Research paper

A tumor-targeted activatable phthalocyanine-tetrapeptide-doxorubicin conjugate for synergistic chemo-photodynamic therapy

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Chemo-photodynamic therapy is a promising strategy for cancer treatments. However, it remains a challenge to develop a chemo-photodynamic therapeutic agent with little side effect, high tumor-targeting, and efficient synergistic effect simultaneously. Herein, we report a zinc(II) phthalocyanine (ZnPc)-doxorubicin (DOX) prodrug linked with a fibroblast activation protein (FAP)-responsive short peptide with the sequence of Thr-Ser-Gly-Pro for chemo-photodynamic therapy. In the conjugate, both photosensitizing activity of ZnPc and cytotoxicity of DOX are inhibited obviously. However, FAP-triggered separation of the photosensitizer and DOX can enhance fluorescence emission, singlet oxygen generation, dark- and photo-cytotoxicity significantly, and lead to a synergistic anticancer efficacy against HepG2 cells. The prodrug can also be specifically and efficiently activated in tumor tissue of mice. Thus, this prodrug shows great potential for clinical application in chemo-photodynamic therapy.

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1. Introduction

Photodynamic therapy (PDT) is a promising mini-invasive therapeutic modality for cancers [1–3]. It destroys target cells through the combined action of a photosensitizer and molecular oxygen upon irradiation with light to generate reactive oxygen species (ROS). Various strategies have been utilized to develop desirable photosensitizers for PDT [4–6]. However, monotherapy for cancers usually could not achieve satisfactory therapeutic outcomes [7,8]. Recently, combination therapies such as chemochemo, chemo-thermal, and chemo-photodynamic therapies have been considered to be promising strategies for cancer treatments, owning to several advantages including enhancement of therapeutic efficacy, decrease of side effects, and remission of drug-resistance problem [9–14].

Development of conjugates of photosensitizers and chemotherapeutic drugs for chem-photodynamic therapy have attracted increasing attention [15–20]. However, almost all of these chemophotodynamic therapeutic conjugates are not specifically activatable prodrugs, and show synergism or antagonism simultaneously to cancer tissue as well as normal tissue. Recently, You et al. developed several visible/far-red light-activatable chemophotodynamic therapeutic prodrugs by taking advantage of the singlet oxygen generated by photosensitizers [21]. The work provided a novel strategy to design activatable chem-photodynamic therapeutic prodrugs, however, the photosensitizing activity of the prodrugs was not shielded. It is possible to cause photosensitizing damage to normal tissue. In addition, the external stimulus-activated prodrugs could not be activated at tumor tissue automatically. Thus, developing a target site-activatable chemo-photodynamic therapeutic prodrug, which can effectively reduce side effects and be selectively activated in tumor microenvironment, is crucial to obtain a desirable outcome of chemo-photodynamic therapy.

Cancer-associated fibroblasts (CAF), which are an important component of tumor stroma, play a critical role in tumor development, including invasion and metastasis [22,23]. Fibroblast activation protein (FAP), a transmembrane serine protease, is highly expressed on CAF in over 90% of human epithelial neoplasms, but it is not expressed in epithelial cancer cells, normal fibroblasts, and other normal tissues [24–27]. With a post-prolyl peptidase activity, FAP is able to cleave substrates with proline as the penultimate
amino acid [28—30]. Thus, FAP has been considered as a potential target for the diagnosis and therapy of numerous carcinomas.

Doxorubicin (DOX) is a well-known broad-spectrum chemotherapeutic drug for various carcinomas, but it is usually restricted in clinical application due to severe side effects, especially cardiotoxicity [31,32]. To circumvent this issue, the DOX-based nanoparticles and conjugates have been widely developed [33—35]. In this work, we report a novel FAP-responsive chemo-photodynamic therapeutic prodrug, ZnPc-TSGP-DOX (5) (Fig. 1), in which a photosensitizer, zinc(II) phthalocyanine (ZnPc) modified with carboxyl group, is covalently conjugated with DOX via a FAP-sensitive peptide with the sequence of Thr-Ser-Gly-Pro. By conjugating the ZnPc with DOX, the photodynamic activity of the photosensitizer can be obviously inhibited, and meanwhile, DOX shows less cytotoxicity. Nevertheless, after being cleaved by FAP, the prodrug shows significantly enhanced cytotoxicities both upon illumination and in the dark, exhibiting an obviously synergistic effect. In addition, a ZnPc-DOX conjugate (6) without the short peptide linker has also been prepared as a negative control. To our best knowledge, this conjugate is the first chemo-photodynamic therapeutic prodrug that can be specifically activated by tumor-associated stimul.

