An unexpected strategy to alleviate hypoxia limitation of photodynamic therapy by biotinylation of photosensitizers

Jing An1, Shanliang Tang1, Gaobo Hong1, Wenlong Chen1, Miaomiao Chen1, Jitao Song2, Zhiliang Li2, Xiaojun Peng1, Fengling Song1,2✉ & Wen-Heng Zheng3✉

The most common working mechanism of photodynamic therapy is based on high-toxicity singlet oxygen, which is called Type II photodynamic therapy. But it is highly dependent on oxygen consumption. Recently, Type I photodynamic therapy has been found to have better hypoxia tolerance to ease this restriction. However, few strategies are available on the design of Type I photosensitizers. We herein report an unexpected strategy to alleviate the limitation of traditional photodynamic therapy by biotinylation of three photosensitizers (two fluorescein-based photosensitizers and the commercially available Protoporphyrin). The three biotyiylated photosensitizers named as compound 1, 2 and 3, exhibit impressive ability in generating both superoxide anion radicals and singlet oxygen. Moreover, compound 1 can be activated upon low-power white light irradiation with stronger ability of anion radicals generation than the other two. The excellent combinational Type I / Type II photodynamic therapy performance has been demonstrated with the photosensitizers 1. This work presents a universal protocol to provide tumor-targeting ability and enhance or trigger the generation of anion radicals by biotinylation of Type II photosensitizers against tumor hypoxia.
Photodynamic therapy (PDT) is an emerging alternative treatment modality for malignant tumors \(^1\text–}^3\), which bases on photosensitizers to transfer light energy into reactive oxygen species (ROS) to induce cell apoptosis and tissue damage \(^4\text–}^6\). Unfortunately, the therapeutic efficacy of PDT is limited by the hypoxic environment in solid tumors \(^7\text–}^8\). Because the dominant mechanism of PDT is based on high-toxicity singlet oxygen (\(^1\text{O}_{2}\)), which is called type II PDT \(^9\text–}^{10}\). Type II PDT highly relies on the surrounding oxygen \(^11\text–}^{12}\), which is in conflict with the inherent properties of tumor hypoxia. Fortunately, recently Type I PDT has been found to be able to perform well under a hypoxic environment \(^13\text–}^{15}\). In contrast to the direct energy transfer from excited photosensitizers to \(\text{O}_2\) in the Type II pathway, the Type I process is that the excited photosensitizers transfer electrons or hydrogen protons to the surrounding substrates, thereby yielding radical species (e.g., superoxide (\(\text{O}_2^{•−}\)) and hydroxyl (OH•) radicals) \(^16\text–}^{17}\). Among these radicals, excessive \(\text{O}_2^{•−}\) is known to be able to react with proteins, DNA, and lipids, causing irreversible damage to cellular components \(^18\text–}^{19}\). Furthermore, \(\text{O}_2^{•−}\) could then participate in superoxide dismutase (SOD) - mediated disproportionation reactions, which would realize the reuse of \(\text{O}_2\) and trigger the formation of other highly toxic ROS \(^20\).

The light source is another important component in photodynamic therapy. White-light PDT has been proposed as an effective treatment for fungal diseases and dermatological lesions (like acne, keratosis, skin tumors, etc.) \(^21\text–}^{23}\). In particular, daylight photodynamic therapy (DL-PDT) has made PDT more widespread, cheaper, and less painful \(^24\). Although some white-light activated photosensitizers have been reported, their absorption profiles mismatch the spectral emission of the white-light source (i.e., solar radiation) \(^25\text–}^{26}\). Furthermore, the majority of those photosensitizers are only able to generate one type of ROS (e.g., \(\text{O}_2^{•−}\)) \(^27\text–}^{28}\).

In this study, three organic photosensitizers with both Type I and Type II mechanisms for white-light activated PDT were developed by biotinylation of typical Type II photosensitizers. In 2018, Peng’s group reported a Type I photosensitizer containing a biotin unit with \(\text{O}_2^{•−}\) generation \(^29\). And the role of the biotin unit was considered to achieve preferential tumor-targeting ability. Inspired by it, the original purpose of introducing biotin into the three photosensitizers was also to provide the photosensitizers with the ability to target tumors, as biotin receptors are over-expressed on the surface of cancer cells or tumor vasculature systems compared with normal tissues \(^30\text–}^{35}\). Unexpectedly, the \(\text{O}_2^{•−}\) generation capacity of all the three photosensitizers (1, 2, and 3) were found to be greatly boosted by biotinylation. Furthermore, compound 1 was studied for a combinational Type I/Type II DL-PDT, which confirmed that the biotinylation strategy can efficiently alleviate the limitation of hypoxia.

