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Trafficking of a Single Photosensitizing Molecule to Different Intracellular Organelles Demonstrates Effective Hydroxyl Radical-Mediated Photodynamic Therapy in the Endoplasmic Reticulum

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Abstract

Photodynamic therapy (PDT) is often used in preclinical and clinical treatment regimens. Reactive oxygen species (ROS) generated by photosensitizers (PSs) upon exposure to light induce cell death via diverse mechanisms. PSs can exert therapeutic effects in different cellular organelles, although the efficacy of organelle-specific PDT has yet to be determined as most previous studies use different PSs in different organelles. Here, we explored how a single PS, chlorin e6 (Ce6), targeted to different organelles altered the effectiveness of PDT. Ce6 intrinsically localizes to the ER after 4 h of incubation. Modification of Ce6 via conjugation with an octapeptide (LS765), a monosubstituted triphenylphosphonium (TPP) derivative (LS897), or a disubstituted TPP derivative (LS909) altered the intrinsic localization. We determined that LS765 and LS897 predominantly accumulated in the lysosomes, but LS909 trafficked equally to both the mitochondria and the lysosomes. Moreover, the conjugation altered the type of ROS produced by Ce6, increasing the ratio of hydrogen peroxide to hydroxyl radicals. Irradiation of identical concentrations of the PSs in solution with 650 nm, 0.84 mW/cm² light for 10 min showed that the TPP conjugates nearly doubled the hydrogen peroxide production from ~0.2 μ M for Ce6 and LS765 to ~0.37 μ M for LS897 and LS909. In contrast, Ce6 produced ~1.5-fold higher hydroxyl radicals than its conjugates. To compare the effect of each PS on cell death, we normalized the intracellular concentration of each PS. Hydrogen peroxide-producing PSs are effective PDT agents in the lysosomes while the hydroxyl-generating PSs are very effective in the ER. Compared to the PSs that accumulated in the lysosomes, only the ER-targeted Ce6 exerted >50% cell death at either

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Supporting Information

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low light power or low intracellular concentration. By delineating the contributions of cellular organelles and types of ROS produced, our work suggests that targeting hydroxyl radical-producing PSs to the ER is an exciting strategy to improve the therapeutic outcome of PDT.

Graphical Abstract

INTRODUCTION

Photodynamic therapy (PDT) is a form of cancer treatment that combines light and light activatable drugs, photosensitizers (PSs), to produce cytotoxic reactive oxygen species (ROS). PDT exerts its therapeutic effects through multiple pathways to induce tumor regression. The effectiveness of PDT arises from the multiple tissue damage pathways, including direct damage to tumor cells resulting in necrosis and apoptosis, disruption of microvessels to deprive tumors of nutrients and oxygen, and activation of the acute and innate immune response.¹⁻³

The intracellular localization of the PS can affect the type of cell damage and therapeutic outcome.⁴ Activation of the PS in the lysosome produces ROS, which oxidize lipids and proteins to induce lysosomal membrane permeabilization (LMP)^{5,6} and the release of cathepsins into the cytosol. Cathepsins B/D/L can activate apoptosis through activation of pro-apoptotic proteins, inhibition of pro-survival proteins, or activation of caspase-8.⁶ Similarly, the activation of a PS in the mitochondria can permeabilize the mitochondrial membrane, releasing cytochrome c and activating apoptosis.^{7,8} Some studies have demonstrated that the activation of PSs in the endoplasmic reticulum (ER) release large stores of calcium, which stimulate apoptosis.⁹ In addition, several reports have shown that

activating ER-localized PSs can induce antitumor immunity,^{10–12} making this organelle an attractive site to activate multiple therapeutic pathways.

Chlorin e6 (Ce6) is widely used in preclinical PDT. It has a high molar extinction coefficient at 660 nm and excellent photostability. A derivative of Ce6, mono-L-aspartyl chlorin e6 (NPe6), has been tested in clinical phase 3 trials to treat hepatocellular carcinoma and colorectal cancer metastasis in the liver.¹³ NPe6 localizes in the lysosomes and has been found to activate mitochondria-mediated apoptosis.^{14–18} Comparing the efficacy of Ce6-mediated PDT in different cellular organelles would provide guidance on the best strategy and timing to optimize therapeutic outcomes. Some studies have attempted to accomplish this goal by comparing multiple PSs that traffic to different organelles, but this fails to isolate the organelle as the single altered variable.^{19–24}

In this study, we conjugated different molecules to the same PS, Ce6. We demonstrate that the conjugates altered the type of ROS produced, internalized in cells at different rates, and trafficked to different cellular organelles. Normalization of the intracellular concentrations at a specific time point allowed us to delineate the contributions of hydrogen peroxide and hydroxyl radicals to PDT efficacy in different organelles. Our results suggest that the activation of hydroxyl radical-generating PSs in the ER, and to a lesser extent the activation of hydrogen peroxide-producing PSs in the lysosomes, is an effective strategy to enhance the therapeutic effect of PDT. For lysosomal PDT, we found that the targeting ligand greatly affected cell death, with LS897 killing more cells than LS765. In addition, after correcting for intracellular PS concentration, we found that lysosomal PDT (LS897) was comparable to dual lysosomal and mitochondrial PDT (LS909). Finally, through normalization of uptake of all four PSs, could isolate that ER PDT resulted in the greatest loss in viability while maintaining the lowest dark toxicity.

RESULTS AND DISCUSSION

Synthesis of Peptide and Small Molecule Conjugates of Ce6.