2. Results and discussion

2.1. Synthesis and characterization

Scheme 1 shows the synthetic route for the ZnPc-TSGP-DOX conjugate. Firstly, a mixed cyclization of carboxyl-modified phthalonitrile 1 [36] and unsubstituted phthalonitrile 2 in the presence of Zn(OAc)2, K2CO3, and 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) in n-pentanol gave the carboxyl-mono-substituted ZnPc 3. After activation with N-hydroxysuccinimide (NHS) under the catalysis of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), the carboxyl group of ZnPc 3 could easily couple with the N terminus of Thr-Ser-Gly-Pro peptide, which was afforded by deprotecting Fmoc group of the commercially available Fmoc-Thr-Ser-Gly-Pro peptide using diethylamine (DEA) in CH2Cl2 solution, to afford compound 4. Finally, the carboxyl group of proline of 4 and DOX underwent an amidation using EDCI and 1-hydroxybenzotriazole (HOBt) as activating agents in the presence of N-methylmorpholine (NMM) to give the final product 5 in a good yield of 81%. Similarly, through an amidation of ZnPc 3 and DOX under EDCI, HOBt, and NMM conditions, the analogue ZnPC-DOX conjugate 6 as a non-cleavable control was prepared in 71% yield (Scheme 2).

All the new compounds were well characterised with various spectroscopic methods, and 4–6 were shown to be pure by HPLC analysis (see Supplementary data).

2.2. Spectroscopic and photosensitizing properties

The electronic absorption spectra of 4–6 were recorded in N,N-dimethylformamide (DMF) and the data are compiled in Table 1. As shown in Fig. S1, all the compounds exhibited a typical spectrum for non-aggregated phthalocyanine, with a B-band at 334—344 nm, a vibronic band at 607—611 nm, and a sharp and intense Q-band at 674—676 nm, which strictly obeyed the Lambert-Beer law. It is worth noting that for the spectra of 5 and 6, there was another relative weak band at 492 nm (Fig. S1b and S1c), which could be assigned to the absorption of DOX. Upon excitation at 610 nm, these compounds showed a fluorescence emission at ca. 684 nm with fluorescence quantum yields ($\phi_F$) of 0.10—0.23 relative to unsubstituted ZnPc ($\phi_F = 0.28$ [37]). Obviously, the $\phi_F$ values of 5 and 6 were lower than that of 4, suggesting the DOX unit could cause some quenching effect on ZnPc unit in the conjugates possibly through an electron-transfer process [17].

The singlet oxygen quantum yields ($\phi_A$) of these compounds were also evaluated in DMF by a steady-state method by using 1,3-diphenylisobenzofuran (DPBF) as a scavenger [38]. The $\phi_A$ values could be determined according to the rates of decay of DPBF. Their $\phi_A$ values ranged from 0.30 to 0.60 relative to unsubstituted ZnPc ($\phi_A = 0.56$ [39]) (Table 1). The photodagradation of DPBF for 5 and 6 was slower than that for 4 (Fig. S2). Therefore, the singlet oxygen generation of 5 and 6 was also partly quenched due to the introduction of DOX.

To investigate the quenching effects in aqueous solution, the spectroscopic properties and singlet oxygen generation efficiency of 4–6 were also measured in phosphate buffered saline (PBS) with 0.1% Cremophor EL. As shown in Fig. 2a, compound 4 still showed intense and sharp Q-band in the aqueous solution, while the Q-bands of 5 and 6 were obviously broader and lowered in intensity, suggesting the two conjugates are more aggregated than 4 in PBS. Upon excitation at 610 nm, 4 showed strong fluorescence emission at 688 nm, but conjugates 5 and 6 exhibited much lower fluorescence emission compared with 4 (Fig. 2b). The singlet oxygen generation efficiency in PBS was also evaluated by comparing the rate of photodgradation of DPBF induced by these compounds (Fig. 2c). It can be seen that upon illumination, 4 could induce the decay of DPBF quickly in PBS. By contrast, both 5 and 6 could not generate singlet oxygen efficiently under the same condition. The results indicate that the quenching effects caused by conjugating with DOX are much more prominent in aqueous solution than in DMF. It could be attributed to not only the quenching of DOX but also the aggregation behavior in the aqueous solution.
2.3. FAP-responsive property

To demonstrate the FAP-responsive property of conjugate 5, the fluorescence change was monitored in the presence or absence of FAP at different incubation time in PBS containing 0.1% Cremophor EL. The fluorescence change of 6 was also recorded for comparison. As shown in Fig. 3a and S3a-b, the fluorescence intensity of 5 hardly changed over 60 h in the absence of FAP, while upon exposure to FAP, its fluorescence intensity increased gradually with time and reached a plateau at 36 h, suggesting the peptide linker of 5 could be cleaved by FAP efficiently, leading to diminishing the fluorescence quenching caused by DOX. By contrast, the fluorescence intensity of the non-cleavable analogue 6 remained almost unchanged over 60 h whether with FAP or not (Fig. 3a and S3c-d).