**Results**

**Synthesis and photophysical properties.** Several fluorescein derivatives with thermally activated delayed fluorescence have been reported as PDT photosensitizers in our previous works \(^36\text–}^{38}\). These fluorescein derivatives were found to be Type II photosensitizers. In this work, two fluorescein photosensitizers 1 and 2 were synthesized from their precursors 4 and 5 by amide condensation with 5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl) pentanehydrazide (Biotin–NH–NH\(_2\), 11), respectively (Fig. 1 and Supplementary Fig. 1). In contrast, we also synthesized a porphyrin photosensitizer 3 by covalently linking compound 11.
with the commercially available Protoporphyrin (PhpIX, compound 6), a typical Type II photosensitizer (Fig. 1 and Supplementary Fig. 1). The introduction of biotin unit to the three photosensitizers was originally aimed to provide them the ability of tumor targeting. Compounds 4, 5, 6, and 11 are employed as control compounds to be discussed in the work. The chemical structures of compound 1, 2, 3, 4, 5, and 11 were fully characterized by 1H NMR, 13C NMR, HRMS, and FTIR (Supplementary Figs. 2–22).

As shown in Fig. 2, compounds 1, 2, 3, 4, 5, and 6 exhibited similar absorbance spectra and emission spectra with their precursors 4, 5, and 6, respectively. In particular, 1 is characterized by broader absorption in 400–700 nm, qualifying it as an ideal PS for white-light harvesting (Fig. 2a and Supplementary Fig. 23). Meanwhile, compound 1 emits near-infrared (NIR) fluorescence centered at 750 nm (Fig. 2b). The biotinylation provides the photosensitizers with the ability of targeting tumor cells. Thus, compound 1 holds application prospects for NIR bioimaging and image-guided white-light-induced PDT for malignant tumors.

PS-Biotin-sensitized ROS generation. According to our previous works, fluorescein derivatives could be used as ideal candidates for Type II PDT. Therewith, 1,3-diphenylisobenzofuran (DPBF) was employed to appraise the generation of 1O2. As indicated by the DPBF decay curves (Fig. 3a and Supplementary Figs. 24 and 25), irradiation of PS-Biotin or its precursor with white light led to a comparable 1O2 generation, implying that the conjugation of biotin moieties hardly matter to the production of singlet oxygen. In other words, biotinylation has little effect on the energy-transfer process from excited photosensitizers to 1O2.

However, the biotinylation was found to have an unexpected effect on the electron transfer process involved the Type I PDT. Herein, the generation of O2−• was measured by the nitrotriazolium blue chloride (NBT). It is known that NBT can be specifically reduced by O2−• to form the insoluble NBT-formazan (Supplementary Fig. 26a) accompanied with the characteristic decrease of the absorbance at 260 nm. According to the results shown in Fig. 3b–d, the production of O2−• in control groups (compound 4 with irradiation, compound 4 and 11 with irradiation) were less than that in compound 1 with irradiation. Similar results were found in compounds 2 and 3 (Supplementary Fig. 27) indicating that the covalently modified by biotin has a positive effect on the O2−• generation of photosensitizers. This inference was supported by massive precipitation observed in the irradiation groups for all the three photosensitizers (Supplementary Fig. 26b–d).