We explored how the intracellular distribution of the same PS to different organelles could alter the effectiveness of PDT by conjugating Ce6 to different molecules (Figure 1). We first conjugated Ce6 to an octapeptide that accumulates in the lysosomes. Standard Fmoc solid phase peptide synthesis was used to prepare the LS765 peptide, followed by conjugation of Ce6. Simultaneous deprotection of all side-chain groups of the amino acid residues and cleavage from the resin afforded LS765 (Figure S1).

Previous reports have shown that TPP facilitates intracellular transport to the mitochondria.^{26–29} We postulated that a Ce6-TPP conjugate will exhibit a similar subcellular distribution. To reduce steric effect and allow direct reaction with the carboxylic acid groups of Ce6, we reacted TPP with a monotrityl ethylenediamine, followed by removal of the trityl group with TFA (Figure S2). Subsequent reaction with Ce6 afforded only one TPP subsituent, LS897 (Figure 1). We altered the synthetic method to increase the number of TPP per Ce6 in anticipation of enhancing mitochondrial localization. Substitution of the ethylene diamine on the TPP with (ethylenedioxy)bis(ethylamine) (Figure S3) afforded a disubstituted TPP-Ce6,

LS909 (Figure 1), but not the trisubstituted analogue. These products were purified by HPLC and characterized by HRMS and spectroscopy.

Other studies have suggested that monosubstitution on one of the three carboxylic acid groups occurs at the propanoic acid group based on steric factors.^{30,31} Although counterintuitive, a recent report provides strong evidence that this reaction favors the ethanoic acid group.³² Thus, activation of Ce6 with CDI forms an anhydride between the ethanoic and methanoic acid groups, which then preferentially reacts with nucleophiles at the more electrophilic ethanoyl group to afford the monosubstituted LS765 and LS897. We were unable to detect disubstituted products, even when excess reactants and coupling agents were used, probably due to steric hindrance caused by the large monosubstituent. To minimize the steric effect, we replaced the ethylenediamine used in LS897 with 2,2-(ethylenedioxy)bis(ethylamine) to obtain the disubstituted LS909, most likely at the less sterically hindered propanoic acid and the ethanoic acid groups. Attempts to obtain the trisubstituted derivative were not successful, probably because of a combination of steric factors and the poor reactivity of the aromatic methanoic acid group. We used the new PSs to determine how these conjugates will alter the intracellular distribution of Ce6 and how the activation of PDT in different organelles will affect cell death.

All compounds had similar spectral profiles as the non-conjugated Ce6 (Figure S4), indicating that we can use identical excitation and emission settings to image and track the subcellular localization of the PSs.

Ce6 Conjugation Modulates ROS Production.

Next, we determined whether conjugation of Ce6 altered the amount and type of ROS produced upon irradiation. We deduced the nature of the ROS generated by the PSs based on the in vitro data (Figure 2). However, the microenvironment of PSs can alter the nature of the ROS produced. Therefore, the ROS produced in cells may differ from what was determined in solutions. Previous studies have shown that even minor modification to PS can alter ROS production.³³ ROS species have different propensities to induce oxidative damage to cells. Both hydrogen peroxide and hydroxyl radicals play key roles in the PDT Type 1 mechanism. Hydroxyl radicals react with biomolecules close to the site of production, which stresses the cell to induce cell death.^{34,35} On the other hand, hydrogen peroxide is more stable and can diffuse through membranes, allowing it to inflict damage across different organelles. Moreover, hydrogen peroxide can convert to singlet oxygen or hydroxyl radicals through the Fenton reaction.³⁶ Delineating how modification of Ce6 alters the production of these ROS will aid in a mechanistic understanding of their efficacy.

To ensure the concentration of each PS was compared, we used the molar extinction coefficients of Ce6 to calculate the concentrations of all PSs. Each PS was irradiated with 0.84 mW/cm² light source at 650 nm. Measurements of hydroxyl and hydrogen peroxide production were accomplished with HPF and Amplex Red assays, respectively. Reference standards were used to determine the concentration or relative amount of each species produced.

In the first 10 min of irradiation, Ce6 and LS765 produced about 0.2 μM of hydrogen peroxide, while LS897 and LS909 produced $\sim 0.37 \mu\text{M}$ hydrogen peroxide, which is 2-fold greater than Ce6 and LS765 (Figure 2a). Interestingly, the rate of the ROS generation mirrored the amount produced. Ce6 and LS765 exhibited similar kinetics, while the TPP conjugates LS897 and LS909 showed similar kinetics within the first 10 min. After 10 min, Ce6 and LS765 exhibited a decrease in the rate of hydrogen peroxide production, although the net concentration continued to increase over time. In contrast, a divergence of the hydrogen peroxide production rate was observed for the TPP conjugates after 10 min. Whereas LS909 continued to generate hydrogen peroxide at a high rate, LS897 rate decreased significantly until 60 min when it recovered.

All four PSs had similar hydroxyl radical production kinetics over 60 min (Figure 2b). Ce6 produced ~ 1.5 -fold higher hydroxyl radicals than its conjugates, which produced similar amounts of hydroxyl radicals.

