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Improved Photodynamic Therapy Efficacy of Protoporphyrin IX Loaded Polymeric Micelles Using Erlotinib Pretreatment

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Abstract

Photodynamic therapy (PDT) has attracted widespread attention in recent years as a non-invasive and highly selective approach for cancer treatment. We have previously reported a significant increase in the 90-day complete response rate when tumor-bearing mice are treated with the epidermal growth factor receptor (EGFR) inhibitor erlotinib prior to PDT with the photosensitizer benzoporphyrin-derivative monoacid ring A (BPD-MA) compared to treatment with PDT alone. To further explore this strategy for anticancer therapy and clinical practice, we tested whether pretreatment with erlotinib also exhibited a synergistic therapeutic effect with a nanocarrier containing the clinically relevant photosensitizer protoporphyrin IX (PpIX). The PpIX was encapsulated within biodegradable polymeric micelles formed from the amphiphilic block copolymer poly(ethylene glycol) - polycaprolactone (PEG-PCL). The obtained micelles were characterized systematically *in vitro*. Further, an *in vitro* cytotoxicity study showed that PDT with PpIX loaded micelles did exhibit a synergistic effect when combined with erlotinib pretreatment. Considering the distinct advantages of polymeric nanocarriers *in vivo*, this study offers a promising new approach for the improved treatment of localized tumors. The strategy developed here has the potential to be extended to other photosensitizers currently used in the clinic for photodynamic therapy.

Graphical abstract

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Supporting Information.

A summary of physical-chemical properties from PpIX loaded micelles and *in vitro* PpIX release.

1. INTRODUCTION

Photodynamic therapy (PDT) is a localized approach for the treatment of malignant or otherwise abnormal tissues that relies on the simultaneous presence of a drug (photosensitizer), oxygen, and drug-related excitation wavelengths of light. In PDT, a photosensitizer is administered either systemically or locally to a patient, followed by local illumination at photosensitizer-absorbing wavelengths of light. Photosensitizer activation leads to the production of cytotoxic reactive oxygen species that produce apoptosis and/or necrosis, shut down the tumor microvasculature, and stimulate the host immune system.¹ Because the photosensitizer accumulates preferentially in malignant or other abnormal tissues in comparison to the surrounding healthy tissues and the light source is directly illuminated on the lesion, PDT can achieve dual selectivity and minimize damage to adjacent healthy structures.

Currently, most clinically approved photosensitizers belong to the porphyrin or chlorin families (*e.g.*, Photofrin, 5-ALA/PpIX, BPD-MA). For example, the first generation photosensitizer Photofrin has worldwide approvals for applications that include treatment of cancers of the lung, esophagus, bile duct, bladder, ovary and brain.^{2, 3} In caring for the Photofrin-sensitized patient, one must be cognizant of its extended period of photosensitivity (measured in weeks). Photosensitivity is of a shorter duration with 2nd generation photosensitizers. In 1999, the US FDA approved the use of 2nd generation photosensitizer BPD-MA as Visudyne® for age-related macular degeneration in ophthalmology.^{4, 5} Also, endogenous protoporphyrin IX (PpIX) induced by exogenous 1,5-aminolevulinic acid (5-ALA) as a precursor is approved by the US FDA for non-oncological PDT treatment of actinic keratosis.⁶ Moreover, the ALA-ester prodrug hexaminolevulinate (Hexvix®) is now widely used for the detection and removal of bladder cancers.⁷ However, both BPD-MA and PpIX are poorly water-soluble drugs and cannot be administered intravenously directly. In addition, minimal dark toxicity with these drugs as well as easy hydrophobic aggregation leads to limited transportation capability within biological media and poor tumor selectivity, further limiting direct clinical applications.⁸ Therefore, optimizing the delivery strategy of these hydrophobic photosensitizers to improve PDT efficacy has been a widely pursued aim.

Nanoparticle-based drug delivery system provides new capabilities to address these problems by improving drug availability in physiological conditions and enhancing tumor accumulation through active and passive targeting.⁹ Currently, a wide range of nanoparticle platforms including liposomes, polymeric micelles/vesicles, dendrimers, and macro- and nanogels, have been tested as drug nanocarriers for cancer treatment.^{10, 11} Among the many nanoparticulate systems, polymeric micelles are of particular interest for encapsulating hydrophobic PDT drugs.^{12, 13, 14, 15} Composed of amphiphilic block copolymers, these micelles have a hydrophilic corona, typically poly(ethylene glycol), and a hydrophobic core into which hydrophobic drugs can be incorporated. These nanocarriers are mostly biocompatible, degradable, and have a prolonged circulation time with enhanced tumor accumulation, allowing them to function as ideal carriers for hydrophobic drugs. Therefore, polymeric micelles are able to overcome certain specific clinical challenges associated with traditional methods of hydrophobic photosensitizer administration.

