE-T-CPS, TPE-PyT-CP and TPE-PyT-PS, respectively. There seemed to be no direct link between lipophilicity and GA targeting ability of these AIEgens. However, it was obvious that the absence of the $-\text{CN}$ group in TPE-PyT-PS made it a poor GA targeting dye, indicating that the $-\text{CN}$ group played an essential role for GA targeting in this AIEgen system.

ROS generation and PDT in tumor cells

The high $^1\text{O}_2$ quantum yield and excellent GA targeting ability of TPE-PyT-CPS encouraged us to investigate its ability to kill cancer cells through PDT. The *in situ* $^1\text{O}_2$ generation capability was evaluated by detecting the fluorescence intensity of Singlet Oxygen Sensor Green (SOSG). As shown in **Figure 5A**, TPE-PyT-CPS and TPE-T-CPS treated HeLa cells showed strong green fluorescence under laser irradiation, while almost no fluorescence was observed under dark condition. Under the same condition, SOSG signal of cells stained with TPE-PyT-CPS was higher than that of TPE-T-CPS treated cells. This was in good accordance with the fact that TPE-PyT-CPS has higher singlet oxygen generation capacity. Subsequently, the PDT effects of TPE-PyT-CPS and TPE-T-CPS were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Upon 532 nm laser irradiation (65 mW cm^{-2}) for 2 min, dose-dependent toxicities were observed and half maximal inhibitory concentrations (IC_{50}) to HeLa cells were determined to be 170 nM and 400 nM for TPE-PyT-CPS and TPE-T-CPS, respectively (**Figure 5B**). However, negligible dark toxicities were observed upon incubation of HeLa cells with TPE-PyT-CPS and TPE-T-CPS (up to $256 \mu\text{M}$) for 24 h, demonstrating exceptionally high phototoxicity index ($\text{PI} = \text{IC}_{50} \text{ dark} / \text{IC}_{50} \text{ light}$) values over 1500 and 640 (**Figure 5C**). The results suggested that TPE-PyT-CPS and TPE-T-CPS could effectively generate ROS and suppress cancer cell growth. Since TPE-PyT-CPS showed better PDT performance *in vitro*, it was selected for the following mechanistic and *in vivo* PDT studies.

To study the cell death mechanism of tumor cells, Annexin V-FITC and propidium iodide (PI) were used to monitor the process of cell apoptosis induced by TPE-PyT-CPS mediated PDT. HeLa cells were incubated with TPE-PyT-CPS and irradiating with 532 nm laser (65 mW cm^{-2} , 2 min), followed by Annexin V-FITC and PI staining, then the fluorescence images were collected using CLSM. As shown in **Figure 5D**, the green and red fluorescence signals were hardly detected with irradiation alone or with TPE-PyT-CPS but without irradiation. However, after TPE-PyT-CPS incubation and light irradiation for 2 minutes, distinct green and red fluorescence were observed in HeLa cells, suggesting the occurrence of cell apoptosis. In addition, compared with the control group, the cells treated with TPE-PyT-CPS and light irradiation, showed significant shrinkage as indicated by the white arrow in the bright fields. Moreover, flow cytometry analysis demonstrated that when the apoptosis rate of HeLa cells reached 56.7% when incubated with TPE-PyT-CPS ($0.2 \text{ }\mu\text{M}$) under light irradiation (**Figure S24**). The results indicated that apoptosis was the main death pathway of tumor cells upon PDT treatment.

PDT induced GA morphology change

TPE-PyT-CPS was confirmed to target GA specifically and generate abundant ROS *in situ*, which could induce severe damage on structural proteins of GA and cause morphological and functional changes of GA.^[32-33] Therefore, we used CLSM and TEM to monitor the ultrastructural alterations of GA in HeLa cells caused by TPE-PyT-CPS (**Figure 6**). When the cells were treated with light only or with TPE-PyT-CPS ($0.2 \text{ }\mu\text{M}$) in the dark, negligible effect on the GA morphology was observed. However, when TPE-PyT-CPS ($0.2 \text{ }\mu\text{M}$) and light were present (i.e., PDT) in unison, the Golgi structure of HeLa cells changed obviously, including intensive swelling and fragmentation of GA cistern. The results confirmed that TPE-PyT-CPS could cause severe damage on GA structure by *in situ* $^1\text{O}_2$ generation during PDT.