Compared with the fluorescein derivatives 4 and 5, the compound 6, porphyrin was well known as a classic Type II PS and did not produce obvious O2−• even under a longer time of irradiation. So, the O2−• generation ability of compound 3 should be attributed to the introduction of biotin (Supplementary Figs. 26d and 27d–f). Such an unexpected strategy of biotinylation suggested that PS-Biotin could serve as an excellent O2−• and 1O2 generator at the same time (Fig. 3e). Due to the hypoxia tolerance of Type I and the high reactivity of Type II, PS-Biotin could realize a combinational PDT through Type I and Type II mechanisms. Considering that compound 1 has a stronger ability of O2−• generation than compound 3, and matches better with the spectral emission of the white-light source than compound 2, compound 1 was chosen for demonstration its PDT performance against tumor hypoxia in the following investigation.
Subcellular colocalization. Before the PDT experiments, the targeting and imaging capacities of compound 1 and 4 were assessed by confocal laser scanning microscopy (CLSM). Compound 1 was incubated with COS-7 (biotin receptor-negative) and MCF-7 (biotin receptor-positive), respectively. As illustrated in Supplementary Fig. 28, the fluorescence intensity of compound 1 in MCF-7 cells significantly increased over incubation time, while almost no fluorescence was observed in COS-7 cells. As for compound 4 (Supplementary Fig. 29), obvious fluorescence was observed in neither COS-7 cells nor MCF-7 cells. These results indicated that compound 1 could specifically bind to biotin receptors, which were overexpressed on the surface of cancer cells rather than normal cells. These results proved that the biotinylation can offer the photosensitizer the targeting ability of tumor cells. Then, the intracellular distribution of compound 1 was investigated using commercial organelle-selective trackers. As shown in Fig. 4a, the red signals of compound 1 nicely overlapped with the green fluorescence of Lyso-sensor Green (Pearson correlation coefficient \( R = 0.83 \)). It evidenced that compound 1 was accumulated selectively in the lysosomes. In contrast, only a small amount of compound 1 was distributed in mitochondria and nucleus (Fig. 4b, c).

**In vitro assessment of PDT efficacy.** In order to evaluate the PDT performance of compound 1, methyl thiazolyltetrazolium...
Importantly, the PDT efficacy in hypoxic (1% O2) environment is very close to that in normoxia. In detail, ~60% of the cells were killed when treated with 20 μM of compound 1, indicating that the photocytotoxicity of compound 1 was mainly attributed to the other types of ROS instead of \( ^1\text{O}_2 \) under hypoxic condition. The generated ROS should be \( \text{O}_2^{•−} \) according to the results shown in Fig. 3. Furthermore, the oxygen concentration had minimal effect on cell survival in the dark (Supplementary Fig. 30). Consequently, the conjugation of biotin moieties successfully endowed compound 1 with outstanding cancer selectivity and the capacity of \( \text{O}_2^{•−} \) generation, which allowing it to achieve targeted phototherapy regardless of oxygen dependence.

The PDT effectiveness of compound 3 and 6 were also assessed. As presented in Supplementary Fig. 31, compound 3 possessed a better photodynamic efficiency both under normoxia and hypoxia than compound 6 because it could produce both \( ^1\text{O}_2 \) and \( \text{O}_2^{•−} \), and the type I mechanism could still function well under depleted oxygen.

The hypoxia-tolerance PDT performance of compound 1 was also confirmed by CLSM experiments. We carried out the live/dead cell staining with Calcein-AM (green fluorescence for live cells) and propidium iodide (PI, red fluorescence for dead cells) to visualize the PDT outcome under both normoxic and hypoxic condition. The CLSM images in Fig. 4f intuitively demonstrated the severe damage to cancer cells by compound 1 exposed to white light under hypoxic condition, and its extensive red PI fluorescence was comparable to that under normal oxygen condition. These results proved that compound 1 could get rid of \( \text{O}_2^{•−} \)-dependence of traditional Type II PDT.

**The PDT mechanism of compound 1.** To further clarify the particular role of biotinylation in the hypoxia-tolerance PDT, MCF-7 cells treated with compound 1 were stained with the nonspecific ROS probe DCFH-DA\(^{11}\). As demonstrated in Fig. 5a–e, upon irradiation, a brightly green fluorescence of DCF was observed, implying the elevated intracellular ROS level by photo-activating compound 1 in MCF-7 cells. The fluorescence intensity was enhanced with the rise of light power from 10 to 20 mW/cm\(^2\), indicating that the production of ROS was related to the light dose. Gratifyingly, conspicuous green fluorescence of DCF was still detected in MCF-7 cells even in the case of hypoxic environment, highlighting that compound 1 was not seriously susceptible to oxygen depletion. The similar results were also verified by compound 3. In marked contrast, there was no fluorescence for compound 6 but obvious fluorescence for compound 3 under hypoxia (Supplementary Fig. 32a). It means that the biotinylation provides other ROS but not \( ^1\text{O}_2 \) to kill the tumor cells.