Erlotinib (Tarceva) is a drug used to treat non-small cell lung cancer (NSCLC), pancreatic cancer and several other types of cancer.¹⁶ It is a reversible tyrosine kinase inhibitor, which acts on the epidermal growth factor receptor (EGFR). To achieve the desired anticancer effect in clinic, erlotinib is typically administered orally at a high dose. It has been extensively reported that the anticancer effects are optimized when it is co-administered with other anticancer chemotherapy agents, such as 5-fluorouracil, doxorubicin (DOX) and especially gemcitabine.¹⁷ Nevertheless, erlotinib remains largely unexplored in combination with PDT. We have recently reported that improved therapeutic outcomes can be achieved when benzoporphyrin-derivative monoacid ring A (BPD-MA)-PDT is combined with erlotinib pretreatment in a xenograft mouse model of non-small cell lung cancer.¹⁸ Given that PDT has previously been combined with erlotinib for anti-angiogenesis,^{19, 20, 21} we proposed that erlotinib may enhance the anti-tumor effect of PDT by the augmentation of PDT vascular effects or increasing photosensitizer retention via tyrosine kinase inhibitor (TKI) pathways. Due to the hydrophobic nature of many photosensitizers, combining free photosensitizer with erlotinib pretreatment does not have a high degree of clinical translation capacity.

Based on the above findings, we designed a biodegradable polymeric micelle platform for delivery of the clinically relevant photosensitizer PpIX. The nanocarriers were prepared from an FDA-approved biocompatible and biodegradable block copolymer, polyethylene oxide-polycaprolactone (PEG-PCL) (Figure 1). The obtained PpIX-loaded micelle was fully characterized. Further, an *in vitro* MTT assay showed that PpIX-loaded micelles demonstrated a similar synergy with erlotinib pretreatment as the combination of free PpIX and erlotinib. Considering the distinct advantages of the polymeric nanocarrier *in vivo*, this study provides a promising strategy for delivering a clinically relevant PDT drug with a clinically used anticancer drug. The polymeric nano-platform may also be extended to other PDT drugs currently used in the clinic for photodynamic therapy.

2. EXPERIMENTAL SECTION

Materials

Poly(ethylene glycol) (4000) - polycaprolactone (3000) copolymer (denoted PEG-PCL) was purchased from Polymer Source (Dorval, Quebec, Canada). Protoporphyrin IX (PpIX) and 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA) were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture medium (Dulbecco's Modified Eagle Medium), penicillin, streptomycin and heat-inactivated fetal bovine serum (FBS) were purchased from Gibco Life Technologies, Inc. (Grand Island, NY, USA). Erlotinib (Genentech, San Francisco, CA) was dissolved in PBS to a stock concentration of 4 mM. All other chemicals were used as received. All of the buffer solutions were prepared with deionized water.

Preparation of PpIX-loaded polymeric micelles

PpIX-loaded polymeric micelles were prepared using a modified oil-in-water emulsion-based self-assembly method as previously described.²² Briefly, PpIX was dissolved in DMSO at 20 mg/mL, and PEG-PCL was dissolved in toluene at a concentration of 50 mg/mL. A combined DMSO/toluene solution (200 μ L) of the PEG-PCL (4 mg) and the

PpIX was added directly to a glass vial containing 4 mL of Millipore water (resistivity=18.2 MΩ cm), and the mixture was sonicated for approximately 5 min in an ultrasonic bath. The PpIX to PEG-PCL in feed weight ratio was varied from 5% to 100%. The emulsions were then allowed to stand overnight to evaporate toluene. The mixture was purified by dialysis (molecular weight cutoff: 3.5 kDa) against water for 36 h to remove free PpIX and the trace amount of DMSO. The water was replaced every 6 h. The resulting brown solution was centrifuged at 4000g for 10 min to remove the large aggregates. The PpIX-loaded polymeric micelles were then filtered through a 0.22 μm cellulose acetate membrane filter (Nalgene, Thermo Scientific). Finally, the PpIX-loaded polymeric micelles were concentrated using 100K MWCO centrifugal filter units (Millipore, Billerica, MA, USA) and exchanged into PBS buffer as necessary.

To determine the loading efficiency of PpIX, PpIX-loaded micelles in H₂O were lyophilized, resuspended in DMSO, and measured by UV-Vis spectrometer (CARY 100 BIO, USA) at 409 nm. All measurements were performed in triplicate. Drug loading efficiency (DLE) and drug loading content (DLC%) were calculated according to the following formulas:

$$\text{DLE (wt. \%)} = (\text{weight of loaded drug} / \text{weight of drug in feed}) \times 100\%$$
$$\text{DLC (wt. \%)} = (\text{weight of loaded drug} / \text{weight of drug loaded micelles}) \times 100\%$$

Characterization of PpIX-loaded micellar formulations

Hydrodynamic diameter (Dh) and polydispersity index (PDI) of the micelles were determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-ZS instrument (Malvern Instruments, Worcestershire, UK) equipped with a 633 nm He-Ne laser at a scattering angle of 173° at 25 °C. The morphology of the polymer micelles was imaged on a Tecnai-12 electron microscope. A drop of the samples were placed on a carbon coated 200-mesh copper grids for 2 min, then washed with Milli-Q water. The grids were stained with 2% phosphotungstic acid. The stain was wicked off using filter paper and the grids were dried. Grids were analyzed at an acceleration voltage of 120 kV.