The FAP-triggered cleavage was also evaluated by measuring singlet oxygen generation efficiency. Fig. 3b compared the rate of photodegradation of DPBF induced by 5 and 6 in the presence or absence of FAP for 30 h in PBS. The singlet oxygen generation efficiency of 5 remained very low without FAP. On the contrary, upon exposure to FAP, its singlet oxygen generation efficiency was enhanced obviously, which also could be attributed to the cleavage of the peptide linker. However, the results of the analogue 6 in both cases were very similar with that of 5 without FAP.

In addition, the cleavage efficiency trigged by FAP was further confirmed by the HPLC analysis, monitored at 680 nm for the absorption of ZnPc part. It can be seen from Fig. 4a that only the chromatographic peak of 5 could be observed with retention time at ca. 2.3 min at 0 h, and after incubation with FAP for 24 h, the peak diminished greatly. Meanwhile, a new peak accounting for 77% with retention time at ca. 8.9 min appeared, which can be assigned to the product of enzymolysis induced by FAP, namely compound 4.

The rate of cleavage, as reflected by the decrease of the percentage of the conjugate, was calculated according to the HPLC chromatograms. As shown in Fig. 4b, in the presence of FAP, the rate of cleavage of 5 increased quickly within 24 h, and after 72 h, the cleavage was more than 90%. However, the cleavage could not be observed basically for the other three cases. The results again suggest that the linker of Thr-Ser-Gly-Pro peptide is highly sensitive to FAP, leading to efficient cleavage of 5.

2.4. In vitro studies

To explore in vitro synergistic anticancer efficiency, the cytotoxicities of these compounds (DOX, 4–6, 5 + FAP, and 6 + FAP)
Fig. 2. (a) UV–vis and (b) fluorescence spectra of 4–6 (all at 2 μM) in PBS with 0.1% Cremophor EL. (c) The rate of photodegradation of DPBF sensitized by 4–6 (all at 5 μM) in the same aqueous solution. The curves of photodegradation of DPBF sensitized by 5 and 6 are overlapped.

Fig. 3. (a) Changes in fluorescence intensity of 5 and 6 (both at 2 μM) in the presence or absence of FAP (2 μg mL⁻¹) in PBS with 0.1% Cremophor EL. (b) Comparison the rate of photodegradation of DPBF (60 μM) sensitized by 5 and 6 (both at 5 μM) in the presence or absence of FAP (5 μg mL⁻¹) for 30 h in PBS with 0.1% Cremophor EL. The curves of photodegradation of DPBF sensitized by 6 in the presence or absence of FAP are overlapped.

Fig. 4. (a) HPLC profiles of 5 incubating with FAP in PBS with 0.05% Cremophor EL at 0 h and 24 h, monitored at 680 nm. (b) Cleavage rates of 5 and 6 monitored by HPLC in the presence or absence of FAP in PBS with 0.05% Cremophor EL. P and P₀ mean the percentage of area of 5 or 6 on HPLC chromatogram at different time intervals and 0 h, respectively.
against HepG2 human hepatocarcinoma cells were evaluated both in the dark and upon illumination (λ > 610 nm) via a colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [40]. The IC\textsubscript{50} values, defined as the drug concentration required to kill 50% of the cells, were summarized in Table 2. Fig. 5a compared the dark toxicities of these compounds. It can be found that, in the absence of light, 4 – 6 were essentially non-cytotoxic toward HepG2 cells at low concentration (1 μM), which could lead to most of cell death upon illumination. DOX (IC\textsubscript{50} = 2.72 μM) exhibited higher cytotoxicity than 5 and 6, particularly 6 (IC\textsubscript{50} = 20 μM). It could be attributed to the inhibition of the binding between DOX and DNA as a result of the modification of amino group on the sugar moiety of DOX [41]. As expected, after pre-treating with FAP for 24 h, the dark toxicity of 5 was remarkably enhanced with over 5-fold reduction of IC\textsubscript{50} value relative to that of 5 alone. Upon illumination, 5 showed apparently lower cytotoxicity than 4 (Fig. 5b). Moreover, both the dark toxicity and photocytotoxicity of 5 pre-treated with FAP are comparable with that of the mixture of 4 and DOX, also suggesting that 5 could essentially be cleaved by FAP (Table 2). Nevertheless, in the presence of FAP, the photocytotoxicity of 5 was significantly enhanced, and it was obviously more cytotoxic than 4, reflecting that 5 could be cleaved efficiently by FAP and released free photosensitizer and DOX at cellular level. By contrast, for 6, whatever pre-treated with FAP or not, it showed comparable dark- and photo-cytotoxicity (Fig. S4 and Table 2).