GA oxidative stress caused apoptosis pathway

The morphological changes of Golgi apparatus can activate the related signal pathways and trigger cell repair or apoptosis.^[34] P115 is a vesicle tethering protein, which functions in Golgi-vesicle tethering and Golgi-cisternal stacking by bridging GM130 and giantin.^[35] To further ascertain whether these structural proteins were affected during GA-targeted PDT process, the expressions of p115 and GM130 in HeLa cells were evaluated by western blot. As shown in **Figure 7A and B**, the expression of GM130 and p115 in HeLa cells was gradually decreased with the extension of irradiation time during PDT, especially for GM130. In addition, we found two new fragments of 30 kD (C-terminal fragment) and 90 kD (N-terminal fragment) proteins which originated from p115 during this process, confirming that p115 was cleaved after GA oxidative stress. Moreover, the western blot experiments demonstrated that p53 and its phosphorylation derivative (Ser15) were significantly upregulated, which suggested that PDT induced GA stress was related to apoptosis.

Studies have suggested that the 30 kD residue of p115 can translocate into the nucleus through sumoylation and then interact with p53, leading to p53 phosphorylation, which leads to apoptosis pathway.^[36-37] To verify this mechanism, we detected the level of p53 upregulated modulator of

apoptosis (PUMA), which was located in the outer membrane of the mitochondria.^[38] Indeed, the dramatically increased expression of pro-apoptotic protein PUMA and Bax was observed, accompanied with a down regulation of anti-apoptotic Bcl-2 in HeLa cells during PDT, resulting an increased Bax/Bcl-2 ratio (**Figure 7C, D**). In addition, an obvious upregulation of cleaved caspase-3 (a mitochondria-apoptotic protein) was also observed.

The activation of Bax can cause its conformational change and then oligomerization in the outer membrane of mitochondria, which will reduce the mitochondrial membrane potential ($\text{MMP}, \Delta\psi_m$).^[39-40] Next, the MMP was evaluated through JC-1 staining assay. As shown in **Figure 8**, the HeLa cells after PDT treatment showed an evident increase in green fluorescence of JC-1 monomers and a significant decrease in red channel of JC-1 aggregates, suggesting a distinct loss of MMP upon PDT. However, HeLa cells treated by light only, dark and “dark + AIEgen” showed intense red fluorescence and weak green fluorescence, indicating intact MMPs. The above results suggested that PDT induced GA oxidative stress could severely affect mitochondria homeostasis and active the apoptosis signal pathway (**Figure 9**).

GA-targeting mediated PDT *in vivo*

The *in vivo* PDT performance of TPE-PyT-CPS was evaluated in a subcutaneous HeLa tumor-bearing mouse model. All animal experiments were carried out in accordance with the regulations of the Institutional Animal Care and Use Committee (IACUC) of Nanjing University. Firstly, we evaluated the imaging ability of TPE-PyT-CPS in mice (**Figure 10A**) and monitored the fluorescence signals at specified intervals. Distinct fluorescence signal in the tumor sites was observed after intratumoral injection of TPE-PyT-CPS (0.1 mM, 120 μL /200 mm^3 tumor) for 3 hours (**Figure S25**), and the fluorescence intensity reached maximum after 18 hours. To gain the fluorescence distribution image of the tumor and other major organs, the mice were sacrificed at 30 h after injection of TPE-PyT-CPS, and the *ex vivo* fluorescence images of isolated organs were shown in **Figure S25**. The results showed that TPE-PyT-CPS could effectively retain in the tumor tissue and exhibit strong fluorescence signal. Moreover, there was almost no fluorescence signals originated from other major organs, including heart, spleen, lung and kidney, except for a weak fluorescence in liver tissue. The results demonstrated that TPE-PyT-CPS could retain in tumor area and serve as an imaging agent.