Critical micelle concentration (CMC) measurement

CMC was measured by fluorescence spectroscopy using a pyrene probe on a SPEX FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon) as described previously.²³ A predetermined amount of pyrene in acetone was added into a series of ampoules, and the acetone was removed with a gentle N₂ flow followed by further drying in a vacuum chamber. A predetermined volume of the PEG-PCL micellar solution and water were added into the ampoules to create solutions with PEG-PCL concentrations ranging from 6.10 × 10⁻⁵ to 1.0 g L⁻¹. The concentration of pyrene in each flask was fixed at 6.0 × 10⁻⁷ mol L⁻¹, slightly lower than the saturation solubility of pyrene in water. These solutions were shaken vigorously and then allowed to equilibrate at 25 °C for at least 24 h. The excitation spectra of pyrene with various PEG-PCL concentrations were measured at the detection emission wavelength (λ_{em} = 390 nm). The CMC value was obtained from the intersection of the horizontal line of I₃₃₇/I₃₃₄ with a relatively constant value and the diagonal line with a rapidly increasing I₃₃₇/I₃₃₄ ratio.

***In vitro* PpIX release in FBS**

In vitro release profile of PpIX from micelles was investigated in fetal bovine serum (FBS). Briefly, 0.5 mL PpIX micelles in water were added to 9.5 mL FBS. The release experiment was initiated by placing the mixture at 37 °C with continuous shaking. At predetermined intervals, 0.5 mL solution was collected. Aliquots were run through PD-10 columns to separate encapsulated PpIX in micelles from released PpIX. The fluorescence intensity of PpIX encapsulated in micelles was measured at an emission wavelength of 632 nm by a microplate reader (Infinite M200 PRO, Tecan) by dissolving separated micelles in DMSO. Finally, the cumulative release percentage of PpIX was plotted as a function of incubation time.

Singlet oxygen detection

The generation of $^1\text{O}_2$ was monitored by following the UV absorbance of 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA) in the PpIX loaded micelle solution in absence and presence of 5% SDS. Briefly, an air-saturated aqueous solution (15 mL) in absence/presence of 5% SDS was prepared with ABDA (50 μM) and fresh PpIX micelles. The solution was then illuminated under a laser ($\lambda = 630 \text{ nm}$, power density = 5 mW/cm^2), and aliquots of sample solution were removed from the irradiated sample at predetermined intervals and subjected to UV-vis absorption measurement. The decreasing absorbance intensity of ABDA at 378 nm was followed by monitoring its absorption over time.

Cell culture

The human breast cancer cell line MDA-MB-231 (ATCC) was cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), supplemented with 100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin at 37 °C with 5% CO_2 .

Cellular uptake measured by fluorescence microscopy

The cellular uptake behavior of PpIX-loaded polymeric micelles was determined by fluorescence microscopy. The MDA-MB-231 cells were seeded in 6-well plates at a density of 5×10^5 cells per well in 2 mL of DMEM and incubated at 37 °C in 5% CO_2 atmosphere for 24 h. After removing culture medium, the cells were incubated for various lengths of time with PpIX-loaded polymeric micelles at an equivalent PpIX concentration of 5 $\mu\text{g mL}^{-1}$ in DMEM. After incubation for a predetermined time at 37 °C, cells were washed three times with cold PBS. All microscopy images were acquired with an Olympus IX81 motorized inverted fluorescence microscope equipped with a back-illuminated EMCCD camera (Andor), an X-cite 120 excitation source (EXFO), and Sutter excitation and emission filter wheels. The relative intensity of PpIX signal from each digital image was processed using Image J software. A region of interest (ROI) was drawn around each individual cell, and the average fluorescence intensity was measured in each region. Similarly, the average fluorescence intensity from an ROI of equal size drawn around a background region was also measured. Numerical values of the background subtracted fluorescence intensity were then acquired.

***In vitro* PDT**

The *in vitro* cytotoxicities of erlotinib, PpIX and PpIX-loaded micelles were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay against MDA-MB231 cells. Briefly, cells harvested in their logarithmic growth phase were seeded in 96-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) at $\sim 5 \times 10^3$ cells per well in 100 μL complete DMEM, and incubated at 37 °C in 5% CO_2 for 24 h. After removing culture medium, free PpIX and PpIX micelles diluted in complete DMEM (100 μL) were added to the wells at concentrations varying from 40 $\mu\text{g/mL}$ to 2.5 $\mu\text{g/mL}$. For the erlotinib group, six erlotinib concentrations (200 μM , 100 μM , 20 μM , 4 μM , 0.8 μM , and 0.16 μM) were tested. After 24h of treatment, the culture medium was removed and the cells were washed with PBS three times. For the PDT condition, cells were illuminated with a light from a Ceralas diode laser (Biolitec AG, Jena, Germany; $\lambda=632\pm 3$ nm) at a power density of 5 mW/cm^2 to the indicated light dose. Light was delivered through microlens-tipped fibers (Pioneer, Bloomfield, CT) to produce fields of uniform intensity and laser output was measured using a power meter (LabMaster, Coherent, Auburn, CA). After irradiation, MDA-MB231 cells were allowed to grow for an additional 3 days in fresh media. Next, 10 μL of 5 mg mL^{-1} MTT assay stock solution in PBS was added to each well. After incubating the cells for 4 h, 100 μL of detergent were added to each well to dissolve the formazan and the plates were returned to the cell culture incubator for another 4 h incubation. Finally, the plates were shaken for 1 min, and the absorbance of formazan product was measured on a Tecan plate reader (Tecan) at 570 nm. Cell viability (%) was calculated as follows: viability = $(A_{\text{sample}}/A_{\text{control}}) \times 100\%$, where A_{sample} and A_{control} denote as absorbance values of the sample and control well, respectively. In all experiments, a number of wells were covered during illumination as an internal negative control. Dark cytotoxicity was assessed in MDA-MB231 cells incubated with drug but unexposed to laser light. Data were graphed from at least two independent experiments performed with six replicates.

