Folate-PEG Conjugates of a Far-Red Light-Activatable Paclitaxel Prodrug to Improve Selectivity toward Folate Receptor-Positive Cancer Cells

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Supporting Information

ABSTRACT: We recently demonstrated the far-red light-activatable prodrug of paclitaxel (PTX), Pc-(L-PTX)2. Upon illumination with a 690 nm laser, Pc-(L-PTX)2 showed combinational cell killing from rapid photodynamic therapy damage by singlet oxygen, followed by sustained chemotherapy effects from locally released PTX. However, its high lipophilicity (log D7.4 > 3.1) caused aggregation in aqueous solutions and has nonselectivity toward cancer cells. To solve these important problems, we prepared folic acid (FA)-conjugated and photoactivatable prodrugs of PTX with a polyethylene glycol (PEG) spacer of various chain lengths: FA-PEGn-Pc-L-PTX [n = 0 (0k, 5), ∼23 (1k, 7a), ∼45 (2k, 7b), ∼80 (3.5k, 7c), or ∼114 (5k, 7d)]. The PEGylated prodrugs had a much improved hydrophilicity compared with the non-PEGylated prodrug, Pc-(L-PTX)2. As the PEG length increased, the hydrophilicity of the prodrug increased (log D7.4 values: 1.28, 0.09, −0.24, and −0.59 for 1k, 2k, 3.5k, and 5k PEG prodrugs, respectively). Fluorescence spectral data suggested that the PEGylated prodrugs had good solubility in the culture medium at lower concentrations (<1 μM), but showed fluorescence quenching due to limited solubility at higher concentrations (>2 μM). Dynamic light scattering indicated that all of the prodrugs formed nanosized particles in both phosphate-buffered saline and culture medium at a concentration of 5 μM. The PEG length affected both nonspecific and folate receptor (FR)-mediated uptake of the prodrugs. The enhanced cellular uptake was observed for the prodrugs with medium-sized PEGs (1k, 2k, or 3.5k) in FR-positive SKOV-3 cells, but not for the prodrugs with no PEG or with the longest PEG (5k), which suggests the optimal range of PEG length around 1k−3.5k for effective uptake of our prodrug system. Consistent with the cellular uptake pattern, medium-sized PEGylated prodrugs showed more potent phototoxic activity (IC50s, ∼130 nM) than prodrugs with no PEG or the longest PEG (IC50, ∼400 nM). In conclusion, we have developed far-red light-activatable prodrugs with improved water solubility and FR-targeting properties compared with the nontargeted prodrug.

INTRODUCTION

Photodynamic therapy (PDT), a clinically approved and minimally invasive technique, has received great attention as a promising method for the treatment of several types of cancers1−4 and other diseases, such as acne and neovascular age-related macular degeneration.5−8 Upon activation of the photosensitizer with the appropriate wavelength of light, reactive and cytotoxic singlet oxygen is produced.9,10 Singlet oxygen has a very short half-life (∼1−2 μs), but showed fluorescence quenching due to limited solubility at higher concentrations (>3 μM). Dynamic light scattering indicated that all of the prodrugs formed nanosized particles in both phosphate-buffered saline and culture medium at a concentration of 5 μM. The PEG length affected both nonspecific and folate receptor (FR)-mediated uptake of the prodrugs. The enhanced cellular uptake was observed for the prodrugs with medium-sized PEGs (1k, 2k, or 3.5k) in FR-positive SKOV-3 cells, but not for the prodrugs with no PEG or with the longest PEG (5k), which suggests the optimal range of PEG length around 1k−3.5k for effective uptake of our prodrug system. Consistent with the cellular uptake pattern, medium-sized PEGylated prodrugs showed more potent phototoxic activity (IC50, ∼130 nM) than prodrugs with no PEG or the longest PEG (IC50, ∼400 nM). In conclusion, we have developed far-red light-activatable prodrugs with improved water solubility and FR-targeting properties compared with the nontargeted prodrug.