Subsequently, the anticancer efficacy of TPE-PyT-CPS was assessed *in vivo*. Tumor (HeLa) bearing Balb/c mice were randomly divided into four groups (n = 5): saline group (saline light -), saline group with laser irradiation group (saline light +), AIEgen group (AIEgen light -) and AIEgen with laser irradiation group (AIEgen light +). After intratumoral injection of TPE-PyT-CPS (0.2 mM, 100 μL) for 18 h, the tumor area of the mice was irradiated with 532 nm laser of 35 mW cm^{-2} for 5 min, labeled as AIEgen (light +) groups. As can be seen from **Figure 10B**, for the control groups (saline light -, saline light+), the tumor volumes increased rapidly regardless of light or dark conditions, suggesting that the tumor growth was not affected in the absence of TPE-PyT-CPS. However, in the presence of our AIEgen, the tumor volumes and weight were effectively suppressed in the light group (**Figure 10C, D**). Additionally, the body weights of mice in “AIEgen light +” group showed no obvious difference from those of control group during PDT

(**Figure 10E**), which clearly demonstrated the minimal side effects of "AIEgen light + " treatment. To understand and verify the different antitumor efficacy and whether TPE-PyT-CPS cause *in vivo* side toxicity of those treatments, tumors and major organs from the four groups are excised and appraised using H&E (hematoxylin and eosin) staining and TUNEL (terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling) assay for histological analysis.^[41] The staining results showed that the TPE-PyT-CPS treated group with laser irradiation (AIEgen light +) exhibited obvious cell apoptosis with pyknotic cells with condensed nuclei (**Figure 10F, Figure S26**), whereas the tumors tissues of mice that treated in control groups (AIEgen light -, saline + light and saline) had massive viable cancer cells. Similar results were obtained in the TUNEL assay, the tumors slice of mice treating with PDT exhibited significantly higher level of cells apoptosis than the control group. Meanwhile, there was no abnormal pathological morphology or prominent tissue damage in the heart, liver, spleen, lung and kidney of mice in all treatment groups (**Figure S27**). Taken together, these results confirmed that TPE-PyT-CPS could efficiently suppress tumor growth by GA targeting mediated-PDT with negligible side effects.

Conclusions

In conclusion, we have designed and synthesized AIEgen based PSs with excellent GA targeting ability and PDT effect. Structure-property relationship studies revealed that the cyano-group played a decisive role in GA targeting, while the introduction of large π spacer pyrene group further enhanced the singlet oxygen quantum yield of the D- π -A system. Large morphological change of GA was observed upon the *in situ* generation of ROS during PDT. Moreover, we found the GA stress can trigger the mitochondria dysfunction during PDT. The GA-mitochondria crosstalk lead to the collapse of MMP and ultimately cell death. TPE-PyT-CPS showed excellent phototoxicity and negligible dark toxicity to HeLa cancer cells with a large PI over 1500. Finally, benefit from its high singlet oxygen generation and effective GA targeting features, TPE-PyT-CPS significantly inhibited the tumor growth of mice without obvious adverse effects on normal tissues *in vivo* after PDT. This work provided a reliable design strategy for the development of AIEgen based GA targeting PSs, which offered a new avenue for precise and efficient PDT through transferring stress signals from Golgi apparatus to mitochondria.

