Synthesis and Photodynamic Activity of Vitamin–Chlorin Conjugates at Nanomolar Concentrations against Prostate Cancer Cells

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ABSTRACT: Phototoxicity response of synthesized vitamin–chlorin conjugates and their zinc and indium complexes was determined in the human PC-3 prostate cancer cell line, which was previously demonstrated to overexpress vitamin receptors on the cell surface. Pantothenic acid (Vit B5) and lipoic acid (or thioctic acid) were covalently linked to methyl pheophorbide (a chlorophyll derivative) and subsequently metallated with zinc and indium. Cell survival assay indicated that the vitamin–chlorin conjugates have better photodynamic activity against the PC-3 prostate cancer line at the nanomolar concentration range than the commercially available starting precursor methyl pheophorbide. Fluorescence and transmission electron microscopy studies indicated some formation of apoptotic cells and cytoplasmic vacuoles of photosensitized prostatic cells. Targeting vitamin receptors in prostatic cancer cells can be utilized to enhance specificity of photosensitizers for photodynamic therapy applications.

INTRODUCTION

Prostate cancer (PC) is the second leading cause of cancer deaths in men next to lung cancer, and accounts for 20% of estimated new cases in the US in 2019. Current treatments for early-stage and advanced PC include radical prostatectomy, brachytherapy (radioactive seed implants), hormone (medical castration or androgen deprivation), cryosurgery, vaccination, and chemotherapy. These treatments can cause mild to severe side effects, complications, and fatal adverse reactions which include menopausal-like symptoms (hot flashes, night sweats and loss of libido), irritability, headache, fatigue, nausea, back pain, osteoporosis, scarring, urinary incontinence, erectile dysfunction, infection, risk of diabetes, obesity, renal failure, and stroke. For less aggressive prostatic tumors especially for older men, active surveillance rather than immediate treatment is the recommended approach.

Focal therapy (FT) is now an emerging treatment for localized PC. Some FTs including high-intensity focused ultrasound, cryotherapy, and radiofrequency have been used for patients with low-risk and localized PC. Photodynamic therapy (PDT) is considered an FT which aims to reduce morbidity associated with radical surgery and radiotherapy, while maintaining oncologic control. PDT is a minimally invasive treatment for cancer of the esophagus, bronchi, gastric, cervix, skin, head, and neck. PDT treatment includes delivery of a fluorophore, known as a photosensitizer (PS), to tumor tissues upon systemic administration and irradiation with harmless visible light (600–800 nm) in the presence of endogenous oxygen. Excitation of PS with light in the red or near infrared (NIR) region generates cytotoxic reactive oxygen species (ROS), including singlet oxygen (\( ^1O_2 \)) causing irreversible damage to tumor cells. PDT for low-risk localized PC can be considered a safe and effective treatment and a tissue-preserving option for men to prevent radical therapy.

From 2002 to 2017, several studies indicated the application of transrectal or abdominal ultrasound-guided PDT for PC. PSs used for PDT consisted of temoporfin,5,9 5-aminolevulinic acid (ALA),10 motexafin lutetium,11 and padaporfin.12 The Gleason score of PC ranged from 6 (3 + 3) to 9 (4 + 5), while the prostate-specific antigen (PSA) prior to PDT ranged from 3.3 to 22.4 ng/mL. Results from a randomized clinical trial indicated that 55% of the patients considered having low-risk localized PC in the study showed negative biopsy post-PDT with a Gleason score of less than 6, and 35% showed a decreased PSA level (<4.0 ng/mL).13 Compared to cryotherapy, brachytherapy, and high-intensity focused ultrasound, PDT for PC seems to show a low rate of side effects.14 Serious adverse side-effects consisted of hematuria, erectile dysfunction, dysuria, prostatic pain, urinary tract infection, and urinary retention being the most common. Even though these studies have demonstrated the efficacy of PDT for PC, this treatment approved 30 years ago remains underutilized in clinical practice and has not received wide acceptance because of necrosis of the entire prostate during clinical trials accompanied by severe inflammation causing extreme pain to...
the patient as a result of nonselective tissue targeting of the FDA-approved PSs (Photofrin and Foscan). Ideal PSs are typically characterized by high selectivity for tumor over normal tissues, a high quantum yield of light-induced triplet state oxygen formation, low dark toxicity, and rapid clearance from the body. The major drawback for the widespread application of PDT in the clinics is mainly due to PS selectivity, and must be improved to optimize anticancer treatment with reduced toxicity.

Cancer treatment utilizing ligands for selective targeted delivery of pharmaceuticals to malignant cells can improve anticancer drugs for treatment and imaging. Drug conjugates or hybrid ligands represent a promising class of anticancer agents to overcome treatment failures because of nonspecific tissue targeting, drug toxicity, and development of resistance. To maintain a high proliferative status, cancer cells demand a variety of nutrient transporters. Utilizing these influx transporters to deliver anticancer drugs into the cells via a transporter-mediated cellular accumulation pathway offers a promising mechanistic route. A sodium-dependent multi-vitamin transporter (SMVT) translocates biotin (BTN; Vit B7), as well as lipoic acid (LA; coenzyme), pantothenic acid (PA; Vit B5), and desthiobiotin (BTN structural analogue) across biological membranes. This transport system, which is upregulated and amplified in malignant tissues as a means to compete with normal healthy cells for survival and propagation, has been utilized for delivering drugs to target cancer cells. Previous studies revealed that the conjugation of BTN to acyclovir, ganciclovir, camptothecin, and doxorubicin caused a significant increase in the accumulation of drug into tumor cells compared to unmodified drug without BTN conjugation.