The differences in the type of ROS generated by Ce6 and its conjugates indicate that conjugation could alter the cell death pathways. The high levels of the reactive hydroxyl radicals produced by Ce6 suggest that the intracellular site of accumulation and light activation will affect PDT efficacy. Thus, activating Ce6 in organelles that express oxidation-sensitive biomolecules would be highly susceptible to PDT. Only LS909 produced higher hydrogen peroxide than Ce6, indicating that it could induce cell death at multiple sites in cells. However, the efficacy of this event relies on the conversion of the hydrogen peroxide to the more reactive hydroxyl or singlet oxygen radicals. If this conversion is mediated by the Fenton reaction, cancer cells which possess higher concentrations of iron than normal cells could be more responsive to PDT. This selectivity can be intentionally induced by using iron transporters such as transferrin to increase the intracellular concentration of iron in cancer cells before initiating PDT. The mechanism by which the Ce6 conjugates decrease hydroxyl radical production is unclear, but may be due to reaction of the trityl and amino acid groups with the hydroxyl radicals which would attenuate the radicals available for reaction with the indicator dyes.

Ce6 Conjugates Alter the Intrinsic Intracellular Distribution of the PS.

Next, we determined the subcellular distribution of Ce6 and its conjugates in the 4T1 murine breast cancer cells. All the PSs localized in the plasma membrane at early time points (Figure S5), which is characteristic of Ce6 binding pattern.^{37,38} By 4 h of incubation, all the PSs predominantly localized in distinct organelles as shown by line plots and Pearson correlation coefficients between the red (PS) channel and green (organelle marker) channel (Figure S9).

Ce6 traffics with its membrane bound component to the ER.³⁹ LS765 internalized through the endosomal pathway and at 4 h resides in the lysosome. LS897, although targeted using TPP which typically delivers cargo to the mitochondria, again resulted in lysosome accumulation.^{40,41} This may be due to the hydrophobicity of Ce6.²⁶ To overcome this, we added a PEG spacer and an additional TPP to Ce6 to obtain, LS909. At 4 h, LS909 resides in both lysosomes and mitochondria as indicated by colocalization with both lysotracker and

mitotracker with Pearson correlation coefficients of 0.5 ± 0.3 and 0.6 ± 0.1 , respectively (Figure 3c and Figure S9).

PDT Efficacy Is Dependent on Intracellular PS Concentration, Distribution Profile, and Type of ROS Produced.

Cellular microscopy showed the selective accumulation of the PSs in different organelles at 4 h time point and ROS generation showed that these light-sensitive drugs produced high levels of different ROS types within 10 min of irradiation. Therefore, we employed these conditions to test the PDT efficacy of Ce6 and its conjugates. Assessment of cell viability in the absence of light showed that the PSs had minimal dark cytotoxicity in both low ($1 \mu\text{M}$) and high ($10 \mu\text{M}$) incubation PS concentrations (Figure 4). None of the PSs showed significant cytotoxicity at the low $1 \mu\text{M}$ concentration and low power of 0.84 mW/cm^2 (Figure 4a). When the PS concentration was increased to $10 \mu\text{M}$, we found that only Ce6 and LS909 were able to kill over 90% of the cells. We then increased the light power to 3.25 mW/cm^2 . The result shows that only Ce6 induced significant cell death at $1 \mu\text{M}$. However, when the concentration of PSs was increased to $10 \mu\text{M}$, cells irradiated with 3.25 mW/cm^2 of light source responded to PDT. Except for LS765, which killed about 50% of the cells, all other PSs induced cell death exceeding 80% (Figure 4b).

Surprised by this discrepancy, we postulated that the differences in cell death response could arise from the amount of the internalized PS, not the initial incubation concentration or subcellular localization. In fact, the intracellular PS concentrations at 4 h with a starting $10 \mu\text{M}$ solution showed significant differences for each PS (Figure 5a). As a result, we altered the starting concentration of each PS to normalize the intracellular concentration to $\sim 0.5 \mu\text{M}$, which corresponds to the lowest measured intracellular concentration found for LS897 using $10 \mu\text{M}$ solution for the incubation (Figure 5b). The cells were then washed and irradiated with a light dose of 3.25 mW/cm^2 (Figure 5c). Although the dark toxicity remained negligible, a stark difference in photoinduced toxicity was observed. Ce6, which localized in the ER and produced the highly reactive hydroxyl radicals, maintained high cell death ($>90\%$). Surprisingly, both LS897 and LS909 exhibited similar cytotoxicity, with cell death $>50\%$, irrespective of the high hydrogen peroxide and mitochondrial localization of LS909 compared to the lysosomal accumulation of LS897. LS765, which exhibited similar ROS production and organelle distribution profile as LS897, was ineffective under these conditions.

The above results highlight several important considerations in comparing organelle specific PDT. Previous studies used different PSs which to different organelles to estimate organelle-based PDT.^{42,43} While this approach is simple, it does not account for the intrinsic differences in the photophysical properties and intracellular concentration of each PS. In this study, we conjugated different molecules to Ce6 to modify its intracellular distribution. The PSs also had different propensities to produce diverse ROS. Therefore, we also explored how different ROS in the same organelle could result in different amounts of cell killing.

For lysosomal PDT, hydrogen peroxide, produced by LS897, proved more lethal than hydroxyl radicals, produced by LS765. Previous studies have shown that the Fenton reaction is more effective under acidic conditions, which stabilizes Fe^{2+} for the conversion of

hydrogen peroxide to reactive ROS.⁴⁴ It is likely that the lysosomal compartment provides the acidic environment needed to transform hydrogen peroxide into cytotoxic radicals. In addition, the diffusion of hydrogen peroxide to distal sensitive organelles from the site of formation can enhance its therapeutic effect. This result suggests that targeting hydrogen peroxide-producing PSs to the lysosomes may be more effective in cell killing than hydroxyl radical-producing PSs.