Methods

Materials and instrument. All chemicals for compounds synthesis were commercially available (Sigma-Aldrich, J&K, Sinopharm, Bidepharma-Tech) and used without further purification. The silica gel (300-400 mesh) was used for column chromatography. The ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX 400 MHz or 600 MHz spectrometer. ESI-MS was determined on a LCQ electrospray mass spectrometer (Thermo Finnigan). High resolution mass spectra (HR-MS) were determined using an Agilent 6540Q-TOF HPLC-MS spectrometer. UV-visible absorption spectra were recorded on a PerkinElmer Lambda 35 spectrophotometer. The transmission electron microscopy (TEM) images were obtained using a JEOL JEM-1011 transmission electron microscope (Japan). Fluorescence confocal imaging was carried out on a laser scanning confocal imaging system (Olympus TH4-200) consisting of ZEISS Laser

Scanning Microscope (LSM 710) and a 20-mW output 488 nm argon ion laser. Flow cytometry analysis was performed with a BD LSRFortessa Cell Analyzer.

Density functional theory (DFT) calculation. Theoretical calculations were adopted to rationalize the ISC process of the TPE-PyT-CPS and TPE-T-CPS using Gaussian 09w. The geometries of TPE-PyT-CPS and TPE-T-CPS were optimized based on the method TD-DFT//B3LYP/6-31G(d). TD-DFT was used to predict the excitation energies for the singlet and triplet excited states of TPE-PyT-CPS and TPE-T-CPS in tetrahydrofuran.

Morphology and Size. Dynamic light scattering (DLS) was used to monitor the size of the TPE-PyT-CPS in water, acetonitrile solution and the morphology was observed by Transmission Electron Microscope (TEM).

Singlet oxygen detection in solution. ABDA (9,10-Anthracenediyl-bis(methylene)-dimalonic acid) was used as an indicator to detect singlet oxygen production capacity since the absorbance of ABDA decreases upon reaction with singlet oxygen, and RB (Rose Bengal) was used as an internal reference. For singlet oxygen detection, the ABDA (50 μM) was mixed with the TPE-PyT-CPS in acetonitrile/water (1:99, v/v) and exposed to 532 nm laser irradiation (10 mW cm^{-2}). The decomposition of ABDA was monitored by the absorbance decrease at 378 nm. The $^1\text{O}_2$ quantum yield of the PS in water was calculated using the following formula (Eq. 1):

$$\Phi_{\text{PS}} = \Phi_{\text{RB}} \frac{K_{\text{PS}} A_{\text{RB}}}{K_{\text{RB}} A_{\text{PS}}} \quad \text{Eq. 1}$$

where K_{PS} and K_{RB} are the decomposition rate constants of ABDA by the TPE-PyT-CPS and RB, respectively. A_{PS} and A_{RB} represent the light absorbed by the different AIEgens (TPE-PyT-CPS, TPE-PyT-CP, TPE-PyT-PS and TPE-T-CPS) and RB, respectively, which are determined by integration of the areas under the absorption bands in the wavelength range of 400-600 nm. Φ_{RB} is the $^1\text{O}_2$ quantum yield of RB, which is 0.75 in water.

Lipophilicity of AIEgens. Solutions of different AIEgens (concentration: 15, 30, 45 μM) were prepared in phosphate buffer (10 mM, pH 7.4) pre-saturated with 1-octanol. Equal volumes (1.5 mL) of the solution and 1-octanol pre-saturated with the phosphate buffer were mixed and placed in a thermostatic ($25.0 \pm 0.1^\circ\text{C}$) air bath orbital shaker at 200 rpm for 4 h. The samples were separated into two phases after centrifugation at 2500 rpm for 15 min. The concentration of the solute in the aqueous phase was determined by spectrophotometry. According to the law of mass conservation, the drug concentration of the 1-octanol phase and the lipohydro partition coefficient $P_{\text{O/W}}$ ($P_{\text{O/W}} = C_{\text{O}}/C_{\text{W}} = A_{\text{O}}/A_{\text{W}}$, A stands for absorbance) were calculated. The average of three parallel experimental data was reported as the final result. The log $P_{\text{O/W}}$ was calculated using the following equation (Eq. 2):

$$\log P_{\text{O/W}} = \log \left[\frac{([C]_{\text{initial}} - [C]_{\text{final}})}{[C]_{\text{final}}} \right] \quad \text{Eq. 2}$$