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To improve the selectivity to cancer cells, we selected a tumor-targeting group, folic acid (FA), which recognizes the folate receptors (FRs). FRs are glycosylphosphatidylinositol-anchored cell surface receptors that tightly bind to the FA or folate. Several researchers have reported that the FRs are overexpressed in numerous types of human cancer, such as ovarian, breast, brain, colorectal, epithelial, and lung cancer, whereas the expression is limited in normal cells or tissues.23−28

Figure 1. Nontargeted (a,b) and targeted (c) prodrugs of CA4 previously synthesized by our group.

Figure 2. Previously synthesized nontargeted prodrug of PTX (a) and folate conjugates of PTX, with or without PEGs, reported in this manuscript (b). FR-mediated cellular uptake and biological effects upon illumination with 690 nm light (c).
Because of the vast difference in the number of FRs in tumors and normal cells or tissues, FRs can be exploited to target folate-conjugated drugs or imaging agents to FR-positive tumors and prevent cellular uptake in normal cells or tissues.29−33 Once the folate tightly binds to the FR receptor, the folate-conjugated drugs or imaging agents can be internalized through endocytosis. Thus, incorporation of FA into the molecule is a popular way to selectively target FR-positive tumor cells. Numerous FA-conjugated prodrugs have been prepared to target FR-overexpressing tumors.34,35 Similarly, inclusion of a polyethylene glycol (PEG) spacer is a popular strategy to increase hydrophilicity and avoid aggregation. Introduction of the PEG spacer increases the hydrophilicity and improves the pharmacokinetic profile of the prodrugs36−40 by reducing kidney elimination, prolonging the plasma circulation time of the prodrugs,41 and protecting the prodrugs from proteolytic enzymes.42 In addition, PEGylation provides passive tumor-targeting via enhanced permeability and retention effect.43,44 PEG can be modified with folates that are recognized selectively by the FR in tumor cells to acquire receptor-mediated active tumor-targeting.45−48 There were several reports that PEGylated prodrugs with hydrophilic PEG or PEG-folate and hydrophobic therapeutic molecules were able to self-assemble to form micelles or aggregates because of their amphiphilic nature.49−53

Previously, our group prepared nontargeted and folate-targeted light-activatable prodrugs of combretastatin A-4 (CA4; Figure 1).16−19 The targeted prodrugs with the appropriate PEG chain length (2k) showed an FR-mediated uptake, selective cytotoxicity, and a more efficient antitumor activity than the nontargeted prodrug.19 Similarly, we successfully showed that the nontargeted far-red light-activatable prodrug of paclitaxel (PTX), upon illumination with 690 nm light, released free PTX and demonstrated promising phototoxicity in SKOV-3 ovarian cancer cells.20 On the basis of these studies, we were determined to develop folate conjugates of PTX, one of the most popular, clinically approved anticancer drugs, with modifications to a previously prepared, nontargeted, far-red light-activatable PTX prodrug (Figure 2a,b). To improve its selectivity toward FR-positive cancer cells (Figure 2c), we replaced one molecule of PTX with an FR-targeting group, FA. FA was conjugated to the photosensitizer either directly or via the PEG spacer.
In the present study, we prepared five folate conjugates of PTX, of which one was non-PEGylated (direct conjugation) and the other four were PEGylated with PEG spacers of different chain lengths (1k, 2k, 3.5k, and 5k). All of the prepared prodrugs were evaluated for in vitro cellular uptake in FR-positive SKOV-3 ovarian cancer cells with or without excess FA. Furthermore, we tested the dark (without light) and phototoxicity (690 nm) of the prepared prodrugs to determine the cytotoxic effects in SKOV-3 cells in vitro. As these prodrugs are polymeric conjugates of the hydrophobic PTX and tend to self-assemble in aqueous solutions, we evaluated the particle size distribution in both phosphate-buffered saline (PBS) and a complete medium.