To further evaluate the synergism efficiency, the combination index (CI), which reflects the interaction effect of dual drugs, was utilized [42,43]. CI could be calculated according to the following equation

\[
CI = \frac{D_A/A\cdot B}{D_A} + \frac{D_B/A\cdot B}{D_B}
\]

(1)

where \(D_A/A\cdot B\) and \(D_B/A\cdot B\) are the dose for DOX part and phthalocyanine part in the conjugate, respectively, which has a 50% cancer cell-killing effect, \(D_A\) and \(D_B\) are the dose for DOX and compound 4, respectively, which has a 50% cancer cell-killing effect. CI < 1, =1, and >1 indicate synergism, additive effect, and antagonism, respectively. The CI values of 5 in the absence and presence of FAP are 1.61 and 0.39, respectively, indicating that an antagonism occurred for 5 alone, while pre-treated with FAP, 5 could be cleaved and exhibited a synergistic chemo-photodynamic therapy.

The FAP-responsive cleavage of 5 was further investigated at cellular level by using confocal laser scanning microscopy. HepG2 cells were incubated with 4, 5, and 6 + FAP for 24 h, respectively, and then the intracellular fluorescence images were detected. As shown in Fig. 6, compound 4 showed strong intracellular fluorescence, while for 5, nearly no intracellular fluorescence could be observed. However, after pre-treatment with FAP, the intracellular fluorescence of 5 was enhanced 4 folds, and the fluorescence intensity was mostly restored, suggesting that 5 could be activated efficiently by FAP followed by uptake by the cells.

The subcellular localization of these compounds (4 + DOX, 5, 6, 5 + FAP, and 6 + FAP) in HepG2 cells was investigated by confocal laser scanning microscopy. Fig. 7 shows the fluorescence distribution of the cells incubated with 4 + DOX and 5 + FAP, respectively. For the cells incubated with the mixture of 4 and DOX, the intracellular fluorescence of 4 was well overlapped with the fluorescence of MitoTracker, but not with that of Hoechst 33342, showing that 4 is preferentially localized in mitochondria. However, DOX mainly accumulates in cell nucleus. The observation is consistent with the previously reported result that DOX could bind to DNA of nucleus, leading to cell death [41]. For the cells incubated with 5 + FAP, the distributions of fluorescence caused by phthalocyanine and DOX were obviously different in the cells. The intracellular fluorescence of phthalocyanine was observed mostly in mitochondria but not in nucleus, while the fluorescence of DOX mainly superimposed with that of Hoechst 33342. It is again demonstrated that 5 is FAP-responsive and able to release free photosensitizer and DOX. As shown in Fig. S5, for the cases of 5 alone and 6 in the presence or absence of FAP, the fluorescence caused by phthalocyanine and DOX showed similar distribution in the cells. The fluorescence could not be observed in nucleus, but largely overlapped with that caused by mitochondria, suggesting that both conjugates are essentially intact and mostly localized in mitochondria under these conditions (5 without FAP and 6 were or without FAP). The results confirm that, in the two conjugates, the binding between DOX and DNA was impeded, which would be an important factor for the much lower dark cytotoxicity of 5 and 6 relative to free DOX.

2.5. In vivo study

To evaluate the activation ability of conjugate 5 in tumor tissue, we further performed in vivo fluorescence imaging. The mice bearing H22 murine hepatocellular tumor were intravenously injected with 5 at a dose of 2 μmol kg\textsuperscript{-1} via tail vein. Compound 4, a free photosensitizer, was used as a control. The whole-body fluorescence images were detected continuously for 96 h. As shown in Fig. 8a, shortly after injection, the fluorescence of compound 4 quickly spread throughout the whole body along with the distribution of 4 in the body, and the whole-body fluorescence signal could still be observed until 24 h. By contrast, for the mice injected with 5 (Fig. 8b), there was still no detectable fluorescence signal on the mice at all within 1 h of injection, although 5 had entered the body. After 2 h post-injection, weak fluorescence signal could be observed only in tumor tissue as a result of selective activation of 5 in this region. The signal increased gradually over time, and reached the strongest at 24 h. Interestingly, the fluorescence signal was hardly observed at other parts of the body except the tumor region at all time. The results indicate that, as expected, conjugate 5 is inactivated during circulation in the body, and could be efficiently and specifically activated in tumor tissue.