Several PSs developed for prostate PDT treatment are based on chlorophyll a derivatives or chlorins, which have been shown to exhibit low dark toxicity and generate singlet oxygen species upon light activation. In the present study, the synthesis of new vitamin–chlorin conjugates including their corresponding zinc(II) and indium(III) metal complexes and evaluation of their photodynamic activity against the human PC-3 PC cell line are described. The PC-3 PC cell line was
observed to overexpress vitamin receptors by other investigators. New chlorin derivatives conjugated to PA and LA will aim to target vitamin receptors overexpressed in PC cells. This research will provide an opportunity to improve PSs for PDT for cancer by exploiting the vitamin–receptor transport system in order to enhance the advancement of cancer research and treatment.

RESULTS AND DISCUSSION

Chemistry. As a continuation of our effort to evaluate the photodynamic cellular activity of vitamin–chlorin conjugates for their potential application in cancer research, a series of PSs composed of methyl pheophorbide \( a \) derivatives linked to vitamins or vitamin analogues have been prepared in our research group. Methyl pheophorbide \( a \) (1), a commercially available starting material, was conjugated to LA and PA using a hexyl diamine linker to produce the target chlorin derivatives (5 and 8) in good yield, using a published peptide coupling procedure. The corresponding zinc and indium complexes (6–7 and 9–10) were also prepared following established metallation protocols. Synthesized PSs as illustrated in Scheme 1 were purified by preparative silica gel thin-layer chromatography (TLC) plate and the molecular structures were characterized by \({^1}H, {^{13}}C\), COSY, HSQC NMR, and mass spectrometry spectral data.

Figure 1 shows the UV–vis absorption spectra of the target chlorin–lipoic acid (CLA, 5) and chlorin–pantothenic acid (CPA, 8) conjugates including their zinc (6 and 9) and indium (7 and 10) complexes. Typical red shift of the Soret band and blue shift of the fourth Q band were observed upon metalation.

Characteristic \( ^1H \) NMR peaks for CPA are the presence of 2 unique methyl groups attached to a quaternary carbon at 0.542 and 0.506 ppm, which were observed to shift upfield with a difference, \( \Delta \delta \), of 0.8 ppm in the zinc complex. Additionally, zinc complexes (ZnCLA and ZnCPA) showed considerable broadening of \( ^1H \) NMR signals primarily because of stacking interaction of the chlorin macrocyclic ring.

Conjugation of the PS to the vitamins involved in the linkage of a hexyl six-carbon spacer arm, which provides the length between the macrocyclic chlorin ring and the vitamin ligand for enhanced binding ability to the vitamin receptors expressed over the cancer cell surface. Other vitamin–chlorin conjugates previously synthesized in our laboratory including chlorin–biotin (CBTN) and chlorin–bexarotene (CBX) with their zinc and indium metallated counterparts, as shown in Scheme 2, were previously synthesized and tested in vitro against mouse colon cancer, triple-negative breast, and triple-positive breast cancer cell lines. Results are already published elsewhere.

The vitamins (BTN and PA), coenzyme LA, and bexarotene (BX; Vit A analogue) were covalently appended to the 13\(^1\) position of the chlorin macrocycle via a hexyl diamine linker.

Cellular Biological Activity of Vitamins in the PC-3 PC Cell Line. Vitamins are generally characterized as poorly immunogenic, cost-effective, and for some can be chemically modified fairly easily. These compounds are considered essential constituents in the proper functioning of a mammalian organism. PA (Vit B5) is a water-soluble vitamin which is a precursor for the synthesis of coenzyme A, an important component in numerous biochemical reactions that sustain life. It is ubiquitously found in foods of both plant and animal origin, and dietary deficiency is a rare occurrence. LA, also present in almost all foods, is abundant in spinach, broccoli, yeast extract, kidney, heart, and liver. Its naturally occurring form is the R-enantiomer. LA, though closely associated with the vitamin B complexes, is not considered a vitamin by most researchers now. Its biochemical activity is linked with its function as a cofactor in several enzymatic mechanisms, in particular, the citric acid cycle, including the enzymes \( \alpha \)-ketoglutarate dehydrogenase and pyruvate dehydrogenase complexes that both require the lipoyl group for acyl group transfer reactions. Aside from its role in metabolic as well as cellular signaling pathways, the therapeutic potential of LA has been extended to its neuroprotective properties in the hippocampal neurons of mouse models of Alzheimer’s disease, to its reduction of serum glucose levels in diabetic rabbits, and to its capability to provoke cellular apoptosis in lung cancer cells.

BTN (Vit B7), a water-soluble micronutrient, plays an essential role in a wide array of metabolic processes in humans related to the consumption of fats, carbohydrates, and amino acids, and, thus, is needed in normal mammalian cell growth, function, and development. Intracellularly, the vast majority of BTN is found to localize in mitochondria and the cytoplasm which is consistent with its role as a coenzyme for carboxylases in these cellular compartments. Additionally, BTN is also abundant in the cell nucleus because of its propensity to biotinylate via amide linkage lysine residues of histones in the maintenance of gene expression and DNA repair to promote genomic stability during cell proliferation. BX is a Vit A analogue classified as a retinoid and widely used for treating visual and dermatological diseases, and their effect on cancer treatment and prevention has attracted attention.