In order to further confirm that the generated ROS is \( \text{O}_2^{•−} \) other than \( ^1\text{O}_2 \) under hypoxia, dihydroethidium (DHE), an indicator of \( \text{O}_2^{•−} \), was chosen to co-stain MCF-7 cells with compound 1 under normoxic and hypoxic conditions\(^{42}\). The oxidized product of DHE by \( \text{O}_2^{•−} \) could intercalate into DNA to emit red fluorescence. As expected, remarkable red fluorescence was still detected in MCF-7 cells even in hypoxia (Fig. 5f, g), meaning that compound 1 can produce \( \text{O}_2^{•−} \) by irradiation under hypoxia. This type I PDT mechanism involving \( \text{O}_2^{•−} \) generation would offer PDT more satisfactory efficacy because \( \text{O}_2^{•−} \) could be catalyzed by intracellular SOD and transformed into other highly cytotoxic radicals (e.g., \( \text{OH}^+ \)) through Haber–Weiss reaction and Fenton reaction to realize the reuse of \( \text{O}_2 \), as proved in extensive previous reports\(^{13,18,19,43}\).

It is noteworthy that the ability of \( \text{O}_2^{•−} \) generation should be attributed to the biotinylation of the photosensitizers. This inference can also be supported by the following living-cells experiments of compounds 3 and 6. It is known that photosensitizer 6 is a traditional singlet oxygen generator and could not produce superoxide anions. But 3 can produce \( \text{O}_2^{•−} \) under white-light irradiation in both 21% O\(_2\) and 1% O\(_2\) conditions (Supplementary Fig. 32b). And these cell-experiments results are consistent with the solution-experiments results of Fig. 3 and Supplementary Fig. 26, indicating that the biotinylation induce the ability of \( \text{O}_2^{•−} \) generation in the all three photosensitizers.

**PDT efficacy ability on MCTS and in vivo.** As a tissue model, 3D multicellular tumor spheroid (MCTS) was widely used to mimic the conditions in solid tumors such as a hypoxic center and its proliferation gradients\(^{44–46}\). MCTS with an average diameter of 800 nm was used to evaluate the PDT efficacy of compound 1. MCTS was incubated with compound 1 for 4 h,
facilitates them to accept electrons, which endow them with the potential to produce more $\text{O}_2^-$ by the Type I process. As illustrated in Type I PDT mechanism (Fig. 7d), the triplet PS is transformed into a radical anion by accepting electrons from adjacent substrates and giving external electrons to oxygen to form $\text{O}_2^-$.\textsuperscript{47,49} Based on the results of theoretical calculation (Fig. 7e), the folded conformation of compound 1 support that more efficient electron transfer can happen between the biotin part and PS part. So, the biotinylation offers an electron-rich substrate in an intramolecular way, which should dramatically benefit to the Type I PDT mechanism.

Discussion

In summary, we designed and synthesized three PDT photosensitizers 1, 2, and 3, in which the introduction of biotin moiety was aimed to achieve tumor-targeting ability. Unexpectedly, the biotinylation endowed the three photosensitizers the ability of generating $\text{O}_2^-$. Considering that the three photosensitizers are different in structures and photophysical properties, the biotinylation can be considered as a potential universal strategy to design PDT photosensitizers which can simultaneously have the functions of tumor targeting and hypoxia tolerance. Photosensitizers designed by the strategy of biotinylation can possess the ability of $\text{O}_2^-$ and $\text{O}_2$ generation at the same time, which would resolve the paradox between traditional Type II PDT and hypoxia environment of solid tumors. Among the three photosensitizers, compound 1 exhibits a broad absorption window (400–700 nm), NIR (750 nm) emission and its combinational Type I/Type II PDT application was demonstrated in this work. We verified that the conjugation of electron-donating biotin moiety to photosensitizers could not only induce the tumor-targeting capability, but also Type I reaction for boosting the production of $\text{O}_2^-$ without affecting $\text{O}_2$ production by our experiments. Besides, 3D multicellular tumor spheroid was effectively disintegrated by compound 1 under white-light irradiation, so as to verify the hypoxia-tolerance PDT performance. In brief, the present work provides a strategy for the practicable design of photosensitizers with synergistic Type I/Type II PDT to relieve the limitation of tumor hypoxia.