3. Conclusions

We have successfully designed and synthesized a novel FAP-responsive phthalocyanine-DOX conjugate (5) using a tetrapeptide with the sequence of Thr-Ser-Gly-Pro as a linker for synergistic chemo-photodynamic therapy. The fluorescence and photosensitizing ability of 5 are significantly decreased relative to the phthalocyanine alone as a result of quenching of DOX and more aggregation of the conjugate. Moreover, the cytotoxicity of DOX against HepG2 cells is obviously inhibited in the conjugate. However, once cleaved by FAP, the conjugate releases free photosensitizer and DOX efficiently both in aqueous solution and at cellular level, leading to significant enhancement of fluorescence emission, singlet oxygen generation, dark- and photo-cytotoxicity, and thus

<table>
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<tr>
<th>Compd</th>
<th>IC\textsubscript{50} (μM) with light in dark</th>
<th>Compd</th>
<th>IC\textsubscript{50} (μM) with light in dark</th>
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<tr>
<td>DOX</td>
<td>3.54</td>
<td>4</td>
<td>0.39</td>
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<tr>
<td>5</td>
<td>0.56</td>
<td>4 + FAP</td>
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<tr>
<td>6</td>
<td>0.42</td>
<td>6 + FAP</td>
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<td>4 + DOX</td>
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exhibits a synergistic anticancer efficacy against HepG2 cells. Moreover, study of in vivo fluorescence imaging preliminarily indicates 5 can be activated specifically and efficiently in tumor tissue of mice. Thus, this conjugate can serve as a promising prodrug for combination of chemo-photodynamic dual therapy.

4. Experimental

4.1. Materials

All the reactions were performed under an atmosphere of nitrogen. DMF and n-pentanol were dried by a Deminex EX-SPS-800 solvent purification system. Chromatographic purifications were performed on silica gel columns (100–200 mesh, Qingdao Haiyang Chemical Co., Ltd., China) with the indicated eluents. Size-exclusion chromatography were carried out Bio-Rad Bio-Beads S-X3 beads with the indicated eluents. HOBt was obtained from Shanghai MEDPEP Co., Ltd., China. DBU, NHS, and EDCI were bought from TCI Shanghai, China. DOX and Fmoc-Thr-Ser-Gly-Pro were purchased from IFFECT CHEMPHAR Co., Ltd., China and GL Biochem Ltd., China, respectively. FAP was commercially obtained from Sino Biological Inc., China. Compound 1 was prepared as previously reported[30]. All other solvents and reagents were of reagent grade and used as received.
4.2. Characterization

$^1$H NMR spectra were determined on a Bruker-400 spectrometer (400 MHz) in CDCl$_3$ with a trace amount of pyridine-d$_5$. Chemical shifts were relative to internal SiMe$_4$ ($\delta = 0$ ppm). High-resolution mass spectra (HRMS) were recorded on a Thermo Scientific Exact Mass Plus LC/MS spectrometer. Elemental analyses were performed using Vario Micro equipment. Reversed-phase analytical HPLC experiments were carried out on a LiChrospher RP-18 column (5 μm, 4.0 × 125 mm) using a Shimadzu LC-10AT liquid chromatograph containing a SCL-10A controller and a SPD-M10A diode array detector monitored by absorbance at 680 nm. A mixed solvents containing an aqueous solution of sodium dodecyl sulfate (10 mM) and phosphoric acid (26 mM), acetonitrile and MeOH (250/250/30, v/v/v) were used as solvent A, and DMF was used as solvent B. The flow-rate was fixed at 1.0 mL/min.

4.3. Synthesis of zinc(II) phthalocyanine 3

A mixture of 3-[4-(2-carboxyethyl)phenoxy]phthalonitrile (1) (147 mg, 0.50 mmol), K$_2$CO$_3$ (69 mg, 0.50 mmol) and unsubstituted phthalonitrile (2) (320 mg, 2.5 mmol) in n-pentanol (20 mL) was stirred at 90 °C for 10 min, and then anhydrous zinc acetate (275 mg, 1.50 mmol) and DBU (0.50 mL, 3.35 mmol) were added. The resulting mixture was stirred at 130 °C for 12 h. After a brief cooling, the volatiles were removed in vacuo. The residue was dissolved in DMF (5 mL), and then was acidiﬁed with dilute HCl (1.0 M) until pH~5 to produce a large amount of dark green precipitate, which was collected by ﬁltration and washing with water until pH~7. After drying in vacuo, the crude was further puriﬁed by size-exclusion chromatography by using CH$_2$Cl$_2$/tetrahydrofuran (THF) (5:1, v/v) as eluent to give a blue solid 3 (39.8 mg, 81%). HRMS (ESI): m/z Calcd for C$_{35}$H$_{33}$N$_{13}$O$_9$Zn [M+H]$^+$ 1081.2724, found: 1081.2724. The purity was found to be 95.4% by HPLC analysis.

4.4. Synthesis of ZnPc-peptide conjugate 4

The Fmoc protecting group of Fmoc-Thr-Ser-Gly-Pro was ﬁrst removed by using 50% diethylamine (DEA) in CH$_2$Cl$_2$.