The *in vitro* cytotoxicity of PpIX/erlotinib and PpIX micelle/erlotinib combinations were also assessed with an MTT assay. Briefly, cells harvested in their logarithmic growth phase were seeded in 96-well plates at a density of $\sim 5 \times 10^3$ cells per well and incubated in 100 μL DMEM (2% FBS) for 24 h. The cells were then pretreated with 20 μM erlotinib for 24 h. The medium was replaced with various drug formulations of PpIX/erlotinib and PpIX micelle/erlotinib. All of the conditions containing PpIX were modulated to final equivalent PpIX concentrations ranging from 40 $\mu\text{g/mL}$ to 2.5 $\mu\text{g/mL}$, while the erlotinib concentration was kept constant at 20 μM . The photocytotoxicity and dark cytotoxicity were investigated as described above.

3. RESULTS AND DISCUSSION

3.1 Synthesis and Characterization of PpIX loaded micelles

PpIX-loaded polymer micelles were prepared by encapsulating hydrophobic PpIX within the amphiphilic block copolymer PEG-PCL using a well-established microemulsion method.²² These as-prepared PpIX-loaded micelles were highly water soluble due to the hydrophilic PEG shell. To identify which compositions led to the maximum PpIX loading while maintaining their micellar structure, the PpIX to polymer weight ratio (w/w) was varied

from 0.05 to 1 during micelle preparation. TEM was used to determine the morphology of the PpIX-loaded micelles. As seen in Figure 2, a narrow distribution of fine spherical structures with tightly packed PpIX contained within the hydrophobic core of the micelles was observed when PpIX to polymer weight ratio lower than 0.5 (Figure 2A–C). However, wormlike, spherical, and irregular structures were found to co-exist in the sample when the PpIX to polymer ratio was at 0.5 (Figure 2D–E). In addition, highly irregular morphologies were obtained at a PpIX to polymer ratio of 1 (Figure 2F). As the PpIX to polymer ratio increased from 0.05 to 1, PpIX loading content (DLC%) increased from 1.41% to 39.4% (Figure 3A), and PpIX loading efficiency (DLE%) increased from 28.7% to 72.5% (w/w) (Figure 3B). The highest loading efficiency was achieved when the PpIX to polymer ratio was 0.5. The hydrodynamic diameter of the particles increased from 30.5 nm to 54.1 nm (Figure 3C–D) as the ratio increased from 0.05 to 0.25. This size increase may be due to higher loading amounts of the PpIX. In all cases, no significant PDI change was observed (Figure 3E). A summary of physical-chemical properties from PpIX loaded micelles is also provided (Supporting Information, Table S1). Given the drug loading efficiency and appropriate size, we chose an optimal micelle formulation of PpIX to polymer ratio of 0.25 for further *in vitro* testing.

The value of the critical micelle concentration (CMC) is an important parameter to evaluate the thermodynamic stability of the prepared micelles.²⁴ To further demonstrate the formation of micelles and their stability, the CMC of PEG-PCL micelles was measured using a widely reported pyrene-probe-based fluorescence technique. The excitation spectra of pyrene was first obtained in aqueous solution. As the concentration of the polymer solution increased, the fluorescence intensity also increased. Further, as micelles formed, transfer of pyrene molecules from the aqueous solvent into the hydrophobic micellar core caused a red shift from 334 to 337 nm in the fluorescent spectra (data not shown). From the plot of I_{337}/I_{334} vs. the log of polymer concentration (Figure 3F), we determined that the CMC for the PEG-b-PCL micelles was 6.6×10^{-3} g/L.

Prior to evaluating whether PpIX loaded micelles are a suitable photosensitizing agent in *in vitro* or *in vivo* studies, the stability of PpIX loaded micelles was first evaluated in 10% fetal bovine serum and PBS. No visible precipitates were observed when PpIX-loaded micelles were incubated in 10% serum for 48 h at 37 °C, and the hydrodynamic diameter of the PpIX loaded micelles showed no significant change. Additionally, there was no observable change in the size for the micelle samples in PBS at pH 7.4 for up to one week, suggesting that the micelles are stable.