Vit A mimics are indicated to modulate cell proliferation, differentiation, and apoptosis. BX (Targretin) is a selective modulator of retinoid X receptors (RXRs) and approved for treating advanced-stage cutaneous T-cell lymphoma. Several preclinical studies and phase I/II clinical trials showed that BX exhibits tumor prevention activity against breast cancer, renal carcinoma, and lung cancer.
Vitamins such as BTN and PA, and the coenzyme LA are internalized by receptor-mediated endocytosis, and demonstrated to be uptaken by human PC-3 prostatic cells via a specialized carrier-mediated transport system. The PC-3 cell line is a widely used in vitro cell culture model for PC derived from prostatic adenocarcinoma metastasized to the bone. Human PC-3 cell line has very aggressive characteristics and represents 90% of prostatic adenocarcinoma. Other PC cell lines such as LNCaP and DU-145, which are less aggressive types, have been used as in vitro models. BTN and LA are shown to be taken up by the cells in almost equal amounts, but PA is internalized into the cells and 23% less than BTN and LA. Figure 2 shows the biological activity of PC-3 cells after 24 h treatment with vitamins without light exposure and with a light irradiation of 4.8 J cm⁻² which is the highest intensity used in this study. PA is observed to promote cellular proliferation by about 26% in the dark. No cellular damage was apparent upon treatment with vitamins alone, except for a 15% decreased cell survival for BX-treated cells in the dark and a slight decrease of 8% cell survival upon 5 min light irradiation. This is due to the fact that BX alone has anticancer properties.

**Photodynamic Activity of Vitamin−Chlorin Conjugates in the PC-3 PC Cell Line.** Selective localization of chlorins into tumor cells is primarily due to decreased pH of cancerous tissues, enhanced penetration of tumor vasculature impairing blood supply to the area, presence of heme...
carrier protein, overexpression of low-density lipoprotein (LDL; apoB/E) receptors, impaired lymphatic drainage or the enhanced permeability and retention (EPR) effect, and large amounts of collagen. Mitochondrial accumulation of chlorophyll derivatives is also believed to be mediated by the peripheral benzodiazepine receptor, now referred to as the translocator protein (TSPO), usually located in the outer mitochondrial membrane and characterized by its ability to bind a variety of benzodiazepine-like drugs and the tetrapyrole intermediates of the heme biosynthetic pathway. TSPO ligands can then be useful as anti-cancer agents because it has been observed that increased TSPO expression is associated with an advanced tumor stage and poor prognosis in human astrocytoma, colorectal, and breast cancers. Conjugating a vitamin ligand to chlorins targeting the vitamin receptors also upregulated on the cancer cell surface will provide additional enhancement of PS internalization into tumor cells.

**Free-Base Vitamin—Chlorin Conjugates.** The unmetallated vitamin–chlorin conjugates including the starting precursor methyl pheophorbide caused no dark cytotoxicity against PC-3 PC cells, except for CPA which showed 25% dark toxicity at a higher concentration of 10 μM as shown in Figure 3. Findings in this study indicated that three of the vitamin–chlorin conjugates (CBTN, CLA, and CPA) showed better photocytotoxicity than the starting methyl pheophorbide. CPA caused the most photocytotoxicity by about 31% reduction in cell proliferation at 200 nM and lowest light dose (0.96 J/cm²) compared to MePheo. The order of photocytotoxicity against PC-3 prostate cells in this study is CPA > CBTN > CLA > MePheo > CBX. CPA has hydrophilic PA with primary and secondary hydroxyl groups providing the desired amphiphilic components for enhanced tumor localization and better photodynamic effect more than BTN and LA. The presence of the Vit A analogue BX in the vitamin–chlorin conjugate CBX did not generate enhanced internal accumulation of the PS in the cells for better photodynamic activity, due to the greater hydrophobic nature of its molecular architecture decreasing amphiphility as a necessary parameter for PS cellular internalization. DMSO, used as the vehicle to dissolve the PSs, caused no cell damage at the concentration used corresponding to ≤0.08% (see Figure 3).

**Metallated Vitamin—Chlorin Conjugates.** Previous studies demonstrated that metal coordination with zinc and indium to chlorins enhances in vitro oxidative cell damage and greater singlet oxygen production, respectively. Thus, the corresponding zinc and indium complexes of the chlorin–vitamin conjugates were also prepared using published metallation protocols.

From the results of the cell viability assay as shown in Figure 3B, only InCLA exhibited the highest dark cytotoxicity, while InCBX has dark toxicity only at a higher concentration of 10 μM. Upon cellular photosensitization, InCLA also caused the most photocytotoxicity, and the photocytotoxicity order for the indium complexes is InCLA > InCBX > CPA > CPA > CPA. This trend and based on the molecular nature of the vitamin appended, PA appears to inhibit the photodynamic effect of InCPA, possibly due to an interaction of the hydroxyl groups with indium. The presence of a carbonyl functionality in BTN could also cause an intermolecular indium–carbonyl interaction also affecting the ability of indium in increasing singlet oxygen production upon photosensitization. Because BX only contains nonpolar molecular functionality, no intermolecular interaction with indium can be expected, and thus, the phototoxic behavior of InCBX is next to InCLA, wherein the disulfide entity in LA did not block the photodynamic effect of indium probably because of the cyclic nature of the disulfide linkage in the lipoate moiety which might be too bulky and sterically constrained to interact with indium.

For the zinc complexes in Figure 4A–D, the photodynamic activity in PC-3 cells is similar to the free-base vitamin–chlorin conjugates. There is a slight 22% dark cytotoxicity at 10 μM of
ZnCPA, while the rest of the zinc complexes exhibited no cell inhibition in the dark. The trend $\text{ZnCLA} \geq \text{ZnCPA} > \text{ZnCBTN} \gg \text{ZnCBX}$ seems to be comparable with that of the unmetallated complexes indicating that zinc metal has a minor effect in improving the photodynamic nature of the vitamin–chlorin conjugates by increasing its in vitro oxidative cell damage. Additionally, the zinc complexes are observed to undergo a stacking interaction of the chlorin macrocyclic ring as manifested by the broadening of the proton NMR signals. Increased lipophilicity of the zinc complex in other studies resulted in a higher vesicle and cellular uptake allowing for more efficient membrane photo-oxidation. However, significant photodynamic enhancement with zinc chelation to chlorin was not observed in this study.