### RESULTS AND DISCUSSION

#### Preparation of Non-PEGylated and PEGylated Folate Conjugates of PTX and Phthalocyanine (Pc).

We prepared the folate conjugates of PTX by conjugating silicon Pc with PTX on one side and FA, with or without the PEG spacer, on the other side (Figure 2 and Scheme 1). We used PEG of the following molecular weights (chain lengths): 1k, 2k, 3.5k, and 5k. As our previously reported, non-targeted prodrugs of PTX were highly lipophilic and tended to aggregate, one of our goals was to improve its solubility and prevent aggregation. The PEG chain length affected the physicochemical properties and the cellular uptake of molecules. Therefore, we selected various chain lengths of PEGs to find the appropriate size for a better cellular uptake and cytotoxicity in our system. As reported in our previous study, the most reactive 2’-OH of PTX, which has a critical role in binding with tubulin, was chosen to conjugate with Pc. First, we conjugated the single PTX molecule with Pc by reacting an equimolar concentration of compound 1 and PTX propiolate in tetrahydrofuran (THF) to get a mixture of substituted and monosubstituted PTX derivative (3). The desired compound 3 was obtained in 41% yield after purification with silica gel chromatography. Non-PEGylated folate conjugate (FA-Pc-L-PTX) (5) was prepared by directly reacting compound 3 with the activated FA. Briefly, FA was activated with N-hydroxysuccinimide (NHS) in the presence of N,N’-dicyclohexylcarbodiimide (DCC) for 16 h at room temperature (rt) and was then treated with compound 3. The reaction was stirred at rt for 24 h to obtain the FA-conjugated derivative 5 in 53% yield.

For the preparation of PEGylated folate conjugates (FA-PEG1k−5k-Pc-L-PTX) (7a−d), compound 3 was converted to acid derivative 4 by reacting it with an equimolar concentration of diglycolic anhydride in dimethylformamide (DMF) to get compound 4 in 70% yield. Compound 4 was then activated by reacting it with DCC and NHS for 20–24 h and was finally conjugated with the folate PEG amine (FA-PEG2−5-NH2) (6a−d) in the presence of triethylamine (Et3N) for 36−48 h. PEGylated folate prodrugs were obtained in 29−45% yield. The prodrugs were characterized with proton nuclear magnetic resonance (1H NMR), ultraviolet−visible (UV−vis) fluorescence, and mass spectroscopies. In 1H NMR (Figure S14), we observed the changes in the chemical shift for the protons in the diglycolic linker, adjacent to the conjugation, from 3.9 to 3.7 ppm, which is due to the reduction in the deshielding effect of free carboxylic acid after conjugation. Similarly, after conjugation, for methylene proton in the folate PEG amine, there was a shift from 2.8 to 3.2–3.3 ppm (overlapped with H2O from the solvent). The pattern was similar for the other prodrugs.

#### Photophysical Properties and log D2,4 Values.

UV−vis absorption and fluorescence properties of the prepared PEGylated and nonPEGylated folate conjugates (5 and 7a−d) were measured in dimethylsulfoxide (DMSO) (Table 1 and Figure S23a,c). Experiments were performed in duplicate (Figure S23a,c). The UV−vis spectrum of Pc was not affected by the conjugation with PTX and folate PEGs. All of the folate conjugates showed Pc’s typical sharp Q-band at 678 nm. Similarly, the fluorescence spectra showed the typical pattern of Pc with Amax at 680 for all folate conjugates. This finding clearly indicates that the prepared folate conjugates retained the optical imaging capability of Pc. However, both absorbance and fluorescence (Figures 3 and S23) for all folate conjugates in PBS were reduced compared to those in DMSO. This is due to the aggregation of folate conjugates in PBS as compared to DMSO.