3.2. UV–Vis and fluorescence properties of PpIX-loaded micelles

PpIX has various aggregated states in aqueous solution.²⁵ The absorption spectra of PpIX within micelles was highly dependent on the loaded amount (Figure 4A and Figure 4B). At 5% PpIX loading, a Soret band at 404 nm and a split Soret band at 450 nm were observed, indicating the monomer and extended aggregates co-existed within micelles.²⁵ When the loading content increased to 25%, a greatly broadened shifted Soret band (maxima at 450 nm) of moderate intensity was observed, which correlated with the formation of extended aggregates within micelles (Figure 4B). Fluorescence emission of PpIX-encapsulated

micelles showed a maximum emission at 630 nm with 5% loading density (Figure 4C). At higher loading densities (> 5%), the fluorescence intensity decreased dramatically (Figure 4C), consistent with the formation of PpIX aggregates within the aggregation core. The highly aggregated PpIX cores may be in their highly quenched state, which may not generate sufficient fluorescence or singlet oxygen. However, following cellular uptake, the highly packed PpIX cores may gradually degrade in the complex intracellular environment and release PpIX, leading to a strong fluorescence signal.²⁶ To verify this hypothesis, we tested the absorbance and fluorescence intensity of 25% PpIX loaded micelles in the presence of 5% SDS. Surfactant SDS is capable of disrupting the condensed self-assembled micellar structure, simulating micellar dissociation in cell cytosolic and intracellular conditions.^{27, 28} As seen in Figure 4B, the sharp Soret band at 405 nm reappeared in the UV-Vis spectrum, suggesting that the PpIX was released from the micellar core and was in its monomer state. Furthermore, the fluorescence spectrum of 25% PpIX-loaded micelles, in the presence of SDS, was very similar to that of the unquenched 5% PpIX micelles (Figure 4D), indicating that the optical properties of PpIX can likely be restored after the micelles have been uptaken by the cancer cells and dissociated in a cytosol-like environment.

3.3. *In vitro* PpIX release in FBS

The *in vitro* release behavior of PpIX micelles was monitored in FBS to mimic physiological release conditions. Although initial fast release was observed from the micelles in the first few hours, less than 50% of encapsulated PpIX was released within 48 h (Figure S1). The remaining PpIX in the micelles allows for prolonged blood circulation time with minimal release of PpIX in the initial phase of administration.

3.4. Efficiency of ¹O₂ generation

Porphyrin-based photosensitizers are able to produce reactive oxygen species upon exposure to light. To verify the ability of PpIX to generate singlet oxygen (¹O₂), the absorbance at 378 nm of water-soluble 9,10-anthracenediylbis(methylene)dimalonic acid (ABDA) was monitored in the presence of PpIX-loaded micelles.¹² When irradiated with a laser (5 mW/cm² intensity at 630 nm), the characteristic absorption of ABDA at 378 nm is relatively stable over the course of light treatment (Figure 5A), suggesting that PpIX is strongly quenched within the micellar core. In contrast, the control study, i.e. in the presence of SDS, showed a rapid decrease in absorbance at 378 nm within 30min, with steady state reached after 40 min (Figure 5B). This time-dependent decrease in absorbance by ABDA in 5% SDS indicated that PpIX could be protected by the nanocarrier in circulation and that the photosensitizer is able to generate singlet oxygen and phototoxicity upon light exposure when it is released from micelles in its target environment.

3.5. Intracellular fluorescence of PpIX loaded micelles

In order to determine the optimal micellar incubation time for PDT, cellular uptake and intracellular PpIX release from PpIX loaded micelles were evaluated over time in MDA-MB-231 cells using optical microscopy. As seen in Figure 6A, PpIX loaded micelles did not present any strong red fluorescence signal and showed low cellular uptake within 0.5 h. The fluorescence intensity did not change considerably even over 1 to 4 h (e.g. relative fluorescence intensity (RFI) values (a.u) were 192 ± 30 and 288 ± 49 at 1 h and 4 h,

respectively; Figure 6 B). This was likely due to the self-quenching effect of PpIX molecules in the nanomicelles. However, PpIX loaded micelles showed a dramatic increase in fluorescence intensity from 8 to 24 h (e.g. RFI values were 348 ± 102 and 715 ± 160 at 8 h and 24 h, respectively; $p < 0.001$, Figure 6 B), indicating that PpIX was restored to their release state in cancerous intracellular conditions. The RFI values varied little from 24 h to 48 h (715 ± 160 and 618 ± 176 at 24 and 48 h, respectively; Figure 6 B). Based on this result, we chose the optimal micelle incubation time to be 24 h for further PDT studies in MDA-MB-231 cells.