Methods

This research complies with all relevant ethical regulations.

Materials. All solvents and reagents were purchased and used as received without further purification. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), LysoTracker Green DND-189, Hoechst 33342 and MitoTracker Green FM, Calcein-AM/propidium iodide (PI) Detection Kit, DHE (Dihydroethidium) were purchased from KeyGEN BioTECH Ltd (Nanjing, China). PpIX (Protoporphyrin) and NBT (nitroetetrazolium blue chloride) were purchased from Aladdin Industrial Corporation (Shanghai, China). All other reagents were purchased from Energy Chemical (Shanghai, China). The purity of all reagents purchased is above 95%. Silica gel (200–300 mesh) was used for flash-column chromatography.

Computational details. All calculations were performed by the Gaussian 09 software package. The molecular geometries were optimized by dispersion-corrected density function theory (DFT-D3) at B3LYP level of theory with 6-31G(d) basis set. The solvent effect (water) was taken into account using the PCM model to simulate the electrostatic environment in an aqueous solution. Cartesian coordinates of optimized compound 1 were provided in Supplementary Table 1.

$\text{O}_2$ detection. The $\text{O}_2$ generation of compounds 1 and 4 in ethanol was calculated by using DPBF. The absorbance decrease of DPBF at 411 nm was recorded for different durations of white-light irradiation (20 mW/cm²) to obtain the decomposition rate of the photosensitizing process. And during the measure, the initial value of absorption of 1 and 4 was consistent at 475 nm.

The same operation to compounds 2, 3, 5, and 6.

The mechanistic explanation of the biotinylation effect. Based on the above results, we can conclude that the ability of $\text{O}_2^-$ generation should be attributed to the biotinylation of the photosensitizers. A mechanistic explanation should be proposed. It is well known that Type I photosensitizers have a lower reduction potential owing to their stronger electron-accepting character.\textsuperscript{47,48} We studied the electrochemical properties of compounds 1–6 by cyclic voltammetry with Ferrocene (Fc) as the external standard. As shown in Fig. 7a–c, all the biotinylated three photosensitizers did show lower reductive potential than their counterparts. The anodic shift of the biotinylated photosensitizers was observed in the entire spheroid, which indicates that the MCTS was completely infiltrated by compound 1 (Supplementary Fig. 33). Upon white-light irradiation (40 mW/cm², 20 min), the photocytotoxicity of compound 1 (10 μM) for 4 h (0 d) and then exposed to white-light irradiation (40 mW/cm²) for 20 min each day (1 d, 2 d, 3 d). The experiment was repeated three times independently, with similar results. Scale bar: 400 μm.

Fig. 6 Photocytotoxicity of compound 1 to MCF-7 cells 3D MCTS. The CLSM images of MCTS incubated with compound 1 (10 μM) for 4 h (0 d) and then exposed to white-light irradiation (40 mW/cm²) for 20 min each day (1 d, 2 d, 3 d).
respectively. The solutions were irradiated for 3 min with white light (20 mW/cm²). NBT could react with O₂•− to form a dark insoluble precipitate.

The same operation to compounds 2, 5, 3, and 6.

**Photo-stability of DCF-TFM-Biotin.** The concentrated DMSO solution of compound 1 (1 mM) was diluted with RPMI-1640 cell culture medium containing 10% fetal bovine serum (FBS; GIBCO) and 1% antibiotics (80 U mL⁻¹ penicillin and 0.08 mg mL⁻¹ streptomycin; GIBCO). MCF-7 cells were cultured in RPMI-1640 cell culture medium (GIBCO) containing 10% fetal bovine serum (FBS; GIBCO) and 1% antibiotics (80 U mL⁻¹ penicillin and 0.08 mg mL⁻¹ streptomycin; GIBCO). Cultured cells were incubated in an atmosphere of 5% CO₂ at 37 °C.