Mitochondrial localized PS did not improve cell killing in this study. We had anticipated that the colocalization of LS909 in both the mitochondria and the lysosomes would confer higher PDT effect. Instead, we found that LS897 was equally or slightly more potent than LS909. Similar to LS765, LS909 produced higher hydrogen radicals than LS897, but the hydrogen peroxide generated was identical for both LS909 and LS897. Therefore, our finding demonstrates that hydrogen peroxide is an effective ROS for PDT, and localization to the lysosome results in greater cell death than the mitochondria.

Finally, Ce6, which localized in the ER and produced ~1.5-fold more hydroxyl radicals than its conjugates, consistently showed effective cell killing at both low light dose and low PS concentration. ER is responsible for protein folding and transport in cells. It is logical that the disruption of this process by rapid oxidative reactive from hydroxyl radicals could lead to improperly folded proteins and alter the efficacy of protein transport. This finding indicates that the ER is highly sensitive to PDT for this therapy.^{45–47}

CONCLUSIONS

To determine the efficacy of organelle specific PDT, we synthesized different small molecule conjugates of Ce6 to redirect its intracellular distribution. At 4 h of incubation, the PSs localized in distinct organelles, with Ce6 predominantly in the ER, LS765 and LS897 in the lysosomes, and LS909 in both the lysosomes and the mitochondria. These conjugates altered not only the organelle distribution, but also intracellular concentrations. Normalization of the intracellular concentrations, based on Ce6 content, allowed us to directly compare the cell-killing effects. Whereas hydrogen peroxide-producing PSs are effective PDT agents in the lysosomes, hydroxyl generating PSs are very effective in the ER. We attribute the sensitivity of ER to the rapid reaction of hydroxyl radicals with newly produced proteins in situ. The reaction could sufficiently prevent or disrupt the formation of tertiary structures of proteins to induce oxidative stress and eventual cell death. As a result, targeting hydroxyl radical-producing PSs to the ER is an exciting strategy to improve the therapeutic outcome of PDT. Previous studies of ER PDT with the PS, hypericin, noted that the upregulation of cytokines is potentially involved in the antitumor immunity of this PS.^{10–12} In the future, we will investigate if the ER-mediated PDT using Ce6 will exhibit a similar immune system activation. We used slightly different linkers to improve the synthesis of the mono- and di-TPP conjugates, which could alter the intracellular distribution of the compounds. Preferably, similar linkers should be used in future studies to rule out this possibility. We did not determine the contributions of Type 2 PDT mechanism. Regardless of the cell death pathway, the intracellular location of PS and the type of ROS produced will impact the degree of light-induced cell death. The diverse multicellular environment of tissue dictates

that the PS should reside in the target organelles for long periods of time to ensure that most cells retain the PS in similar organelles before light activation.

EXPERIMENTAL SECTION

Chemicals and Materials.

The fluorenylmethyloxy carbonyl(Fmoc) amino acids and the Fmoc-Lys(Boc)-Wang Resin were purchased from AAPPTec (Louisville, KY, USA). All the other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Chlorin e6 (Ce6) was purchased from Frontier-Scientific (Logan, UT, USA). Water was obtained from Millipore Q3 system (Billerica, MA, USA).

Dulbecco's modified Eagle's medium (DMEM) culture medium, fetal calf serum, and penicillin–streptomycin were purchased from Gibco (Life Technologies, NY, USA). LysoTracker green, mitotracker green, ER tracker green, HPF, Amplex Red assay, and Calcein AM were purchased from Thermo Fisher Scientific.

Cell Culture.

4T1/luc murine breast cancer cells (Sibtech, Brookfield, CT) were cultured in DMEM supplemented with 10% FBS, and 1% penicillin–streptomycin in a humidified incubator at 5% CO₂ balanced with air at 37 °C. The cells were kept at 50–80% confluence.

Synthesis of LS765.

The linear GRD peptide, H-DCys(Acm)-Gly-Arg(Pbf)-Asp(tBu)-Ser(tBu)-Pro-Cys(Acm)-Lys(Boc)-OH, was prepared via a CEM Liberty microwave peptide synthesizer (Matthews, NC, USA) on the Fmoc Lys(Boc)-wang resin (Figure S1a). The resin (0.1 mmol) was swollen in DCM for 1 h before use. Fmoc-amino acids (0.5 mmol, 5 equiv), coupling reagent (HBTU, 0.5 mmol, 5 equiv), and DIEA (1 mmol, 10 equiv) were added to the resin and the mixture was allowed to react for 15 min under MW irradiation (50 W, 75 °C). The resin was washed three times with DMF. Treatment of the resin with 20% piperidine/DMF for 5 min under MW irradiation (50 W, 75 °C) was sufficient to remove the Fmoc. At the completion of the peptide synthesis, the peptidyl resin was washed and cyclized with thallium trifluoroacetate (1.2 equiv) in DMF for 90 min to form an intramolecular disulfide bridge. Subsequent addition of Ce6 (3 equiv) to the cyclic peptide on solid resin in the presence of HATU (5 equiv) and DIEA (5 equiv) in DMF afforded LS765 peptidyl resin. The Ce6–peptide conjugate was cleaved from the resin by mixing with a cocktail of TFA:thioanisole:phenol:water (85:5:5:5, v/v/v/v) for 90 min at room temperature. The cleaved peptide product was concentrated *in vacuo*, then purified on reverse-phase HPLC. LS765 (Ce6-cyclic (DCys-Gly-Arg-Asp-Ser-Pro-Cys)-Lys-OH), M/W 1441, ESI-MS observed 1442 (M+1) and 722 (M+2/2) (Figure S1b). Extinction coefficient: $\epsilon_{408\text{ nm}} = 135,500\text{ M}^{-1}\cdot\text{cm}^{-1}$, $\epsilon_{660\text{ nm}} = 42,500\text{ M}^{-1}\cdot\text{cm}^{-1}$.