3.6. PDT efficacy in MDA-MB-231 cancer cells

To evaluate the efficacy of PDT alone or in combination with erlotinib pre-treatment, in inducing cell death on MDA-MB-231 breast cancer cells overexpressing EGFR receptors,²⁹ we performed MTT assays (Figure 7). Cell viability was measured as the percentage of the viable cells over the untreated cell control (i.e. without drug incubation and laser exposure). First, we investigated the cell viability for the free drug erlotinib with or without 632 nm laser light at 5 mW/cm^2 , as shown in Figure 7A. No significant cytotoxicity was observed in cells treated with erlotinib alone at concentrations of up to $20 \mu\text{M}$. Moreover, no cell death was observed regardless of whether untreated control cells were irradiated with light. This is likely due to erlotinib's inability to generate singlet oxygen or toxic radicals. In contrast, MDA-MB-231 cells treated with PpIX showed significant dose-dependent phototoxicity upon light irradiation at 5 mW/cm^2 for ~ 16 mins (5 J/cm^2). No apparent toxicity was observed in cells treated by PpIX in the dark (Figure 7 B).

Next, the cytotoxicity of combined erlotinib and PpIX therapy against MDA-MB-231 cells was evaluated (Figure 7 B). Cells were first pretreated with $20 \mu\text{M}$ of erlotinib for 24 h. Next, free PpIX was added and co-incubated with erlotinib for another 24 h. After PDT, cells were allowed to grow for additional 72 h for the MTT assay. As shown in Figure 7B, the PpIX and erlotinib combination showed slightly higher dark cytotoxicity than PpIX alone; however, the PpIX and erlotinib combination with light irradiation was significantly more effective against MDA-MB-231 cells. The combination treatment with light irradiation was also significantly more effective than just PpIX with light irradiation, indicating that this combination has a synergistic effect for PDT.

Micelle samples were evaluated next. Empty PEG-PCL micelles did not show any observable phototoxicity or dark toxicity against MDA-MB-231 cells (data not shown). The combination of erlotinib and PpIX micelles showed a slight increase in dark toxicity against cancer cells compared to PpIX micelles or erlotinib alone (Figure 7C). However, the efficacy was significantly increased when MDA-MB-231 cells incubated with PpIX micelles and erlotinib were irradiated with light. The combination treatment with light irradiation was also significantly more effective than just PpIX micelles with light irradiation. This mirrors what was observed with free PpIX, and similarly suggests that this combination has a synergistic effect for PDT, regardless of whether the PpIX is encapsulated in micelles or not.

The PpIX micelles and erlotinib combination also showed a dose dependent response to light when the light dosage was increased from 2.5 J/cm^2 to 10 J/cm^2 . As shown in Figure 7D, cell viability decreased from 80% to 40% when the light dose increase from 2.5 J/cm^2 to

10 J/cm², while micelles alone at a dose of 5 µg/mL showed no significant cytotoxicity, regardless of light exposure.

4. CONCLUSION

In summary, this study systematically investigated a more effective strategy for photodynamic therapy by using a PEG-PCL micellar formulation to load hydrophobic PpIX combined with erlotinib pretreatment. An oil-in-water emulsion encapsulation technique was employed to incorporate PpIX into PEG-PCL micelles, which yielded PpIX in the micellar core. The obtained PpIX-loaded micelles (25% w/w) assumed a fine spherical structure with a mean diameter of approximately 54 nm. The PpIX exists in its quenched state inside the micellar core in solution, but can be released in cancerous intracellular conditions. An *in vitro* MTT assay showed that PpIX micelles have enhanced efficacy for photodynamic therapy when combined with erlotinib pretreatment. These results contribute to the establishment of a nanoplatform combined with erlotinib pretreatment to improve PDT efficacy for subsequent *in vivo* evaluation in animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Castano AP, Mroz P, Hamblin MR. Photodynamic therapy and anti-tumour immunity. *Nat Rev Cancer*. 2006; 6(7):535–545. [PubMed: 16794636]
2. Castano AP, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy: part one- photosensitizers, photochemistry and cellular localization. *Photodiagn Photodyn*. 2004; 1(4):279–293.
3. Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, Gollnick SO, Hahn SM, Hamblin MR, Juzeniene A, Kessel D, Korbelik M, Moan J, Mroz P, Nowis D, Piette J, Wilson BC, Golab J. Photodynamic Therapy of Cancer: An Update. *Ca-Cancer J Clin*. 2011; 61(4):250–281. [PubMed: 21617154]
4. Schmidt-Erfurth U, Miller JW, Sickenberg M, Laqua H, Barbazetto I, Gragoudas ES, Zografos L, Piguet B, Pournaras CJ, Donati G, Lane AM, Birngruber R, van den Berg H, Strong HA, Manjuri U, Gray T, Fsadni M, Bressler NM. Photodynamic therapy with verteporfin for choroidal neovascularization caused by age-related macular degeneration - Results of retreatments in a phase 1 and 2 study. *Arch Ophthalmol-Chic*. 1999; 117(9):1177–1187.
5. Bressler NM, Degeneration TARM. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin - One-year results of 2 randomized clinical trials - TAP report 1. *Arch Ophthalmol-Chic*. 1999; 117(10):1329–1345.
6. Juarranz A, Jaen P, Sanz-Rodriguez F, Cuevas J, Gonzalez S. Photodynamic therapy of cancer. Basic principles and applications. *Clin Transl Oncol*. 2008; 10(3):148–154. [PubMed: 18321817]
7. Jichlinski P, Guillou L, Karlsen SJ, Malmstrom PU, Jocham D, Brennhovd B, Johansson E, Gartner T, Lange N, van den Bergh H, Leisinger HJ. Hexyl aminolevulinate fluorescence cystoscopy: A new diagnostic tool for the photodiagnosis of superficial bladder cancer - A multicenter study. *J Urology*. 2003; 170(1):226–229.