**Confocal fluorescence imaging of cells.** COS-7 cells and MCF-7 cells were planted onto 35-mm confocal dishes at a density of 1 × 10⁵ cells. After incubation for 24 h, the medium was refreshed and washed with PBS twice. Fresh medium containing compound 1 (10 μM) was added and incubated for different times at 37 °C. COS-7 cells and MCF-7 cells were co-cultivated with compound 1 (10 μM) for 4 h at 37 °C. After removal of the medium and PBS washing for three times and the addition of fresh medium, cell imaging was conducted using the confocal laser scanning microscopy (CLSM). The excitation wavelength was 635 nm. Collection wavelength was from 690 nm to 790 nm for compound 1.

**Intracellular ROS detection.** A compound based on 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was employed as the intracellular ROS indicator and a compound was converted to DCF and emits bright green fluorescence in the presence of ROS. MCF-7 cells were plated onto 35 mm confocal dishes at a density of 1 × 10⁵ cells and cultured for 24 h at 37 °C under 5% CO₂. The cells were then incubated with 10 μM compound 1 for 4 h. After rinse with PBS, the cells were incubated with 1 μM DCFH-DA for another 30 min. The cells were washed with PBS and exposed to irradiation for 10 min with a LED lamp (400–800 nm, 20 mW/cm²). After irradiation, confocal fluorescence imaging was used to observe the intracellular ROS level. The excitation wavelength for DCF was 488 nm and emission wavelength was collected from 500 nm to 550 nm. In order to simulate hypoxic environment (1% O₂), Anaero Pack-Antaero and Anaero Pack-Micro Aéro (Mitsubishi Gas Chemical Company, Japan) were used. MCF-7 cells were plated onto 35 mm confocal dishes at a density of 1 × 10⁵ cells and cultured for 16 h under normoxic condition, and then the cells were incubated for another 8 h at 37 °C under hypoxic. Other operations were same to that in normoxic condition.

**Intracellular O₂•− detection.** The detection of O₂•− was performed using the similar procedure described for the detection of ROS, except that DHE (10 μM) was used as the O₂•−-specific probe. The red fluorescence signal of cells was collected by CLSM.

**Subcellular colocalization assay.** MCF-7 cells were planted onto 35 mm confocal dishes at a density of 1 × 10⁵ cells and incubated with 10 μM compound 1 for 4 h at 37 °C under 5% CO₂. The cells were further stained by 1 μM LysoSensor™ Green DND-189 for 30 min or 1 μM Hoechst 33342 or 1 μM Rho 123 for 10 min. Cells were then visualized with laser confocal microscopy. The excitation wavelength for compound 1 was 635 nm, while the excitation wavelength for LysoSensor™ Green DND-189 and Rho 123 was 488 nm, for Hoechst 33342 was 405 nm. The emission wavelength was collected from 500 nm to 550 nm. The emission wavelength was collected from 690 nm to 790 nm for compound 1, 500–550 nm for LysoSensor™ Green DND-189, and Rho 123, 440 to 480 nm for compound 4.

**Calcine-AM/PI staining of MCF-7 cells.** MCF-7 cells were planted onto 35 mm confocal dishes at a density of 1 × 10⁵ cells for 24 h at 37 °C under 5% CO₂. MCF-7 cells incubated with different following treatments were used: group 1, untreated; group 2, incubated with 10 μM compound 1 for 4 h without light irradiation; group 3, incubated with 10 μM compound 1 for 4 h followed by 400–800 nm LED light at a light dose of 20 mW/cm² for 10 min. Before imaging, each group was stained with 2 μM Calcine-AM and 8 μM PI. The fluorescence images of Calcine-AM/PI within MCF-7 cells were detected using confocal microscopy with the excitation wavelength of 488 nm, capture emission region from 500 nm to 550 nm for green channel, 600–640 nm for red channel.
In vitro PDT cytotoxicity assay. The MCF-7 or COS-7 cells were planted in 96-well plate (5000 per well) for 16 h, and another 8 h under normoxic (21% O2) or hypoxic (1% O2) atmosphere. After 24 h, the compound 1 at different concentrations was added and continued to incubate 4 h under normoxic (21% O2) or hypoxic (1% O2) atmosphere. After that, the cell culture media was replaced with 100 μL fresh medium. Subsequently, the cells were irradiated upon white light for 10 min (20 mW/cm2). After irradiation, the cells were again incubated for 24 h. Then, 10 μL MTT (0.5 mg/mL) was added to each well. After 4 h of incubation, the medium was removed carefully, and 150 μL DMSO was added to each well to dissolve the produced blue formazan. The absorbance value of each well was recorded with a microplate reader at 540 nm. The cell viability ratio was calculated by the following equation:

\[
\text{Cell viability} = \frac{OD_{	ext{pg}} - OD_{	ext{blank control}}}{OD_{	ext{Control}} - OD_{	ext{blank control}}} \times 100\% \tag{1}
\]