Subcellular organelle imaging. The HeLa cancer cells were used in the following experiments and cultured using DMEM culture medium containing 10% FBS and 1% penicillin-streptomycin in an artificial environment (5% CO₂ at 37 °C). The HeLa cells were regularly checked for mycoplasma contamination and then conducted when the cells were grown to 80% confluence in the culture dish. The HeLa cancer cells were cultured in the special confocal chambers at a density of 10⁵ cells mL⁻¹ in culture medium. After 24 h, when cells have reached the desired confluence TPE-PyT-CPS (10 µM), TPE-PyT-CP (10 µM), TPE-T-CPS (10 µM) and TPE-PyT-PS (10 µM) in FBS free medium were added and incubated with HeLa cancer cells at 37 °C for 6 h.

Mitochondrial Imaging. The cells were then washed three times carefully using 1×PBS, followed by addition of commercial Mito Tracker Green FM (50 nM) and incubation for another 30 min. Subsequently, the cells were washed three times using 1×PBS and visualized by confocal laser scanning microscopy at 488 nm excitation for TPE-PyT-CPS, TPE-PyT-CP, TPE-T-CPS, TPE-PyT-PS (10 µM) and 488 nm excitation for Mito Tracker Green FM, respectively. The signal collections of CLSM were 600-750 nm for different AIEgens and 495-535 nm for Mito Tracker Green.

Lysosome imaging. The cells were then washed three times carefully using 1×PBS, followed by addition of commercial LysoSensorTM Green DND-189 (75 nM) and incubation for another 60 min under growth conditions appropriate for the particular cell type. Subsequently, the cells were washed three times using 1×PBS and visualized by confocal laser scanning microscopy at 488 nm excitation for TPE-PyT-CPS, TPE-PyT-CP, TPE-T-CPS, TPE-PyT-PS (10 µM) and 488 nm excitation for LysoSensorTM Green DND-189, respectively. The signal collections of CLSM were 600-750 nm for different AIEgens and 495-535 nm for LysoSensorTM Green DND-189.

Golgi apparatus imaging. The cells were then washed three times carefully using 1×PBS, followed by addition of commercial Golgi Green (5 µM) and incubation for 30 min under 4 °C. After, washing the cells three times using 1×PBS growth and incubation for another 30 min under 37 °C. Subsequently, visualized by confocal laser scanning microscopy at 488 nm excitation for TPE-PyT-CPS, TPE-PyT-CP, TPE-T-CPS, TPE-PyT-PS (10 µM) and 488 nm excitation for Golgi Green, respectively. The signal collections of CLSM were 600-750 nm for different AIEgens and 495-535 nm for Golgi Green.

Endoplasmic reticulum imaging. The cells were then washed three times carefully using 1×PBS, followed by addition of commercial ER-TrackerTM Blue (1 µM) and incubation for another 45 min under growth conditions (37 °C) appropriate for the particular cell type. Subsequently, the cells were washed three times using 1×PBS and visualized by confocal laser scanning microscopy at 488 nm excitation for TPE-PyT-CPS, TPE-PyT-CP, TPE-T-CPS and TPE-PyT-PS (10 µM) and 405 nm excitation for ER-TrackerTM Blue, respectively. The signal collections of CLSM were 600-750 nm for different AIEgens and 455-520 nm for ER-TrackerTM Blue.

Intracellular ROS detection. The capability of TPE-PyT-CPS and TPE-T-CPS for intracellular ROS production were assessed using singlet oxygen sensor green (SOSG) as an ROS indicator. The HeLa cancer cells were cultured in the special confocal chambers and incubated with TPE-PyT-CPS (0.2 μM) or TPE-T-CPS (0.4 μM) for 6 h at 37 °C. Subsequently, the cells were washed three times with 1×PBS, followed by addition of singlet oxygen sensor green (SOSG, 15 μM) in FBS free culture medium and incubation for 30 min. The above-mentioned process was performed in dark. Then, the cells were exposed to white light irradiation (25 mW cm^{-2}) for 2 min, followed by imaging with CLSM. For CLSM imaging, the excitation of SOSG was 488 nm with a collection of fluorescence signal at 525 ± 20 nm.