Partition coefficients, as anticipated, showed that the hydrophilicity of the folate conjugates increased on increasing the PEG chain length from 1k to 5k. Folate conjugate 7d, with the longest chain length, showed the lowest log D2,4 (−0.59), whereas folate conjugate 7a, with the shortest chain length, showed the highest log D2,4 (1.28). Folate conjugate 5, without PEG, was the most lipophilic, log D2,4 = 2.22. Compared with the non-targeted prodrug [Pc-(L-PTX)2, log D2,4 > 3.1], all of the folate conjugates, and in particular, the PEGylated folate conjugates showed much improved hydrophilicity.

#### Influence of the PEG Chain Length on the Cellular Uptake of Folate Conjugates (5 and 7a−d).

From the cellular uptake study (Figure 4) of folate conjugates 5 and 7a−d to SKOV-3, we found that 7a−c showed a better cellular uptake than 5 and 7d. At 24 h (Figure 4f), there was more than 12-fold increase in the intracellular prodrug accumulation of 7a−7c compared with prodrug 5. The introduction of the PEG chain length from 1k to 3.5k (7a−c) enhanced the cellular uptake; however, the uptake was dramatically reduced when the PEG chain length reached 5k (prodrug 7d).

The folate conjugates were expected to be internalized by FRs on the cell membrane. By adding FA (0.5 mM) to the culture medium, we expected a competition between FA and FA-conjugated prodrugs for the same receptors. 7a, 7b, and 7c demonstrated a decreased uptake of 55, 62, and 69% compared with prodrug 5. The introduction of the PEG chain length from 1k to 3.5k (7a−c) enhanced the cellular uptake; however, the uptake was dramatically reduced when the PEG chain length reached 5k (prodrug 7d).
demonstrated by fluorescence quenching. Although all of the prodrugs have the same fluorophore (Pc), the fluorescence of prodrug 5 in PBS was mostly quenched compared with other prodrugs (fluorescence: 238 950 vs 1 056 130 arb unit, Figures 3d and S23d). Furthermore, we compared the concentration-dependent fluorescence of prodrugs 5, 7b, and 7d in a complete medium (0.039–40 μM) using a plate reader (Figures 5 and S24). The fluorescence of prodrug 5 was much lower compared with those of prodrugs 7b and 7d, supporting the observation that prodrug 5 was more aggregated than 7b and 7d, even in the complete medium. Prodrugs 7b and 7d showed fluorescence quenching at higher concentrations (>5 μM).

The low cellular uptake of prodrug 7d is probably due to both high water solubility and steric hindrance by longer PEG spacers. We speculate that an excessively long PEG chain (5k for 7d) not only hindered the interaction of the FA moiety and FRs, reducing FR-mediated uptake, but also rendered prodrug 7d too hydrophilic, reducing the nonspecific uptake. It was
reported that PEG conjugates with a longer chain length (5k) could not be taken up by the cells, whereas PEG conjugates with a shorter chain length (2k) were able to enter the cells. Therefore, selection of PEG with an appropriate chain length is vital for attaining a better cellular uptake. Therefore, we hypothesized that the low fluorescence of 7b in DMSO was probably because of the quenching of the singlet oxygen state of Pc through the photoinduced electron transfer process. Therefore, we protonated 7b in DMSO solution with acid (HCl) and measured the fluorescence. We found that the fluorescence increased dramatically with protonation. This finding confirms that the prodrugs remain mostly in the monomeric form, without fluorescence quenching, in acidic solution. Comparing the fluorescence of prodrug 7b in the complete medium, DMSO with HCl, and 5% Tween 80 in PBS, fluorescence was significantly reduced in the complete medium, which is probably due to quenching by the formation of micelles. This result further supports our observation that all of the prodrugs form micelles in the complete medium.