For dark toxicity measurement of compound 1, no light irradiation was applied to this experiment, and other steps were the same.

3D multicellular tumor spheroid (MCTS) model of MCF-7 cells. Multicellular tumor spheroids (MCTSs) from MCF-7 cells were obtained using hanging drop technique. 18 μL cell suspension was seeded on the lid, and then inverted onto the confocal dish and cultured for 3–6 days, consequently aggregating into tumor spheroids.

In vitro penetration. When the size of MCTSs reached about 800 μm in diameter, MCTSs were treated with compound 1 (10 μM) for 4 h. Optical sections of MCTSs were imaged under a CLSM from top to bottom with 25 μm per section.

PDT efficacy ability on MCTS. MCTSs were treated with compound 1 (10 μM) for 4 h. The spheroids were irradiated with 40 μW/cm² white light for 20 min. PDT experiments of MCTS last for 3d. The control group was only irradiated for 20 min/day.

Animals and tumor model. All animal experiments involved in this study have been approved by the local ethics committee of Dalian Medical University to establish a breast cancer model. In brief, 1 × 10⁶ 4T1 cells were subcutaneously injected into the right hind leg to establish 4T1-bearing BALB/c mice. The tumor volume of 4T1-bearing mice was allowed to grow to 25 mm³. Then the solvents were evaporated in vacuum. The crude product was purified by column chromatography (methanol/dichloromethane = 1:100–1:8). Compound 4 was obtained as black solid (100 mg, 24.4% yield). ¹H NMR (400 MHz, DMSO-d₆) δ 6.35 (d, J = 15.2 Hz, 1 H, 2 H), 7.82 (d, J = 7.2 Hz, 2 H), 7.59 (d, J = 7.2 Hz, 2 H), 7.07 (s, 1 H), 7.00 (d, J = 8.2 Hz, 2 H), 6.52–6.46 (m, 3 H), 6.26 (dd, J = 7.2 Hz, 2 H), 6.14 (d, J = 7.2 Hz, 2 H), 5.98–5.92 (m, 2 H), 5.79 (d, J = 8.2 Hz, 2 H), 4.80 (dd, J = 7.2 Hz, 2 H), 4.71 (d, J = 7.2 Hz, 2 H), 4.14 (m, 2 H), 3.86 (m, 3 H), 3.72 (m, 3 H), 3.52 (m, 3 H), 3.38–3.29 (m, 3 H), 2.95–2.90 (dd, J = 10.0, 5.0 Hz, 1 H), 2.71 (d, J = 10.0, 5.0 Hz, 1 H), 1.78 (t, J = 7.2 Hz, 2 H), 1.60–1.46 (m, 4 H), 1.49–1.29 (m, 2 H), 1.25 (t, J = 7.2 Hz, 3 H), 1.28 (m, 2 H), 0.86 (m, 2 H), 0.78 (m, 1 H), 0.75 (m, 2 H). ¹³C NMR (100 MHz, DMSO-d₆) δ 172.7, 163.4, 160.5, 154.9, 150.9, 130.9, 129.9, 129.3, 128.7, 127.1, 118.2, 115.8, 115.0, 107.9, 106.0, 105.3, 19.8. HRMS (m/z) [M-H]⁻ found, 783.0805; calcd. for C₉₈H₇₅Cl₄N₄O₉, 783.0787.