A mixture of compound 3 (44.6 mg, 0.06 mmol), NHS (13.8 mg, 0.12 mmol), and EDCI (23.0 mg, 0.12 mmol) in DMF (4 mL) was stirred at 0 °C for 2 h, and another 6 h at room temperature. The volatiles were removed in vacuo, and the residue was puriﬁed by silica gel column chromatography by using CH$_2$Cl$_2$/tetrahydrofuran (THF) (5:1, v/v) as eluent to give a crude. Then, a solution of the Fmoc-deprotected Thr-Ser-Gly-Pro tetrapeptide in DMF (3 mL) was added to the NHS-activated crude and stirred in the presence of N,N-Diisopropylethylamine (DIEPA) (0.6 mL) at room temperature in the dark overnight. The reaction mixture was treated with ice water (300 mL), and acidiﬁed with HCl (1.0 M) to produce a large amount of dark green precipitate, which was collected by ﬁltration and washing with water until pH~7. After drying in vacuo, the crude was further puriﬁed by size-exclusion chromatography by using DMF as the eluent to give a blue solid 4 (29.0 mg, 44%). HRMS (ESI): m/z Calcd for C$_{55}$H$_{53}$N$_{13}$O$_9$Zn [M+H]$^+$ 1608.4515, found: 1608.4482. The purity was found to be 96.9% by HPLC analysis.

4.5. Synthesis of ZnPc-TSGP-DOX conjugate 5

A mixture of compound 4 (32.5 mg, 0.03 mmol), HOBt (12.2 mg, 0.09 mmol), and EDCI (17.3 mg, 0.09 mmol) in DMF (3 mL) was stirred at 0 °C for 20 min, and then DOX (19.1 mg, 0.033 mmol) and NMM (15.0 μL, 0.10 mmol) were added. The mixture was further stirred at room temperature overnight. The reaction mixture was treated with water (40 mL) to produce a greenish-gray precipitate, which was collected by ﬁltration and washing with a solution of citric acid (pH 5–6) and water. The crude was re-dissolved in DMF (3 mL) and then precipitated by the addition of diethyl ether (~30 mL), followed by centrifugation to remove the supernatant. This puriﬁcation procedure was repeated three times and the product was dried in vacuo to obtain a greenish-gray solid 5 (39.8 mg, 81%). HRMS (ESI): m/z Calcd for C$_{82}$H$_{73}$N$_{13}$O$_{19}$Zn [M+H]$^+$ 1608.4515, found: 1608.4482. The purity was found to be 96.9% by HPLC analysis.

4.6. Synthesis of ZnPc-DOX conjugate 6

A mixture of compound 3 (37.1 mg, 0.05 mmol), HOBt (20.3 mg,
0.15 mmol, and EDCI (28.8 mg, 0.15 mmol) in DMF (3 mL) was stirred at 0 °C for 20 min, and then DOX (29.0 mg, 0.050 mmol) and NMM (15.0 μL, 0.10 mmol) were added. The mixture was further stirred overnight at room temperature. The reaction mixture was treated with water (40 mL) to give greenish-gray precipitate, which was collected by filtration and washing with water. After drying in vacuo, the crude was purified by silica gel column chromatography by using CH2Cl2/MeOH (1:1, v/v) as eluant to give a greenish-gray solid 6 (45.0 mg, 71%). HRMS (ESI): m/z Calcd for C40H40O12Zn [M − H]− 1264.2820, found: 1264.2836. ¹H NMR (CDCl3 with a trace amount of pyridine-d₅, 400 MHz): δ 12.57 (s, 1 H, OH), 12.37 (s, 1 H, OH), 9.32–9.37 (m, 3 H, Pc-H), 9.23–9.26 (m, 2 H, Pc-Hz), 9.11 (d, 1 H, J = 7.6 Hz, Pc-H), 7.87 (d, 1 H, J = 7.2 Hz, Pc-H), 8.05–8.07 (m, 4 H, Pc-Hz), 7.93–7.98 (m, 2 H, Pc-Hz); 7.84 (t, 1 H, J = 7.6 Hz, Pc-Hz); 7.61 (d, 1 H, J = 7.6 Hz, Pc-Hz); 7.47 (d, 2 H, J = 8.0 Hz, Ar-H); 7.17 (virtual d, 2 H, J = 8.0 Hz, Ar-H), 7.05 (t, 1 H, J = 8.0 Hz, Ar-H), 6.90 (d, 1 H, J = 7.2 Hz, NH), 6.69 (d, 1 H, J = 8.0 Hz, Ar-H), 6.18 (d, 1 H, J = 7.2 Hz, Ar-H), 4.93 (s, 1 H, OH), 4.56–4.63 (m, 3 H, CHz, CH), 4.29 (s, 1 H, OH), 3.74–3.82 (m, 2 H, CH), 3.47 (s, 3 H, OCH3), 3.45 (s, 1 H, OH), 2.81–2.86 (m, 1 H, CHz), 2.68–2.79 (m, 2 H, CHz), 2.22–2.40 (m, 3 H, CHz), 2.22 (d, 1 H, J = 14.8 Hz, CHz), 1.99 (d, 1 H, J = 15.2 Hz, CHz), 1.15–1.55 (m, 4 H, CHz), 1.01 (d, 3 H, J = 6.0 Hz, CH3). The purity was found to be 95.5% by HPLC analysis.