Synthesis of LS897.

The synthetic scheme is illustrated in Figure S2. Briefly, (3-caboxypropyl)triphenylphosphonium bromide (TPP; 86 mg, 0.2 mmol) was dissolved in 1

mL of anhydrous DMF, followed by the addition of 1,1-carbonyldiimidazole (CDI; 65 mg, 0.4 mmol) and N-Boc-ethylenediamine (38 μ L, 0.24 mmol). The mixture was vortexed for 6 h and the product was precipitated in diethyl ether, dried, and treated with 95% TFA to remove the Boc-protection. The resultant TPP-amine was dried and dissolved in 1 mL of anhydrous DMF before adding to a mixture of Ce6 (60 mg, 0.1 mmol), 4-dimethylaminopyridine (DMAP; 12 mg, 0.1 mmol), and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate, (HATU; 76 mg, 0.2 mmol). The reaction mixture was vortexed overnight at room temperature and the final product was precipitated with diethyl ether and purified on reverse-phase HPLC. LS897, M/W 970. ESI-MS: observed 917(M+1) and 486 (M+2/2). ¹H NMR (400 MHz, CD₃OD): δ 10.40 (s, 1H), 10.28 (s, 1H), 9.67–9.64 (m, 1H), 8.49–8.43 (m, 1H), 8.04–7.82 (m, 15H), 6.65–6.52 (m, 2H), 5.80–5.65 (m, 2H), 4.92–4.79 (br, 2H), 4.58 (br, 1H), 4.19 (br, 3H), 3.81–3.77 (br, 7H), 3.63 (br, 7H), 3.13 (br, 2H), 2.94–2.84 (m, 2H), 2.69–2.60 (m, 2H), 2.46 (br, 1H), 2.21–2.04 (m, 5H), 1.84 (br, 4H) (Figure S7). Extinction coefficient: $\epsilon_{407\text{ nm}} = 145,000\text{ M}^{-1}\cdot\text{cm}^{-1}$. $\epsilon_{660\text{ nm}} = 40,500\text{ M}^{-1}\cdot\text{cm}^{-1}$.

Synthesis of LS909.

The synthetic scheme is illustrated in Figure S3. Briefly, TPP (86 mg, 0.2 mmol) was dissolved in 1 mL of anhydrous DMF, followed by adding CDI (65 mg, 0.4 mmol) and 2,2-(ethylenedioxy)bis(ethylamine) (87 μ L, 0.58 mmol), which was vortexed for 6 h. The product was precipitated with diethyl ether and dried before dissolving in anhydrous DMF (1 mL). This solution was added to Ce6 (30 mg, 0.05 mmol), DMAP (6 mg, 0.05 mmol), and HATU (36 mg, 0.1 mmol). The reaction mixture was vortexed overnight at room temperature. The final product was precipitated with diethyl ether and purified on a reverse-phase HPLC. LS909, M/W 1519. ESI-MS: observed 760 (M+2/2) and 507 (M+3/3). ¹H NMR (600 MHz, CD₃OD): δ 10.11 (br, 1H), 9.99 (br, 1H), 9.30 (br, 1H), 8.29–8.23 (m, 1H), 7.79–7.65 (m, 30H), 6.43 (d, 1H, *J* = 18 Hz), 6.31 (d, 1H, *J* = 12 Hz), 5.54 (d, 1H, *J* = 18 Hz), 5.34 (d, 1H, *J* = 18 Hz), 4.70–4.66 (m, 1H), 4.51 (br, 1H), 4.02–3.90 (m, 3H), 3.63 (br, 4H), 3.60–3.57 (m, 3H), 3.55 (br, 4H), 3.53 (br, 2H), 3.49 (br, 5H), 3.45–3.39 (m, 13H), 3.20–3.18 (m, 6H), 2.54–2.49 (m, 1H), 2.32–2.12 (m, 6H), 1.86–1.81 (m, 3H), 1.78–1.71 (m, 4H), 1.70–1.64 (m, 4H). (Figure S8) Extinction coefficient: $\epsilon_{407\text{ nm}} = 147,500\text{ M}^{-1}\cdot\text{cm}^{-1}$. $\epsilon_{660\text{ nm}} = 41,000\text{ M}^{-1}\cdot\text{cm}^{-1}$.

Colocalization Imaging.

Colocalization studies were performed on a MicroTime200 (MT200) fluorescence lifetime (PicoQuant) or FV1000 confocal (Olympus) microscope. MT200 imaging was performed with a 100 $\times$ objective using filters for the PSs based on Ce6 spectral properties (red; ex/em 640/655–725 nm) and organelle stain (green; ex/em 488/510–530 nm). Similarly, confocal imaging was conducted with a 60 $\times$ objective using filters for the PSa (ex/em 633/650/750 nm) and organelle stain (ex/em 488/510–530 nm).

All PSs were incubated with cells for 4 h at 5 μ M. For lysosome staining, lysotracker green (75 nM) was incubated with cells for 2 h. For mitochondrial staining, mitotracker (50 nM) was incubated with cells for 15 min. For ER staining, ER-tracker green (1 μ M) was incubated with cells for 15 min.

Imaging was conducted in DMEM without phenol red. Line plots were generated by selecting a line of interest in the image to produce to a profile plot via ImageJ. The intensities were normalized to the max value of each line. Pearson correlation coefficients were calculated using the ImageJ (NIH, USA) plug-in JACoP, with at least 3 representative images.²⁵

ROS Measurements.