**Fluorescence and Electron Microscopy.** PDT can lead to various forms of cell death pathways, either apoptosis, necrosis and/or autophagy. Apoptosis, referred to as programmed cell death pathway, is a complex multistep highly controlled process characterized by several biochemical and morphological changes associated with the demise of a living cell. It is the preferred cell death mechanism because of the absence of inflammation causing less pain to the patients compared to necrosis (accidental and unprogrammed cell death mechanism characterized by acute tissue injury, cytoplasmic cell swelling, and disintegration of cellular membranes), or autophagy (autodegradation characterized by the presence of vesicles and double-membrane structures called autophagosomes surrounding the target region). Recently, paraptosis, which is a term to describe extensive single-membrane bounded vacuole formation because of misfolded proteins in the endoplasmic reticulum, has been implicated as a response to PDT damage. Morphological

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**Figure 5.** Fluorescence images of fixed human PC-3 PC cells with Hoechst 33258 nuclear dye. Morphology of untreated unirradiated (A) and irradiated cells (B); 24 h treatment with 500 nM CBTN (C), CBX (E), CLA (G), CPA (I), and MePheo (K) unirradiated; and, after 24 h treatment with 500 nM CBTN (D), CBX (F), CLA (H), CPA (J), MePheo (L), BTN (M), BX (N), LA (O), and PA (P), followed by 650 nm light exposure (power of 16 mW/cm$^2$ and fluence rate of 1.92 J/cm$^2$). Shrunk cells and sparse cell density (D,F,J,H,L) are observed without significant apoptosis characterized by nuclear fragmentation, except for a few in CBX-treated cells (F). Insets are enlarged views.

**Figure 6.** Fluorescence images of fixed human PC-3 PC cells after 24 h treatment with 500 nM of metallated chlorin–vitamin complexes and stained with Hoechst 33258 nuclear dye. Morphology of cells treated with InCBTN (A), InCBX (B), InCLA (C), InCPA (D), ZnCBTN (E), ZnCBX (F), ZnCLA (G), and ZnCPA (H), then exposed to 650 nm light (power of 16 mW/cm$^2$ and fluence rate of 1.92 J/cm$^2$). Shrunk cells and reduced cell density (D,F,H) are observed without substantial apoptosis except for InCLA (C) showing few chromatin fragments. Insets are enlarged views.
examination using fluorescence microscopy has provided detailed structural features characterizing apoptotic cell death which are mainly cell shrinkage, rounding, membrane blebbing, and apoptotic bodies. DNA fragmentation has been observed to be present in both apoptotic and necrotic cells and, therefore, is not an entirely definitive indication to unambiguously identify apoptosis alone. PDT response is dependent on the cell type, types of PSs utilized, subcellular localization of the PS, and light dose for activation. It is now accepted that a high PS concentration or high fluence rate of light introduced typically will cause necrotic cell death, while the administration of low doses of PS and light predispose tumor cells to undergo apoptosis. However, PDT has the ability to activate multiple cell death pathways. Results in our study from fluorescence light microscopy experiments revealed only cell shrinkage and rounding, but the presence of apoptotic bodies, nuclear margination, and DNA fragmentation were not evident to a large extent. At 500 nM concentration and a light dose of 1.92 J/cm², only CBX-sensitized PC-3 cells indicated a few apoptotic cells (Figure 5F). Significant reduction of cell density after photosensitization was apparent for the unmetallated vitamin–chlorin conjugates CBTN, CLA, and CPA as compared to precursor MePheo as shown in Figure 5D, H, J, L, and this observation is consistent with the cell survival assay depicted in Figure 3 in which CPA, CBTN, and CLA (at 500 nM and 0.96 J/cm²) reduced cell survival by about 58, 50, and 30%, respectively, more than precursor MePheo.

For the In complexes at 500 nM concentration and a light dose of 1.92 J/cm², InCBTN and InCBX showed intact cells with very little cellular damage (Figure 6A,B). InCLA revealed evidence of apoptosis with the appearance of chromatin fragments and reduction of cell density (Figure 6C), while the InCPA indicated combination of necrosis due to cell swelling and apoptosis due to some nuclear condensation (Figure 6D). Fluorescence microscopy results matched the cell viability assay data shown in Figure 3B in which InCLA exhibited the most phototoxic effect. For the zinc complexes at the same concentration and light dose, only intact cells were observed (Figure 6F–H) consistent with the results from the cell proliferation assay in which cell damage was apparent starting at the micromolar range (5 μM). For PC-3 cells treated with vitamins alone and photo-irradiated, no cellular damage was detected (Figure 5M–P), and, in fact, vitamins promoted cell growth.

Transmission electron microscopy (TEM) revealed ultrastructural images of PC-3 cells before and after treatment with PS and light. Figure 7AB shows images of control cells with round and well-preserved cytoplasm and plasma membrane. Similarly, when cells (Figure 7C) were treated with 500 nM CLA in the dark, a large nucleolus and well-defined plasma membrane with the cell adhering to its neighbor is observed. Upon 24 h treatment with CLA and after photosensitization with 1.92 J/cm², cells as shown in Figure 7D–F started to retract, and degeneration of nuclear and cytoplasmic structures, ruffling, blebbing, budding, and vacuole formation were indicative of cellular injury caused by irradiation. Degradation of intracellular components as a response to insults seem to be taking place in discrete steps including sequestration of cytoplasmic structures. Aside from the appearance of shrunk and irregularly shaped cells, a striking feature in Figure 7F is the formation of vacuoles which can be described as an example of autophagic vesicles whereby the intracellular components are completely unrecognizable with the cell compartments enclosed into a somewhat thicker or perhaps a double-membrane structure characteristic of autophagosomes. From the TEM results, CLA-induced photosensitization appears to promote autophagy as one of its mechanistic route for cellular degradation.