4.7. Photophysical and photochemical studies

Electronic absorption spectra were measured on a Shimadzu UV-2450 UV−vis spectrophotometer. Fluorescence spectra were taken on an Edinburgh FL9000/FS9000 spectrofluorimeter.

The fluorescence quantum yields (Φf) were determined in DMF by the equation: Φf(sample) = (Fl(sample)/Fl(ref)) × (Aref/A(sample)) × Φf(ref), where F and A are the measured fluorescence (area under the fluorescence spectra) and the absorbance at the excitation position (610 nm), respectively. The unsubstituted zinc(II) phthalocyanine (ZnPc) in DMF was used as the reference (Φf(ref) = 0.28) [37].

The Φf were determined in DMF by a steady-state method using DPBF as the scavenger. A solution of the photosensitizer (compound 4, 5 or 6) (4 μM) containing DPBF (40 μM) were prepared in DMF in the dark and irradiated with red light immediately, then DPBF degradation at 412 nm was monitored along with irradiation time. The Φf was calculated by the equation: Φf(4, 5 or 6) = (Ik(sample)/Ik(ref)) × (Aref/A(sample)) × Φf(ref), where Φf(4, 5 or 6) is the singlet oxygen quantum yield of the reference (unsubstituted ZnPc) in DMF (Φf(ref) = 0.56) [39]. Ik(sample) and Ik(ref) are the photobleaching rates of DPBF in the presence of the samples and reference, respectively; A(sample) and A(ref) are the absorbance at Q band (area under the absorption spectra in 610−750 nm) of the samples and reference, respectively. The light source consisted of a 150 W halogen lamp, a water tank for cooling, and a color glass filter cut-on 610 nm. The fluence rate (λ > 610 nm) was 1.0 mW cm⁻².

4.8. Studies of FAP-triggered cleavage

For fluorescence measurements, conjugates 5 and 6 were dissolved in DMF to give 1 mM solution, respectively, which was diluted to 2.5 μM with PBS (with 0.1% Cremophor EL). After 2 h (to make the solutions reach an equilibrium state), the PBS without FAP (0.5 mL) or a FAP solution (10 μg mL⁻¹, 0.5 mL) in the PBS was then mixed with the solutions of 5 and 6, respectively, to give the solution of 5 or 6 (2 μM with 0.1% Cremophor EL), respectively, as a solution of FAP and 5 or 6 (containing [FAP] = 2 μg mL⁻¹) [5] or [6] = 2 μM and 0.1% Cremophor EL). These solutions were stirred at 25 °C in the dark continuously. Their fluorescence spectra (λex = 610 nm, λem = 620−800 nm) were recorded with time.

For singlet oxygen measurements, the solution of 5 or 6 (1 mM in DMF) was diluted with PBS (with 0.1% Cremophor EL) and the FAP solution (10 μg mL⁻¹, respectively, to give the solution of 5 or 6 (10 μM with 0.1% Cremophor EL), as well as a solution of FAP and 5 or 6 (containing [FAP] = 10 μg mL⁻¹, [5] or [6] = 10 μM, and 0.1% Cremophor EL). After stirring at 25 °C in the dark for 30 h, these solutions (1 mL) were mixed with a solution of DPBF (120 μM with 0.1% Cremophor EL, 1 mL), respectively, to give a mixture of DPBF and 5 or 6 (containing [DPBF] = 60 μM, [5] or [6] = 5 μM, and 0.1% Cremophor EL). The mixture was irradiated with red light immediately, and then DPBF degradation at 415 nm was monitored along with irradiation time.

For HPLC analysis, conjugate 5 or 6 was first dissolved in dimethyl sulfoxide (DMSO) to give 4 mM solution, which was formulated with Cremophor EL and then diluted with PBS to 10 μM containing 0.05% Cremophor EL. The solution of 5 (or 6) (10 μM, 1 mL) was incubated with FAP (10 μg) and then stirred at 25 °C. Meanwhile, the solutions without FAP were used as a control in the same condition. The solutions were monitored using HPLC by measuring the absorbance at 680 nm at different time intervals.

4.9. Cell culture and compounds

HepG2 human hepatocarcinoma cells (from ATCC) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (50 units mL⁻¹), and streptomycin (50 μg mL⁻¹) at 37 °C in a humidified 5% CO₂ atmosphere.