Cytotoxicity Study. Briefly, 100 μL of HeLa cell suspension were added into each well of 96-well plate at a density of 8×10^4 cells mL^{-1} . After 80% confluence, fresh culture medium (the concentration of DMSO lower than 0.5%) containing a series of concentrations of TPE-PyT-CPS was added and incubated with the HeLa cancer cells for 6 h at 37 °C in dark. Subsequently, the cells were exposed to 532 nm laser irradiation (65 mW cm^{-2}) for 2 min. Alternatively, the TPE-PyT-CPS-treated HeLa cancer cells were kept in dark without light exposure. At 24 h post irradiation, the wells were replaced with freshly prepared 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; 2.5 mg mL^{-1} in PBS) solution. After 4 h incubation, the solution in each well was carefully removed and 150 μL of DMSO was added to each well to dissolve the formazan. The plate was gently shaken for 10 min at room temperature and then the absorbance of MTT at 595 nm was monitored by the microplate reader (Thermo Scientific Varioskan Flash) in order to determine the cell viability. Cell viability rates (%) and IC_{50} values were calculated on the data of three parallel tests.

Annexin V-FITC/Propidium Iodide Co-Staining Assay. HeLa cells pretreated with TPE-PyT-CPS (0.2 μM) for 30 min were stained with both annexin V-FITC and PI following the standard protocol (Life Technologies) and imaged after irradiation of 532 nm laser (65 mW cm^{-2} , 2 min). For control experiments, HeLa cells preincubated with TPE-PyT-CPS (0.2 μM), annexin V-FITC, and PI were imaged without light irradiation. The excitation wavelengths for annexin V-FITC and PI were 488 and 559 nm, respectively, and the emissions for annexin VFITC and PI were collected in the range of 500–530 and 600–630 nm, respectively.

Detection the expression of p115, GM130, P53, ser15, PUMA, Bcl-2, Bax and Caspase-3. The HeLa cancer cells were seeded into 6 cm plates at the density of 1×10^5 cells mL^{-1} . After adherence, the HeLa cancers cells were incubated with 0.2 μM of TPE-PyT-CPS, for 6 h at 37 °C, respectively. For the dark control group, there is no need to light, and for the light control group, there is no need to add photosensitizer, only light for 20 minutes (25 mW cm^{-2}). Next, the treated cells were washed and irradiated by 532 nm laser irradiation for 4, 6, 8, 10 and 20 min. After 6 h, the cells were collected and then conducted with centrifugation using 12,000 rpm for 15 min at 4 °C. Protein concentrations were measured using protein assay reagents, and equal amounts of protein per lane were separated on SDS-PAGE gel and transferred to a PVDF membrane. The membrane was incubated with Anti-p115-RhoGEF (1:100, Abcam, ab223759), Anti-GM130 (1:1500, Abcam, ab52649), Anti-p53 (1:2000, Abcam, ab26), Anti-p53 (phospho S15) (1:2000,

Abcam, ab26), Anti-PUMA (1:2000, Abcam, ab33906), Bcl-2 (1:200, Abcam, ab182858), Bax (1:5000, Abcam, ab32503), caspase-3 (1:200, Abcam, ab184787) and Anti-GAPDH (1:10000, Abcam, ab8245) followed by incubation with the peroxidase-labeled goat antirabbit HRP secondary antibody. Western blots were visualized by enhanced chemiluminescence detection system. Relative grayscale was calculated by ImageJ.