For both hydroxyl radical and hydrogen peroxide assays, the PS (50 μL , 10 μM) was irradiated with light (650 nm, 0.84 mW/cm²). For hydroxyl radical assays, hydroxyphenyl fluorescein (HPF; 10 μM) was added to the PS, followed by irradiating the solution in a 96 well plate for 0–60 min. The fluorescence of each sample was measured on a plate reader (BioTek, USA), with filters set at ex/em 485/518–538 nm. Triplicates of each sample were used for each PS.

For hydrogen peroxide measurements, the Amplex Red assay was used to quantify hydrogen peroxide concentration after irradiation. Fluorescence intensity was measured on a plate read with filters set at ex/em 545/580–600 nm. A reference standard of known hydrogen peroxide concentration was used to calculate the concentration of hydrogen peroxide produced.

PDT and Intracellular PS Concentration.

4T1 cells were seeded in a 96-well plate at a density of 5000 cells (100 μL) per well 24 h before PDT experiments. Cells were treated with 0–100 μM Ce6, LS765, LS897, or LS909 for 4 h and washed. The culture media were replaced with phenol-free DMEM, and their fluorescence was recorded on a plate reader (BioTek, USA) using ex/em 390/645 nm filter to quantify the amount of PS that internalized into the cells. These cells were irradiated with 650 nm light for 10 min at either 0.84 mW/cm² or 3.25 mW/cm². The cells were then cultured for another 24 h before determining their viability. Morphology of the cells were monitored with a bright field microscope.

To normalize the intracellular concentration of each PS, cells were treated with different starting concentrations of PS: 6.35 μM (Ce6), 4.68 μM (LS765), 10 μM (LS897), and 3.11 μM (LS909) (Figure S3, Figure 5a). PDT assays were conducted as described above.

Cell Viability.

Cells were washed three times with PBS before incubating with Calcein AM (100 μL , 3.33 μM in PBS) per well for 30 min. Calcein AM fluorescence was measured on a plate reader with filters set at ex/em 485/518–538 nm. Fluorescence was normalized to the untreated control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

PDT	photodynamic therapy
PS	photosensitizer
ROS	reactive oxygen species
TPP	triphenylphosphonium
Ce6	Chlorin e6
LS765	Ce6-octapeptide conjugate
LS897	Ce6-mono-TPP conjugate
LS909	Ce6-di-TPP conjugate

REFERENCES

- (1). Castano AP, Mroz P, and Hamblin MR (2006) Photodynamic therapy and anti-tumour immunity. *Nat. Rev. Cancer* 6, 535–545. [PubMed: 16794636]
- (2). Wilson BC, and Patterson MS (2008) The physics, biophysics and technology of photodynamic therapy. *Phys. Med. Biol* 53, R61–109. [PubMed: 18401068]
- (3). Pandey RK (2000) Recent advances in photodynamic therapy. *J. Porphyrins Phthalocyanines* 4, 368–373.
- (4). Srivatsan A, Pera P, Joshi P, Wang Y, Missert JR, Tracy EC, Tabaczynski WA, Yao R, Sajjad M, Baumann H, et al. (2015) Effect of chirality on cellular uptake, imaging and photodynamic therapy of photosensitizers derived from chlorophyll-a. *Bioorg. Med. Chem* 23, 3603–3617. [PubMed: 25936263]
- (5). Brunk UT, and Ericsson JLE (1972) Cytochemical evidence for the leakage of acid phosphatase through ultrastructurally intact lysosomal membranes. *Histochem. J* 4, 479–91. [PubMed: 4120446]
- (6). Aits S, and Jaattela M (2013) Lysosomal Cell Death at a Glance. *J. Cell Sci* 126, 1905–1912. [PubMed: 23720375]
- (7). Tait SW, and Green DR (2010) Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat. Rev. Mol. Cell Biol* 11, 621–632. [PubMed: 20683470]
- (8). Orrenius S, and Zhivotovsky B (2005) Cardiolipin oxidation sets cytochrome c free. *Nat. Chem. Biol* 1, 188–189. [PubMed: 16408030]
- (9). Kim I, Xu W, and Reed JC (2008) Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat. Rev. Drug Discovery* 7, 1013–1030. [PubMed: 19043451]
- (10). Buytaert E, Matroule JY, Durinck S, Close P, Kocanova S, Vandenheede JR, de Witte PA, Piette J, and Agostinis P (2008) Molecular effectors and modulators of hypericin-mediated cell death in bladder cancer cells. *Oncogene* 27, 1916–1929. [PubMed: 17952126]
- (11). Garg AD, Krysko DV, Vandenabeele P, and Agostinis P (2012) Hypericin-based photodynamic therapy induces surface exposure of damage-associated molecular patterns like HSP70 and calreticulin. *Cancer Immunol. Immunother* 61, 215–221. [PubMed: 22193987]