These compounds (DOX, 4, 5, or 6) were first dissolved in DMSO to give 2 mM solutions, which were diluted with an aqueous solution containing 1% Cremophor EL to 200 μM, followed by dilution with PBS to 10 μM (except the dark cytotoxicity test using the concentration at 20 μM). Conjugates 5 and 6 (10 μM, 1 mL) were further mixed with FAP (10 μg), respectively. All the samples (4–6, 5 + FAP, and 6 + FAP) were incubated for 24 h at 37 °C in a humidified 5% CO₂ atmosphere. After that, the solutions were diluted with the culture medium to the desired concentrations before incubated with the cells. For the dark cytotoxicity test with the concentration at 20 μM, the above aqueous solutions of phthalocyanine (200 μM) were directly diluted with the culture medium to the desired concentrations before incubated with the cells.

4.10. In vitro photocytotoxicity

HepG2 cells (about 1 × 10⁴ cells per well) were maintained in 96-well plates overnight at 37 °C in a humidified 5% CO₂ atmosphere. The cells were then incubated with 100 μL of the samples (DOX, 4–6, 5 + FAP, and 6 + FAP), respectively, at various concentrations in the dark for 24 h. After removing the samples, the cells were rinsed with PBS and re-fed with 100 μL of the culture medium before illumination at ambient temperature. The light source consisted of a 500 W halogen lamp, a water tank for cooling, and a colored glass filter cut-on 610 nm. The fluence rate (λ > 610 nm) was 15 mW cm⁻². An illumination of 30 min led to a total fluence of 27 J cm⁻².

Cell viability was determined by the colorimetric MTT assay [40]. A MTT (Sigma) solution in PBS (20 μL, 5 mg mL⁻¹) was added to each well followed by incubation for 4 h under the same condition. 150 μL of DMSO was then added to each well. The 96-well plate was agitated on a microplate reader (Tecan M200Pro) at ambient temperature for 20 s before the absorbance at 490 nm at each well was taken. The average absorbance of the blank wells, which did not contain the cells, was subtracted from the readings of the other wells. The cell viability was then determined by the equation: Cell Viability (%) = [Σ(Ai/Acontrol) × 100]/n, where Ai is the absorbance of the ith data (i = 1, 2, …, n), Acontrol is the average absorbance of the control wells, in which the phthalocyanine was
absent, and n (≥3) is the number of the data points.

4.11. Intracellular fluorescence imaging

About 1 × 10^5 HepG2 cells in RPMI 1640 medium were seeded in confocal dishes and incubated overnight at 37 °C under a humidified 5% CO2 atmosphere. After removing the medium, the cells were incubated with the samples (4, 5, and 5 + FAP) (2 μM, 400 μL) respectively, for 24 h under the same condition. The cells were then rinsed with PBS twice and imaged using a Leica laser fluorescence confocal microscope. The samples were excited at 635 nm and monitored at 640–750 nm. The images were then digitized and analyzed by using the SPE ROI Fluorescence Statistics software. The average intracellular fluorescence intensities (a total of 50 cells for each sample) were also determined.

4.12. Subcellular localization

About 1 × 10^5 HepG2 cells in the culture medium were seeded on a confocal dish and incubated overnight at 37 °C with 5% CO2. After removing the medium, the cells were incubated with the solutions of the samples (4 + DOX, 5, 6, 5 + FAP, and 6 + FAP) in the medium (2 μM, 400 μL) for 23 h under the same condition, and then Mito-Tracker Green (5 μM, 20 μL) for further 30 min co-incubation, followed by Hoechst 33342 for another 15 min co-incubation, leading to a total incubation time of ca. 24 h for the samples, 45 min for Mito-Tracker Green, and 15 min for Hoechst 3342. The cells were rinsed with PBS and viewed with a Leica laser fluorescent confocal microscope. The Mito-Tracker Green and Hoechst 3342 were excited at 498 and 405 nm, and monitored at 499–529 and 415–454 nm respectively. The photosensitizers and DOX were excited at 635 and 532 nm, and monitored at 640–750 nm and 552–617 nm respectively. The subcellular localization of the samples was revealed by comparing the intracellular fluorescence images caused by Mito-Tracker, Hoechst 3342, DOX, and the photosensitizer.

4.13. In vivo imaging

H22 murine hepatoma cells were obtained from the China Center for Type Culture Collection (CCTCC, Wu Han, China); Female KM mice were purchased from Wushi Laboratory Animal Co., Ltd, China. All animal studies were performed in compliance with guidelines of the Animal Care Committee of Fuzhou University. H22 cells (~5 × 10^5 cells in 200 μL) were inoculated subcutaneously on the flank of the mice (~20 g). When the tumors had grown to 100–200 mm3, an aqueous solution of compound 4 or conjugate 5 in saline (both at 200 μM, 200 μL) was intravenously injected into the tail vein of the tumor-bearing mice. In vivo fluorescence imaging of the mice was performed from 690 nm at different time points with SI Imaging AmiX imaging system (excited at 605 nm).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.12.056.
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