5-(2-oxo-2,5-dihydro-1H-thiophene-3-ylidene)malononitrile (4). Compound 11 was prepared by a modification of previous method52. SOCl₂ (0.30 mL, 40 mmol) was added to the suspension of bisoxime (300 mg, 1.23 mmol) in MeOH (30 mL). 10% Pd/C was added under ambient overnight at room temperature in a clear solution. After hydrogenation of the solvent and excess PdO under reduced pressure, biotin methyl ester (29 mg) was obtained and used directly for the next reaction. Biotin methyl ester (296 mg, 1.14 mmol) was dispersed in MeOH (2.5 mL) and hydrazine (0.48 mL, 10 mmol) was added with stirring. The mixture was refluxed for 16 h at 70°C. Cooled to room temperature, excess methanol was added for washing, extraction and drying to give Biotin–NH–NH₂ (279.4 mg, 95% yield) as a yellow solid: ¹H NMR (400 MHz, D₂O) δ 5.15–5.00 (m, 2 H), 3.82–3.74 (m, 3 H), 3.54 (m, 3 H), 3.18 (m, 3 H), 2.88–2.78 (m, 4 H), 1.30 (m, 2 H), 0.88 (m, 2 H), 0.78 (m, 2 H). ¹⁳C NMR (100 MHz, D₂O) δ 157.4, 157.0, 134.6, 110.0, 109.5, 108.9, 108.7, 50.0, 48.6, 48.1, 47.9, 46.8, 46.4, 45.4, 44.7, 42.6, 31.6, 31.5, 29.8, 27.9, 27.7, 27.6, 24.1, 22.8, 21.9, 14.0. HRMS (m/z) [M–H]⁻ found, 763.0885; calcd. for C₉₆H₇₅Cl₄N₄O₈, 763.0787.

Syntheses of compounds

4-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthene-9-yl) benzoic acid (7). Compound 7 (400 mg, 1.0 mmol) and hexamethylenetetramine (1.0 g, 5.0 mmol) were dissolved in triluoroacetic acid (15 mL) and magnetically stirred at 90°C overnight. Then, cooled to room temperature. 2 N hydrochloric acid was added with stirring until the orange solid did not precipitate and the cake was washed with deionized water until the filtrate became colorless. After drying, the crude product 8 (429.2 mg) was obtained and used directly in subsequent reactions.

2-(3-cyano-4,5,5-trimethylfuranyl-2(H)-ylidene) malononitrile (9). 3-hydroxy-3-methylbutan-2-one (1.02 g, 10.0 mmol), malononitrile (1.98 g, 30.0 mmol) and magnesium ethoxide (1.25 g, 11.0 mmol) were dissolved in ethanol and magnetically stirred at 60°C overnight. Then the solvent was evaporated in vacuum. After the addition of 10 mL of dichloromethane was added to the reaction flask, sonicated, extracted and the filter cake was washed with dichloromethane (10 mL × 3). The filtrate was evaporated in vacuum to obtain a yellow-brown crude product 9 (1.91 g) and used directly in subsequent reactions.

4-(2,6-dimethyl-4H-pyranyl-4-ylidene)malononitrile (10). 2,6-dimethyl-4H-pyran-4-one (0.62 g, 5.0 mmol) and malononitrile (0.53 g, 5.0 mmol) were added to a 100 mL round bottom flask. 10 mL of acetic anhydride was added to the mixture and stirred at 140°C for 6 h. The heating and stirring were stopped and the reaction solution was stood overnight, then filtered. The filter cake was washed with n-hexane (15 mL × 3) and dried to give a brown crude product 10 (0.832 g).

5-(2-oxo-2,5-dihydro-1H-thieno[3,4-d]imidazol-4-yl)-pentanehydrazide (11). Compound 11 was prepared by a modification of previous method52. SOCl₂ (0.30 mL, 40 mmol) was added to the suspension of bisoxime (300 mg, 1.23 mmol) in MeOH (30 mL). 10% Pd/C was added under ambient overnight at room temperature in a clear solution. After hydrogenation of the solvent and excess PdO under reduced pressure, biotin methyl ester (29 mg) was obtained and used directly for the next reaction.
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Author contributions
F.L.S. designed and guided the overall research project. J.A. as the first author designed the experiments and wrote the manuscript. S.L.T. and G.B.H. assisted the synthesis. W.L.C. and M.M.C. involved cellular and mice experiments. I.T.S., Z.L.L., X.J.P., and W.H.Z. provided intellectual input and revised the manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Fengling Song or Wen-Heng Zheng.

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