**CONCLUSIONS**

Vitamin transporters upregulated in cancer cells are targets for improving cellular accumulation of poorly permeable highly potent anticancer drugs including the delivery of PSs for PDT.
applications. Findings in our study demonstrated that BTN, LA, and PA in the vitamin–chlorin conjugates provided a means for increased photodynamic activity at the nanomolar range to disrupt cancer cell proliferation of human PC-3 prostatic cells. The presence of indium provided additional PDT efficacy in vitro compared to the unmetallated chlorin–vitamin conjugate and the zinc complexes. BX, a Vit A analogue, did not enhance photodynamic activity of the synthesized PS in the PC-3 PC cell line. Further research is required to determine if the vitamin-conjugated PSs synthesized in this study will provide selectivity in vivo to internalize PSs in specific types of cancer that exhibit overexpression of vitamin receptors including ovarian, breast, renal, lung, and leukemia cell lines. PDT is slowly being used as a viable option in the treatment of cancer, specifically, the aggressive type. More work is needed to create PSs that would be more specific to the tumor, thereby inhibiting toxicity to normal healthy cells.

**EXPERIMENTAL SECTION**

**Chemical Synthesis. General.** Solvents and reagents were purchased mainly from Sigma-Aldrich Chemical Co. (St. Louis MO, USA). All air and moisture sensitive reactions were performed in anhydrous solvents under a nitrogen atmosphere. Chromatographic purifications were performed in the normal phase preparative TLC plate (Analtech). Reactions were monitored spectrophotometrically or by TLC using a polyester-backed normal phase analytical plate (Merck, Silica gel 60 F254 precoated 200 μm) and detected with UV light (λ = 254 nm). Visible spectra were recorded with a ThermoScientific Genesys 150 UV–visible spectrophotometer. NMR spectra were acquired with a Bruker AVANCE NMR spectrometer (400 MHz for 1H and 100 MHz for 13C). Chemical shifts are reported in δ ppm referenced according to the deuterated solvent used as the internal standard: CDCl3, 7.24 ppm (1H), 77.23 ppm (13C). High-resolution mass spectroscopy (HRMS) data were obtained on a Bruker microTOF-II ESI mass spectrometer. All compounds synthesized were isolated and purified in ≥99% purity as confirmed by 1H, 13C, 2D COSY (correlated spectroscopy), DEPT 90/135 (distortion-less enhancement by polarization transfer), HMQC (heteronuclear single quantum correlation) NMR spectra. Sample purity was also checked using ThermoScientific Ultimate 3000 high-performance liquid chromatography (HPLC) equipped with a diode-array four channel variable UV–visible detector, an autosampler, and a fraction collector using a reverse-phase column (C-18, 4.6 × 50 mm2, 3.5 μm) in the isocratic mobile phase (100% acetonitrile or 100% methanol) visualizing at λ = 405 and 665 nm with a flow rate of 1 mL min−1.