Mitochondrial membrane potential (MMP) assay. MMP was evaluated by confocal imaging via JC-1 staining. HeLa cells were seeded in a glass bottom cell culture dish at 40% confluence and cultured in media containing TPE-PyT-CPS (0.2 μ M) respectively. After the incubation of 6 h, then cells were stained with JC-1 (Beyotime Biotechnology) following the manufacture's protocol. After rinse three times with incomplete culture medium, the cells irradiated with a laser of 532 nm (25 mW cm^{-2} , 2 min), and confocal imaging was immediately carried out with Leica SP8. The imaging band path for green channel of JC-M was 520-550 nm (λ_{ex} , 490 nm), while that for red channel of JC-A was 570-640 nm (λ_{ex} , 525 nm).

***In vivo* fluorescent imaging.** *In vivo* fluorescent imaging of TPE-PyT-CPS in HeLa tumor-bearing nude mice model was tested by using the IVIS Lumina III *in vivo* Imaging System (PerkinElmer). Nude mice at 4-5 weeks old were purchased at pushing medical. TPE-PyT-CPS (100 μ M, 120 μ L) was injected intratumorally and fluorescent images were captured by the Lumina III at 0, 3, 6, 12, 18 and 30 hours after injections (Ex: 530 nm, Em:690 nm). The mice were sacrificed at designated time points (24 h) and the tissues including heart, liver, spleen, lung, kidney, were excised and imaged by IVIS[®] Lumina III fluorescence imaging system.

***In vivo* PDT.** HeLa tumors bearing Balb/c mice were randomly divided into four groups (n = 5): saline group (saline light -), saline group with laser irradiation group (saline light +), AIEgen group (AIEgen light -) and AIEgen with laser irradiation group (AIEgen light +). After intra-tumoral injection of TPE-PyT-CPS (0.2 mM, 100 μ L) for 18 h, the tumor area of the mice was irradiated with 532 nm laser of 35 mW cm^{-2} for 5 min. The tumor size was measured every 3 days and calculated as follows: volume = 1/2(tumor length) $\times$ (tumor width)².

Histological analysis. All mice of different groups were sacrificed on day 22, and major organs and tumors were separated and made into slices for H&E or TUNEL staining.^[3] Major organs were collected and fixed in 4% paraformaldehyde, which were then embedded into paraffin, sliced at thickness of 5 μ m. The tissue slices were stained with H&E or TUNEL and then imaged by optical microscopy and assessed by 3 independent pathologists.

Declarations

Competing interests

The authors declare no competing financial interest.

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Author contributions

M.L., Y.C., W.H. and Z.G. designed the study. M.L. synthesized probes; C. W. performed the DFT calculations. M.L., S. Y. and S. J. performed the spectroscopic and cellular experiments; M.L., Y. G. and H. Y. performed the in vivo experiments; M.L. and Y.C. co-wrote the manuscript, Y.C. and H. F. revised the manuscript. All authors discussed the results and commented on the paper. All authors have given approval to the final version of the manuscript.

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Figures

Figure 1

(A) The chemical structures of AIEgens with different GA targeting (PCC values) and ROS generation abilities. (B) Schematic illustration of the GA targeting AIEgen induced GA stress and the crosstalk of GA and mitochondria for cell apoptosis upon PDT.

Figure 2

AIE property of TPE-PyT-CPS and its ROS generation ability in different solvents. (A) Emission spectra of TPE-PyT-CPS in acetonitrile/water mixtures with varied water fractions (f_w). (B) Plot of I/I_0 (dark dot) and the maximum emission wavelength (red dot) of TPE-PyT-CPS in different f_w of the solvent mixture, I_0 represents the emission intensity in acetonitrile. (C) Particle sizes of TPE-PyT-CPS (10 μ M) in water and acetonitrile dynamic light scattering, Inset: TEM images of TPE-PyT-CPS in water (D), (E) Changes of UV-vis spectra of ABDA (50 μ M) in the presence of TPE-PyT-CPS (10 μ M) in acetonitrile and water under different durations of light irradiation (25 mW cm^{-2}). (F) The decomposition rates of ABDA in different solvent with TPE-PyT-CPS under light irradiation, respectively.