- (12). Barathan M, Mariappan V, Shankar EM, Abdullah BJ, Goh KL, and Vadivelu J (2013) Hypericin-photodynamic therapy leads to interleukin-6 secretion by HepG2 cells and their apoptosis via recruitment of BH3 interacting-domain death agonist and caspases. *Cell Death Dis* 4, No. e697. [PubMed: 23807226]
- (13). Wang S, Bromley E, Xu L, Chen JC, and Keltner L (2010) Talaporfin sodium. *Expert Opin. Pharmacother* 11, 133–140. [PubMed: 20001435]
- (14). Aggarwal N, Santiago AM, Kessel D, and Sloane BF (2015) Photodynamic therapy as an effective therapeutic approach in mame models of inflammatory breast cancer. *Breast Cancer Res. Treat* 154, 251–262. [PubMed: 26502410]
- (15). Kessel D (2016) Photodynamic therapy: Promotion of efficacy by a sequential protocol. *J. Porphyrins Phthalocyanines* 20, 302–306.
- (16). Liu L, Zhang Z, and Xing D (2011) Cell death via mitochondrial apoptotic pathway due to activation of Bax by lysosomal photodamage. *Free Radical Biol. Med* 51, 53–68. [PubMed: 21530645]
- (17). Kimani S, Ghosh G, Ghogare A, Rudshteyn B, Bartusik D, Hasan T, and Greer A (2012) Synthesis and characterization of mono-, di-, and tri-poly(ethylene glycol) chlorin e6 conjugates for the photokilling of human ovarian cancer cells. *J. Org. Chem* 77, 10638–10647. [PubMed: 23126407]
- (18). Bachor R, Shea CR, Belmonte SJ, and Hasan T (1991) Free and conjugated chlorin E6 in the photodynamic therapy of human bladder carcinoma cells. *J. Urol* 146, 1654–1658. [PubMed: 1942362]
- (19). Kessel D (2017) Subcellular targeting as a determinant of the efficacy of photodynamic therapy. *Photochem. Photobiol* 93, 609–612. [PubMed: 27935055]
- (20). Patel N, Pera P, Joshi P, Dukh M, Tabaczynski WA, Sifers KE, Kryman M, Cheruku RR, Durrani F, Missert JR, et al. (2016) Highly effective dual-function near-infrared (NIR) photosensitizer for fluorescence imaging and photodynamic therapy (PDT) of cancer. *J. Med. Chem* 59, 9774–9787. [PubMed: 27749069]
- (21). Srivatsan A, Ethirajan M, Pandey SK, Dubey S, Zheng X, Liu TH, Shibata M, Missert J, Morgan J, and Pandey RK (2011) Conjugation of cRGD peptide to chlorophyll a based photosensitizer (HPPH) alters its pharmacokinetics with enhanced tumor-imaging and photosensitizing (PDT) efficacy. *Mol. Pharma ceutics* 8, 1186–1197.
- (22). Rizvi I, Obaid G, Bano S, Hasan T, and Kessel D (2018) Photodynamic therapy: Promoting in vitro efficacy of photodynamic therapy by liposomal formulations of a photosensitizing agent. *Lasers Surg. Med* 50, 499–505. [PubMed: 29527710]
- (23). Rajora MA, Lou JWH, and Zheng G (2017) Advancing porphyrin's biomedical utility via supramolecular chemistry. *Chem. Soc. Rev* 46, 6433–6469. [PubMed: 29048439]
- (24). Shi JY, Liu TWB, Chen J, Green D, Jaffray D, Wilson BC, Wang F, and Zheng G (2011) Transforming a targeted porphyrin theranostic agent into a PET imaging probe for cancer. *Theranostics* 1, 363–370. [PubMed: 21938264]
- (25). Bolte S, and Cordelieres FP (2006) A guided tour into subcellular colocalization analysis in light microscopy. *J. Microsc* 224, 213–232. [PubMed: 17210054]
- (26). Zhang CJ, Hu Q, Feng G, Zhang R, Yuan Y, Lu X, and Liu B (2015) Image-guided combination chemotherapy and photodynamic therapy using a mitochondria-targeted molecular probe with aggregation-induced emission characteristics. *Chem. Sci* 6, 4580–4586. [PubMed: 28717475]
- (27). Zielonka J, Joseph J, Sikora A, Hardy M, Ouari O, Vasquez-Vivar J, Cheng G, Lopez M, and Kalyanaraman B (2017) Mitochondria-targeted triphenylphosphonium-based compounds: syntheses, mechanisms of action, and therapeutic and diagnostic applications. *Chem. Rev* 117, 10043–10120. [PubMed: 28654243]
- (28). Mahalingam SM, Ordaz JD, and Low PS (2018) Targeting of a photosensitizer to the mitochondrion enhances the potency of photodynamic therapy. *ACS Omega* 3, 6066–6074. [PubMed: 30023938]
- (29). Tarhouni M, Durand D, Onal E, Aggad D, Isci U, Ekineker G, Bregier F, Jamoussi B, Sol V, Gary-Bobo M, et al. (2018) Triphenylphosphonium-substituted phthalocyanine: Design, synthetic strategy, photoproperties and photodynamic activity. *J. Porphyrins Phthalocyanines* 22, 552–561.