**Synthesis of 13’-Hexamethylenediaminyl-pantothenylchlorin ε5 Dimethyl Ester, CPA, 8.** In a dry round-bottom flask containing a mixture of 13’-hexamethylenediaminylchlorin ε5 DME 2 (40 mg, 0.055 mmol), PA 4 (120 mg, 0.548 mmol), EDC (151 mg, 0.786 mmol), DIPEA (0.2 mL), and HBTU (120 mg, 0.316 mmol) was stirred in dry N,N-dimethylformamide (DMF; 8 mL) under nitrogen overnight for 12 h. The reaction was monitored by TLC (5% methanol in CH2Cl2) until the reaction showed disappearance of the starting amine. The solvent was evaporated and the residue was purified by the preparative TLC plate using the same solvent system to afford 23 mg (0.025 mmol, 45% yield) of 13’-hexamethylenediaminyl-pantothenylchlorin ε5 DME, CPA 8 (C55H45N6O6S2). UV–vis (CH3Cl2): λmax (ε/M−1 cm−1) 663 (35,818), 605 (4506), 528 (3909), 402 (113,013); 1H NMR (CDCl3, 400 MHz): δ 9.66 (s, 1H, 10-meso H), 9.61 (s, 1H, 5-meso H), 8.81 (s, 1H, 20-meso H), 8.03–7.96 (dd, J = 17.88, 11.48 Hz, 1H), 3.14 (3CH = CH2), 6.84 (br s, 1H, −NHCH(CH2)2CH2NH−), 6.31–6.26 (d, J = 17.88 Hz, 1H, trans 3CH = CH2), 6.13–6.12 (d, J = 11.70 Hz, 1H, cis 3CH = CH2), 5.63 (br t, 1H, −NHCH(CH2)2CH2NH− in LA ring), 3.72 (3H, 15CO2CH3), 3.55 (s, 3H, 17CO2CH3), 3.45 (s, 3H, 11CH3), 3.43 (br s, 2H, −NHCH(CH2)2CH2NH− and, 3H, 21CH3), 3.23 (br t, 2H, −NHCH(CH2)2CH2NH−, and, 3H, 21CH3), 2.96–2.84 (m, 2H, −CH2CH2− in LA ring and 2H, −NHCO−), 2.49–2.45 (br m, 1H, −CH2CHSS− in LA ring), 2.14 (br m, 2H, NHCOCH2−, 2H, and 1H, −CH2CHSS− in LA ring), 1.76 (br m, 2H, 17CH2CH2 and 2H, 17CH2), 1.70 (br d, 3H, 18−CH3), 1.62–1.57 (br t, 3H, 8−CH3 and 6H−pH in LA alkyl chain), 1.39–1.28 (br m, 8H, −NHCH(CH2)2CH2NH−), −2.08 (br s, 2H, ring NH); 13C NMR (CDCl3, 100 MHz): δ 173.8 (17C−CO2CH3 and 15C−CO2CH3), 173.2 (13−CONH), 170.6 (hexyl-NHCO), 165.5 (19), 162.6 (1), 160.0 (4), 151.2 (6), 147.2 (14), 146.2 (16), 145.4 (3), 144.0 (8), 139.6 (9), 135.9 (11), 135.4 (2), 134.7 (12), 130.6 (7), 129.3 (3), 129.3 (15), 122.4 (30), 104.0 (13), 103.1 (10), 101.0 (5), 94.6 (20), 60.4 (17), 56.3 (C8H3, LA ring), 53.4 (15CO2CH3), 52.2 (17CO2CH3), 49.4 (18), 40.3 (−NH−CH2(CH2)2CH2−NHCO−LA), 40.1 (C8H3, LA alkyl chain), 39.0 (−NH−CH2(CH2)2CH2−NHCO−LA), 38.8 (C8H3, LA ring), 37.9 (15), 36.2 (−NH−(CH2)2CH2CH2−NHCO−LA), 34.4 (C8H3, LA alkyl chain), 31.1 (C8H2, LA ring), 29.7 (−NH−CH2(CH2)2CH2−NHCO−LA), 29.4 (17), 29.1 (17), 28.7 (C8H2, LA alkyl chain), 26.5 (−NH−(CH2)2CH2−CH2−NHCO−LA), 26.2 (−NH−(CH2)2CH2−CH2−NHCO−LA), 25.6 (C8H2, LA alkyl chain), 23.1 (18), 19.7 (8), 17.5 (8), 12.2 (12), 12.1 (2), 11.4 (7); HPLC (100% MeOH), τR: 1.590 min (96%); HRMS (MALDI-TOF) m/z: 911.4552 [M]+, calc for C55H45N6O6S2, 911.4558.
2H, 15$^1$CH$_3$), 5.19 (s, 1H, −OH in PA), 4.42−4.36 (q, 1H, 18-H), 4.28−4.25 (m, 1H, 17-H), 3.69 (s, 3H, 15$^2$CO$_2$CH$_3$), 3.64−3.62 (br q, 2H, 8$^2$-CH$_2$), 3.52 (s, 3H, 17$^2$CO$_2$CH$_3$) overlapping m, 4H$_{15}$C$_6$H$_{14}$CH$_2$NH− and 1H$_{15}$−CH$_2$−CH$_2$NH− in PA), 3.38 (s, 6H, 12$^1$CH$_3$ and 2$^1$CH$_3$), 3.34 (s, 1H, −OH in PA), 3.19 (s, 3H, 7$^1$-CH$_3$), 3.01 (br m, 4H, −NH$_2$(CH$_2$)$_4$CH$_2$NH−), 2.67 (s, 1H, OH in PA), 2.09 (br m, 2H$_{16}$−CH$_2$− in PA and 2H, 17$^2$-CH$_2$), 1.61 (br t, 8H, 18$^1$, 8$^2$-CH$_2$ and 17$^2$-CH$_2$), 1.28−1.17 (br m, 6H, −NH$_2$(CH$_2$)$_4$CH$_2$NH−), 0.542 and 0.506 (two s, 6H$_{15}$B, −C(CH$_3$)$_3$ in PA), −2.03 (br m, 2H, ring NH); 13$^C$ NMR (CDCl$_3$, 100 MHz): δ 174.6 (172$^2$-CO$_2$CH$_3$), 173.6 (13-CN=O), 173.5 (hexyl-NHCO), 171.0 (CO−PA), 167.9 (NHCO in PA), 165.9 (19), 162.6 (1), 158.5 (4), 154.2 (3), 149.1 (14), 144.8 (16), 145.4 (3), 144.0 (8), 138.9 (9), 136.2 (11), 135.0 (2), 134.9 (12), 134.7 (7), 129.8 (15), 128.5 (3$^1$), 121.3 (3$^2$), 102.0 (13), 101.2 (10), 98.9 (5), 93.7 (20), 90.7 (18), 3.78 (−N − H − C (− H)$_2$ C (− H)$_2$ N − HCCO− PA), 3.54 (−CH$_2$CH$_2$NH− in PA), 3.38 (N(−(−CH$_2$)$_6$C(CH$_3$)$_3$−NH), 3.10 (−(−CH$_2$)$_6$C(NH)$_2$−NH), 2.92 (17$^2$−CH$_2$CN=O$^{−}$NH), 2.91 (17$^2$−CH$_2$), 2.64 (−(−CH$_2$)$_6$C(−H)$_2$−CH$_2$NH−), 2.61 (−(−CH$_2$)$_6$C(−CH$_2$)$^{−}$NH), 2.31 (18$^1$), 20.2 and 21.0 (−C(C$_6$H$_5$)$_{15}$H), in PA), 19.6 (8$^1$), 17.7 (8$^2$), 12.1 (12$^1$), 11.9 (2$^1$), 11.3 (7$^1$); HPLC (100% MeOH), t$_{R}$: 1.467 min (96%); HRMS (MALDI-TOF) m/z: 924.5230 [M$^+$], calcd for C$_{90}$H$_{64}$Cl$_{15}$N$_{10}$O$_{6}$S$_{2}$ 925.4360.