Figure 3

Calculated frontier molecular orbitals and energy levels for TPE-T-CPS and TPE-PyT-CPS. (A) HOMO–LUMO electronic cloud distributions of TPE-T-CPS and TPE-PyT-CPS by DFT calculations. (B) Energy levels (S1–S3, T1–T3) of TPE-T-CPS and TPE-PyT-CPS calculated by the vertical excitation of the optimized structures.

Figure 4

Subcellular co-localization of TPE-PyT-CPS. CLSM of HeLa cells stained with different commercial probes (Mito-Green: 495-535 nm, λ_{ex} , 488 nm. ER-Blue:455-520 nm, λ_{ex} , 405 nm. Golgi-Green: 495-535 nm, λ_{ex} , 488 nm. Lyso-Green:495-535 nm, λ_{ex} , 488 nm) and TPE-PyT-CPS (10 μ M, 600-750 nm, λ_{ex} , 488 nm). Scale bar: 20 μ m for ER and Golgi channel, 10 μ m for Mito and Lyso channel.

Figure 5

Intracellular ROS, cytotoxicity and cells apoptosis induced by PDT. (A) Intracellular 102 detection by CLSM after HeLa cells were incubated with TPE-PyT-CPS (0.2 μM) and SOSG under laser irradiations (25 mW cm^{-2} for 2 min, scale bar: 20 μm). Cell viabilities of HeLa cells after incubation with varied concentrations of TPE-PyT-CPS (B) with or (C) without 532 nm laser irradiation (65 mW cm^{-2} , 2 min). (D) Apoptosis of HeLa cells treated with TPE-PyT-CPS (0.2 μM) under 532 nm laser (65 mW cm^{-2} for 2 min, scale bar: 50 μm).

Figure 6

Morphological changes of Golgi apparatus observed by CLSM (stained with Gogli Green) and TEM imaging in HeLa cells after treatment with light only, TPE-PyT-CPS (0.2 μM) with or without light irradiation (25 mW cm^{-2} , 2 min).

Figure 7

Effect of PDT on Golgi apparatus and mitochondria associated protein expression. The expressions of p115, p115 (90 kD), p115 (30kD), GM130, p53, ser15 in Golgi apparatus (A) and PUMA, Bcl-2, Bax, caspase-3 in mitochondria (C) after different treatment with TPE-PyT-CPS (0.2 μ M) under light irradiation (25 mW cm⁻², 2 min). (B) and (D) normalized protein expression changes for different irradiation time, the protein expression in the dark was taken as 1.

Figure 8

Confocal fluorescence imaging of MMP in HeLa cells in the absence and presence of TPE-PyT-CPS (0.2 μ M) via JC-1 staining assay under different conditions. Scale bar: 25 μ m.

Figure 9

Proposed mechanism for the cell apoptosis through the crosstalk between Golgi apparatus and mitochondria in GA-targeted photodynamic therapy.

Figure 10

Therapeutic effect of TPE-PyT-CPS in vivo. (A) Fluorescent image of a mouse after intratumoral injection of TPE-PyT-CPS (0.1 mM, 120 μ L) for 18 h. (B) Photographs of excised tumors on the 22th day. (C) Changes of tumor volume and weight (D) after PDT. (E) Body weight curves of mice during PDT

treatment employing TPE-PyT-CPS (0.2 mM, 120 μ L) as the PS and 532 laser irradiations of 35 mW cm⁻² for 5 min at different time points post-treatment in different groups: saline (light -), saline (light +), TPE-PyT-CPS (light -) and TPE-PyT-CPS (light +) groups. (F) H&E staining (scale bar: 50 μ m) and apoptotic analysis of TUNEL staining (scale bar: 100 μ m) images of tumor slices from mice after treatment with saline and TPE-PyT-CPS under 532 laser irradiations.

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