8. Fernandez JM, Bilgin MD, Grossweiner LI. Singlet oxygen generation by photodynamic agents. *J Photoch Photobio B*. 1997; 37(1–2):131–140.
9. Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. Nanocarriers as an emerging platform for cancer therapy. *Nat Nanotechnol*. 2007; 2(12):751–760. [PubMed: 18654426]
10. Mura S, Nicolas J, Couvreur P. Stimuli-responsive nanocarriers for drug delivery. *Nat Mater*. 2013; 12(11):991–1003. [PubMed: 24150417]
11. Yan LS, Li XD. Biodegradable Stimuli-Responsive Polymeric Micelles for Treatment of Malignancy. *Current pharmaceutical biotechnology*. 2016; 17(3):227–236. [PubMed: 26873075]
12. Xu JS, Zeng F, Wu H, Hu CP, Wu SZ. Enhanced Photodynamic Efficiency Achieved via a Dual-Targeted Strategy Based on Photosensitizer/Micelle Structure. *Biomacromolecules*. 2014; 15(11):4249–4259. [PubMed: 25329523]
13. Tian JW, Zhou JF, Shen Z, Ding L, Yu JS, Ju HX. A pH-activatable and aniline-substituted photosensitizer for near-infrared cancer theranostics. *Chem Sci*. 2015; 6(10):5969–5977.
14. Feng XY, Jiang D, Kang T, Yao JH, Jing YX, Jiang TZ, Feng JX, Zhu QQ, Song QX, Dong N, Gao XL, Chen J. Tumor-Homing and Penetrating Peptide-Functionalized Photosensitizer-Conjugated PEG-PLA Nanoparticles for Chemo-Photodynamic Combination Therapy of Drug-Resistant Cancer. *Acs Appl Mater Inter*. 2016; 8(28):17817–17832.
15. Gibot L, Lemelle A, Till U, Moukarzel B, Mingotaud AF, Pimienta V, Saint-Aguet P, Rols MP, Gaucher M, Violleau F, Chassenieux C, Vicendo P. Polymeric Micelles Encapsulating Photosensitizer: Structure/Photodynamic Therapy Efficiency Relation. *Biomacromolecules*. 2014; 15(4):1443–1455. [PubMed: 24552313]
16. Sridhar SS, Seymour L, Shepherd FA. Inhibitors of epidermal-growth-factor receptors: a review of clinical research with a focus on non-small-cell lung cancer. *Lancet Oncol*. 2003; 4(7):397–406. [PubMed: 12850190]
17. Lee MJ, Ye AS, Gardino AK, Heijink AM, Sorger PK, MacBeath G, Yaffe MB. Sequential Application of Anticancer Drugs Enhances Cell Death by Rewiring Apoptotic Signaling Networks. *Cell*. 2012; 149(4):780–794. [PubMed: 22579283]
18. Gallagher-Colombo SM, Miller J, Cengel KA, Putt ME, Vinogradov SA, Busch TM. Erlotinib Pretreatment Improves Photodynamic Therapy of Non-Small Cell Lung Carcinoma Xenografts via Multiple Mechanisms. *Cancer Research*. 2015; 75(15):3118–3126. [PubMed: 26054596]
19. Nowak-Sliwinska P, Weiss A, van Beijnum JR, Wong TJ, Ballini JP, Lovisa B, van den Bergh H, Griffioen AW. Angiostatic kinase inhibitors to sustain photodynamic angio-occlusion. *J Cell Mol Med*. 2012; 16(7):1553–1562. [PubMed: 21880113]
20. Weiss A, van Beijnum JR, Bonvin D, Jichlinski P, Dyson PJ, Griffioen AW, Nowak-Sliwinska P. Low-dose angiostatic tyrosine kinase inhibitors improve photodynamic therapy for cancer: lack of vascular normalization. *J Cell Mol Med*. 2014; 18(3):480–491. [PubMed: 24450440]
21. Weyergang A, Selbo PK, Berg K. Sustained EKR inhibition by EGFR targeting therapies is a predictive factor for synergistic cytotoxicity with PDT as neoadjuvant therapy. *Bba-Gen Subjects*. 2013; 1830(3):2659–2670.
22. Al Zaki A, Joh D, Cheng ZL, De Barros ALB, Kao G, Dorsey J, Tsourkas A. Gold-Loaded Polymeric Micelles for Computed Tomography-Guided Radiation Therapy Treatment and Radiosensitization. *ACS nano*. 2014; 8(1):104–112. [PubMed: 24377302]
23. Yan LS, Yang LX, He HY, Hu XL, Xie ZG, Huang YB, Jing XB. Photo-cross-linked mPEG-poly(gamma-cinnamyl-L-glutamate) micelles as stable drug carriers. *Polymer Chemistry*. 2012; 3(5):1300–1307.
24. Owen SC, Chan DPY, Shoichet MS. Polymeric micelle stability. *Nano Today*. 2012; 7(1):53–65.
25. Ding HY, Sumer BD, Kessinger CW, Dong Y, Huang G, Boothman DA, Gao JM. Nanoscopic micelle delivery improves the photophysical properties and efficacy of photodynamic therapy of protoporphyrin IX. *Journal of Controlled Release*. 2011; 151(3):271–277. [PubMed: 21232562]
26. Lee SJ, Koo H, Lee DE, Min S, Lee S, Chen XY, Choi Y, Leary JF, Park K, Jeong SY, Kwon IC, Kim K, Choi K. Tumor-homing photosensitizer-conjugated glycol chitosan nanoparticles for synchronous photodynamic imaging and therapy based on cellular on/off system. *Biomaterials*. 2011; 32(16):4021–4029. [PubMed: 21376388]