**Synthesis of Zn(II)-13$^1$-Hexamethylenediaminyl-pantothenylchlorin e$_g$ Dimethyl Ester, ZnClA, 9.** The procedure for In insertion was followed as above using CPA 8 (20 mg, 0.002 mmol). The crude product was purified by the preparative TLC plate using 9% methanol−dichloromethane (DCM) to afford 9 mg (0.009 mmol, 42% yield) of the title compound ZnClA 9. UV−vis (CH$_2$Cl$_2$): λ$_{max}$ (ε/M$^{-1}$ cm$^{-1}$) 637 (42 758), 592 (7106), 412 (113 855); $^1$H NMR (CDCl$_3$, 400 MHz): δ 9.46 (br s, 2H, 10- and 5-s-meso H), 8.53 (s, 1H, 19-meso H), 8.01 (very br s, 1H, 3$^2$CH$_3$), 7.25 (br t, 1H, −NHCO in PA), 6.85 (br s, 1H, −NH$_3$(CH$_3$)$_3$CH$_2$NH−), 6.19 (d, 2H, 2$^2$CH$_3$), 5.95 (very br s, 1H, −NH−CH$_2$(CH$_3$)$_3$CH$_2$NH−), and 2H, 15$^2$CH$_3$, 5.32 (br m, 1H, 18-H), 5.30 (s, 1H, −OH in PA), 5.0 (br m, 1H, 17-H), 3.74 (s, 3H, 15$^2$CO$_2$CH$_3$), overlapping m, 2H, 8$^2$-CH$_2$, 3.63 (s, 3H, 17$^2$CO$_2$CH$_3$) with overlapping m, 4H$_{15}$C$_6$H$_{14}$CH$_2$NH− and 1H$_{15}$−CH$_2$−CH$_2$NH− in PA), 3.48−3.34 (s, 6H, 12$^1$-CH$_3$ and 2$^1$-CH$_3$), 1H, −OH in PA; 3H, 7$^1$−CH$_3$; 4H, −NH$_2$(CH$_3$)$_3$CH$_2$NH−, 2.71 (br s, 1H, OH in PA), 2.56−2.16 (br m, 2H$_{16}$−CH$_2$− in PA and 2H, 17$^2$CH$_3$, 1.65−1.59 (br m, 8H, 18$^1$, 8$^2$-CH$_2$ and 17$^2$-CH$_3$), 1.39−1.21 (br m, 6H, −NH$_2$(CH$_3$)$_3$CH$_2$NH−), 0.253 and 0.314 (two s, 6H$_{15}$B, N=C(CH$_3$)$_3$PA); HPLC (100% MeOH), t$_{R}$: 1.400 min (94%); HRMS (MALDI-TOF) m/z: 985.4274 [M$^+$], calcd for C$_{90}$H$_{64}$Cl$_{15}$N$_{10}$O$_{6}$S$_{2}$ 985.4286.

**Synthesis of 13$^1$-Hexamethylenediaminyl-pantothenylchlorin e$_g$ Dimethyl Ester Indium(III) Chloride, InClA-Cl, 10.** The procedure for In insertion was followed as above using CPA 8 (13 mg, 0.004 mmol). The crude product was purified by the preparative TLC plate using 10% methanol−DCM to afford 4 mg (0.0037 mmol, 27% yield) of the title compound InClA-Cl 10. UV−vis (CH$_2$Cl$_2$): λ$_{max}$ (ε/M$^{-1}$ cm$^{-1}$) 640 (46 568), 595 (65 852), 412 (133 619); $^1$H NMR (CDCl$_3$, 400 MHz): δ 9.70−9.68 (s, 2H, 10- and 5-s-meso H), 8.69 (s, 1H, 20-meso H), 8.04−7.97 (dd, J = 11.97, 6.74 Hz, 1H, 3$^2$CH$_3$), 7.16 (br t, 1H, −NHCO in PA), 6.78 (br s, 1H, −NH$_2$(CH$_3$)$_3$CH$_2$NH−), 6.27−6.22 (d, J = 17.87 Hz, 1H, −CH$_2$(CH$_3$)$_3$CH$_2$NH−), 6.14−6.11 (d, J = 11.66 Hz, 1H, cis 3$^2$CH$_3$=CH$_2$), 5.58 (br t, 1H, −NH−CH$_2$(CH$_3$)$_3$CH$_2$NH−), 5.48−5.43 (br m, 2H, 15$^2$CH$_3$), 5.3 (s, 1H, −OH in PA), 4.50−4.45 (q, 1H, 18-H), 4.39−4.37 (m, 1H, 17-H), 3.83−3.80 (br s and overlapping m, 3H, 15$^2$CO$_2$CH$_3$ and 2H, 8$^2$-CH$_2$, 3.68−3.62 (s, 3H, 17$^2$CO$_2$CH$_3$, overlapping m, 4H$_{15}$C$_6$H$_{14}$CH$_2$NH− and 1H$_{15}$−CH$_2$−CH$_2$NH− in PA), 3.44 (s, 6H, 12$^1$-CH$_3$).
CH1 and 2'-CH3), 3.36 (s, 3H, 7'-CH3 and 1H, –OH in PA), 3.26 (br m, 4H, –NHCH2(CH3)2CH2NH–), 2.65 (s, 1H, OH in PA), 2.39 (br m, 2H, –CH=– in PA, and 2H, 17'-CH3), 1.87 (br t, 3H, 18'-CH3), 1.75 (t, 3H, 82'-CH3), 1.60–1.54 (br m, 6H, –NH((CH2)6)2CH2NH–, and 2H, 17'-CH3), 0.68 and 0.64 (two s, 6H, –C(CH3)2 in PA); HPLC (100% ACN), tR: 1.297 min (96%); HRMS (MALDI-TOF) m/z: 1036.3516 [M – Cl–], calculated for C36H42ClN6O9, 1071.7751.