- (30). Guo X, Wang L, Wang S, Li Y, Cao L, Cai R, and Zhao W (2017) Synergistic antiproliferative effect of chemo-phototherapy: Synthesis and photodynamic activity evaluation of novel Chlorin e6 artesunate conjugates as antiproliferative agents. *Bioorg. Med. Chem. Lett* 27, 4548–4551. [PubMed: 28882481]
- (31). Yumita N, Iwase Y, Nishi K, Ikeda T, Komatsu H, Fukai T, Onodera K, Nishi H, Takeda K, Umemura SI, et al. (2011) Sonodynamically-induced antitumor effect of mono-l-aspartyl chlorin e6 (NPe6). *Anticancer Res* 31, 501–506. [PubMed: 21378330]
- (32). Chen H, Waruna Jinadasa RG, Jiao L, Fronczek FR, Nguyen AL, and Smith KM (2015) Chlorin e6 13(1):15(2)- Anhydride: A key intermediate in conjugation reactions of chlorin e6. *Eur. J. Org. Chem* 2015, 3661–3665.
- (33). Roxin A, Chen J, Paton AS, Bender TP, and Zheng G (2014) Modulation of reactive oxygen species photogeneration of bacteriopheophorbide a derivatives by exocyclic E-ring opening and charge modifications. *J. Med. Chem* 57, 223–237. [PubMed: 24328124]
- (34). Dai TH, Fuchs BB, Coleman JJ, Prates RA, Astrakas C, St Denis TG, Ribeiro MS, Mylonakis E, Hamblin MR, and Tegos GP (2012) Concepts and principles of photodynamic therapy as an alternative antifungal discovery platform. *Front. Microbiol* 3, 120. [PubMed: 22514547]
- (35). Ochsner M (1997) Photophysical and photobiological processes in the photodynamic therapy of tumours. *J. Photochem. Photobiol., B* 39, 1–18. [PubMed: 9210318]
- (36). Fenton HJH (1894) Oxidation of tartaric acid in presence of iron. *J. Chem. Soc., Trans* 65, 899–910.
- (37). Mojzisova H, Bonneau S, Vever-Bizet C, and Brault D (2007) Cellular uptake and subcellular distribution of chlorin e6 as functions of pH and interactions with membranes and lipoproteins. *Biochim. Biophys. Acta, Biomembr* 1768, 2748–2756.
- (38). Mojzisova H, Bonneau S, Vever-Bizet C, and Brault D (2007) The pH-dependent distribution of the photosensitizer chlorin e6 among plasma proteins and membranes: a physico-chemical approach. *Biochim. Biophys. Acta, Biomembr* 1768, 366–374.
- (39). Li Y, Yu Y, Kang L, and Lu Y (2014) Effects of chlorin e6- mediated photodynamic therapy on human colon cancer SW480 cells. *Int. J. Clin. Exp. Med* 7, 1867–1876. [PubMed: 25126192]
- (40). Lv W, Zhang Z, Zhang KY, Yang H, Liu S, Xu A, Guo S, Zhao Q, and Huang W (2016) A mitochondria-targeted photosensitizer showing improved photodynamic therapy effects under hypoxia. *Angew. Chem., Int. Ed* 55, 9947–9951.
- (41). Hu Z, Sim Y, Kon OL, Ng WH, Ribeiro AJ, Ramos MJ, Fernandes PA, Ganguly R, Xing B, Garcia F, et al. (2017) Unique triphenylphosphonium derivatives for enhanced mitochondrial uptake and photodynamic therapy. *Bioconjugate Chem* 28, 590–599.
- (42). Gyenge EB, Luscher D, Forny P, Antoniol M, Geisberger G, Walt H, Patzke G, and Maake C (2013) Photodynamic mechanisms induced by a combination of hypericin and a chlorin based-photosensitizer in head and neck squamous cell carcinoma cells. *Photochem. Photobiol* 89, 150–162. [PubMed: 22882495]
- (43). Rizvi I, Nath S, Obaid G, Ruhi MK, Moore K, Bano S, Kessel D, and Hasan T (2019) A combination of visudyne and a lipid-anchored liposomal formulation of benzoporphyrin derivative enhances photodynamic therapy efficacy in a 3D model for ovarian cancer. *Photochem. Photobiol* 95, 419–429. [PubMed: 30499113]
- (44). Kremer ML (2003) The Fenton reaction. dependence of the rate on pH. *J. Phys. Chem. A* 107, 1734–1741.
- (45). Buytaert E, Callewaert G, Hendrickx N, Scorrano L, Hartmann D, Missiaen L, Vandenheede JR, Heirman I, Grooten J, and Agostinis P (2006) Role of endoplasmic reticulum depletion and multidomain proapoptotic BAX and BAK proteins in shaping cell death after hypericin-mediated photodynamic therapy. *FASEB J* 20, 756–758. [PubMed: 16455754]
- (46). Gomes-da-Silva LC, Zhao L, Bezu L, Zhou H, Sauvat A, Liu P, Durand S, Leduc M, Souquere S, Loos F, et al. (2018) Photodynamic therapy with redaporfin targets the endoplasmic reticulum and Golgi apparatus. *EMBO J* 37, No. e98354. [PubMed: 29807932]
- (47). Moserova I, and Kralova J (2012) Role of ER stress response in photodynamic therapy: ROS generated in different subcellular compartments trigger diverse cell death pathways. *PLoS One* 7, No. e32972. [PubMed: 22403731]

Figure 2.

(a) Hydrogen peroxide or (b) hydroxyl radical production by PS irradiation with 650 nm, 0.84 mW/cm² light. Bar plot shows comparison at 10 min, the time used for in vivo PDT. *n* = 3.

Figure 3.

(a) Subcellular localization experiments where each PS (red) was coincubated with an organelle stains (green). Composite images show colocalization (yellow) between the two dyes. (b) Normalized intensity of each dye along a line of interest (shown in white). (c) Pearson correlation coefficient between the red and green channel for each PS and organelle staining. $n = 3$ images.

Figure 4.

4T1/luc cells were incubated with 1 or 10 μ M of each PS for 4 h, wash, and irradiated with 650 nm: (a) 0.84 mW/cm² or (b) 3.25 mW/cm² light for 10 min. $n = 3$.

Figure 5.

(a) Intracellular concentration of PS after 10 μM of PS was incubated with cells for 4 h. (b) Incubation of cells with different starting concentrations of respective PS, to normalize intracellular concentration of PS after incubating for 4 h. (c) Cell viability after PDT, when all PS had the same intracellular concentrations. Each experiments were completed in triplicate.