27. Chen H, Kim S, He W, Wang H, Low PS, Park K, Cheng JX. Fast release of lipophilic agents from circulating PEG-PDLLA micelles revealed by in vivo Forster resonance energy transfer imaging. *Langmuir*. 2008; 24(10):5213–5217. [PubMed: 18257595]
28. Vangeyte P, Leyh B, Auvray L, Grandjean J, Misselyn-Bauduin AM, Jerome R. Mixed self-assembly of poly(ethylene oxide)-b-poly(epsilon-caprolactone) copolymers and sodium dodecyl sulfate in aqueous solution. *Langmuir*. 2004; 20(21):9019–9028. [PubMed: 15461482]
29. Park EJ, Min HY, Chung HJ, Hong JY, Kang YJ, Hung TM, Youn UJ, Kim YS, Bae K, Kang SS, Lee SK. Down-regulation of c-Src/EGFR-mediated signaling activation is involved in the honokiol-induced cell cycle arrest and apoptosis in MDA-MB-231 human breast cancer cells. *Cancer Lett*. 2009; 277(2):133–140. [PubMed: 19135778]

Figure 1. Schematic illustration of PpIX loaded PEG-PCL micelles for photodynamic therapy with erlotinib pretreatment. (A) Hydrophobic PpIX was incorporated into hydrophobic core of PEG-PCL micelle. (B) Following cellular uptake, the PpIX-loaded micelle gradually degraded in the complex intracellular environment and released PpIX. Under light irradiation, the singlet oxygen generated by the PpIX caused the phototoxicity to the cells. A synergistic effect was observed when PDT was combined with erlotinib pretreatment.

Figure 2. Representative transmission electron microscopy (TEM) images: (A) PpIX/PEG-PCL (5%); (B) PpIX/PEG-PCL (10%); (C) PpIX/PEG-PCL (25%); (D, E) PpIX/PEG-PCL (50%); (F) PpIX/PEG-PCL (100%). The scale bar is 50 nm.

Figure 3. Characterization of PpIX loaded PEG-PCL micelles. (A) Drug loading content, (B) Drug loading efficiency, (C) Hydrodynamic size by dynamic light scattering (DLS), (D) Mean diameter and (E) PDI of micelles as a function of PpIX to polymer ratio, and (F) Critical micelle concentration (CMC) of PEG-PCL micelles.

Figure 4.

Absorbance and fluorescence curves of PpIX loaded PEG-PCL micelles. (A) Typical absorbance spectra of PpIX loaded micelles (PpIX to polymer ratio at 5%). (B) Absorbance spectra of PpIX loaded micelles (PpIX to polymer ratio at 25%) with or without 5% SDS. (C) Fluorescence spectra of micelles loading with different amount PpIX. (D) Fluorescence spectra of PpIX loaded micelles (PpIX to polymer ratio at 25%) with or without 5% SDS.

Figure 5.

UV-vis spectra of ABDA in the presence of PpIX loaded micelles (PpIX to polymer ratio at 25%) as a function of laser irradiation time ($\lambda=630$ nm, power density = 5 mW/cm^2) in the absence of 5% SDS (A) and in the presence of 5% SDS (B), respectively.

Figure 6. (A) Optical images of MDA-MB231 cancer cells incubated with PpIX loaded micelles (PpIX to polymer ratio at 25%) over time. (B) Quantitative analysis of optical images.

Figure 7.

(A) Cell viability of MDA-MB 231 cells after 24 h of incubation with erlotinib in darkness and irradiation upon light. (B) Cell viability of MDA-MB cells after treatment with PpIX, erlotinib/PpIX, PpIX/PDT, or erlotinib/PpIX/PDT. (C) Cell viability of MDA-MB cells after treatment with micelle, erlotinib/micelle, micelle/PDT, or erlotinib/micelle/PDT. (D) Cell viability of MDA-MB cells after treatment with micelle, erlotinib/micelle, micelle/PDT, or erlotinib/micelle/PDT upon different light dose. For A–C, 630 nm laser light at 5 J/cm² was used for PDT. For cell pretreatment, 20 μM of erlotinib was used.