**Photocytotoxicity Assay.** General.** The human PC-3 PC cell line was purchased from the American Type Culture Collection (ATCC CRL-1435). PC-3 cells were cultured in F-12 medium (ATCC CC-30-2006) supplemented with 10% fetal bovine serum and were grown to 80–100% confluence in 75 cm² culture flasks (Corning) for about a week (5–6 days) in a humidified incubator (Fisher Scientific Isotemp) with 5% CO2 at 37 °C. Culturing and subculturing procedures were followed according to the ATCC Protocol available online for the PC-3 PC cell line.69

**Cell Survival Assay.** Cells were grown to confluence in a 96-well plate (4 × 10⁴ cells/well) and treated for 24 h with compounds or PSs (0.02, 0.05, 0.1, 0.5, 1, 5, 10 μM) in growth media from a stock solution of 10 mM in DMSO (Fisher). After 24 h treatment, cells were gently replaced with fresh media and positioned below a noncoherent Lumacare LC-122650 nm light source and subsequently irradiated for 1, 2, and 5 min at an energy fluence rate of 16 mW/cm² (measured using a Newport optical power meter model 840). Untreated cells served as control samples. Cells were gently washed with prewarmed phosphate-buffered saline (PBS) the next day, and MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma, 0.3 mg/mL) in PBS was added to each well. Samples were incubated for 2 h. DMSO was added to each well and plates were shaken at room temperature for 1 h to dissolve the purplish-blue formazan crystals. Absorbance values at 490 nm were measured on a microplate reader (BioRad 550). Absorbance readings were calculated based on values at 490 nm were measured on a microplate reader.

**Fluorescence Microscopy.** Cells (1 mL aliquots) obtained from a diluted cell suspension were seeded into each well (1.7 cm², ~1000 cells/well) of a 4-well culture slide (BD Biosciences) and grown to confluence in 5% CO2 at 37 °C. After aspirating the old media, 1 mL of the compound or PS (0.5 μM) in fresh prewarmed media at 37 °C was added to each well. After PS treatment for 24 h, cells were washed with 1 mL fresh growth media, and then irradiated with light using Lumacare LC-122 as described above. Cells were stained in the dark with 1 mL of 0.1 mg/mL Hoechst 33258 (Molecular Probes) in prewarmed media for 15 min at 37 °C, washed twice with 1 mL filtered PBS, then fixed with 1 mL filtered prewarmed 4% paraformaldehyde for 10 min in the incubator. Wells were removed after liquid aspiration and slides were allowed to dry in the dark for 1 h. Slides were then protected with coverslips, sealed with nail polish, and allowed to dry in the dark for another 30 min. Images were recorded by fluorescence microscopy [4',6'-diamidino-2-phenylindole (DAPI) for Hoechst 350–390 nm excitation and 460–490 nm emission filters] using an upright fluorescence microscope with a Retiga imaging 2000R (Nikon Optiphot-2, 20X and 40X) and image processing Nikon NIS-Elements V4.0 Qimaging software.

**Transmission Electron Microscopy.** PC-3 prostatic cells were cultured to confluence in a Petri dish (50 cm in diameter), treated for 24 h with 500 nM of PS, and then irradiated for 2 min as above. After photosensitization 24 h later, cells were scraped gently in the dark, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, then postfixed with 1% osmium tetroxide (containing 0.8% ferricyanide), treated with 2% aqueous uranyl acetate, and subsequently dehydrated in gradient concentrations (50–100%) of varying ethanol/water mixtures. The resulting pellets were embedded in resin and consequently cut with an ultramicrotome to a 70 nm thickness, and then viewed using a Tecnai G2 20 transmission electron microscope.

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**Author Contributions**
Biological experiments, data analysis, and manuscript preparation were done solely by the corresponding author. All authors have given approval to the final version of the manuscript. The co-author (D.M.H.) assisted in some of the synthetic work in this research study.

**Notes**
The authors declare no competing financial interest.

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**ABBREVIATIONS**
PC, prostate cancer; FT, focal therapy; PDT, photodynamic therapy; PS, photosensitizer; NIR, near infrared; ROS, reactive oxygen species; ALA, 5-aminolevulinic acid; SMVT, sodium-dependent multivitamin transporter; TLC, thin-layer chromatography; COSY, correlated spectroscopy; DEPT, distortionless enhancement by polarization transfer; HSQC, heteronuclear single quantum correlation; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; CLA, chlorin–lipoic acid; CPA, chlorin–pantothenic acid; CBX, chlorin–baxeroten; CBTN, chlorin–biotin; MePheo, methyl pheophorbide a; LA, lipoic acid; PA, pantothenic acid; BTN, biotin; BX, baxeroten; DCM, dichloromethane; Boc, tert-butoxy carbonyl; TFA, trifluoroacetic acid; DMTMM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride;
DME, dimethyl ester; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; DAPI, 4',6-diamidino-2-phenylindole; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DIPEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; HBTU, 2-((1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; $\lambda_{\text{max}}$, maximum wavelength; LDL, low-density lipoprotein; EPR, enhanced permeability and retention; RXR, retinoid X receptors; TSPO, translocator protein; DNA, deoxyribonucleic acid; TEM, transmission electron microscopy

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