Dark and Phototoxicity of Folate Conjugates (5 and 7a–d). The folate conjugates were evaluated for cytotoxicity after illumination (phototoxicity; Figure 7b) with a 690 nm laser. After illumination, 7a–c demonstrated higher phototoxicity than 5 and 7d, which was consistent with the findings from the uptake study (Figure 4). The IC50 values of 7a–c (124–137 nM) was about 3 times less than those of prodrugs 5 and 7d (415 and 406 nM, respectively, Table S1). Furthermore, prodrugs 7a–c showed a higher maximum inhibition (Emax = 90%) than prodrug 5 (80%). Emax and IC50 values for 7a, 7b, and 7c were similar. Notably, prodrug 7b showed a broader dose–response curve with a hill slope of 0.8 (Table S1) compared with prodrugs 7a and 7c, suggesting that prodrug 7c produces antitumor activity over a wide concentration range. Without illumination (dark), low toxicity was observed for all folate conjugates (5 and 7a–d). More than 90% of cells survived after 72 h of incubation with 7a–d (1k–5k) at a concentration of 500 nM (Figure 7a). For non-PEGylated prodrug 5, 40% of cells were killed at a concentration of 1000 nM after 72 h of incubation.

### CONCLUSIONS
In summary, we successfully prepared folate conjugates of PTX with (or without) PEG spacers of various chain lengths ranging from 1k to 5k. As observed from the log D24 values, the PEGylation of far-red light-activatable PTX prodrug has increased the hydrophilicity, thereby reducing the tendency found that the fluorescence of 7b was highly quenched in PBS, indicating that the micelle or aggregate formation is higher in PBS than in other solvents (DMSO, complete medium, 5% Tween 80 in PBS). The fluorescence was higher at low concentrations of <5 μM in the complete medium than it was in DMSO, whereas the fluorescence decreased as the concentration increased. We expected that the prodrug would mostly remain as a monomer in DMSO. However, the fluorescence was not higher than that observed in the complete medium. As was previously reported, 58 we hypothesized that the low fluorescence of 7b in DMSO was probably because of the quenching of the singlet oxygen state of Pc through the photoinduced electron transfer process. Therefore, we protonated 7b in DMSO solution with acid (HCl) and measured the fluorescence. We found that the fluorescence increased dramatically with protonation. This finding confirms that the prodrugs remain mostly in the monomeric form, without fluorescence quenching, in acidic solution. Comparing the fluorescence of prodrug 7b in the complete medium, DMSO with HCl, and 5% Tween 80 in PBS, fluorescence was significantly reduced in the complete medium, which is probably due to quenching by the formation of micelles. This result further supports our observation that all of the prodrugs form micelles in the complete medium.

### Table 2. Mean Diameter, zp, and Poly Dispersity Index (PDI) of Folate Conjugates (5 and 7a–d) in PBS and Complete Medium

|          | size (nm) | zp (mV) | PDI |
|----------|-----------|---------|-----|
|          | medium    | PBS     | medium | PBS | medium | PBS |
| 5        | 155 ± 10  | 193 ± 8.9 | −14.1 ± 2.9 | −12.4 ± 1.5 | 0.345 ± 0.03 | 0.312 ± 0.02 |
| 7a       | 180 ± 14  | 190 ± 10 | −19.4 ± 2.7 | −21.1 ± 2.1 | 0.354 ± 0.03 | 0.276 ± 0.02 |
| 7b       | 140 ± 22  | 174 ± 13 | −13.1 ± 2.4 | −7.6 ± 1.6  | 0.328 ± 0.05 | 0.303 ± 0.03 |
| 7c       | 121 ± 26  | 183 ± 17 | −2.5 ± 1.3  | −12.6 ± 1.8 | 0.314 ± 0.04 | 0.295 ± 0.03 |
| 7d       | 108 ± 9.4 | 175 ± 9.3 | −10.3 ± 3.5 | −7.8 ± 2.5  | 0.292 ± 0.04 | 0.286 ± 0.02 |
to aggregate randomly. Another advantage is the amphiphilic nature of these prodrugs. Because of the presence of both hydrophobic (PTX) and hydrophilic (FA-PEG) moieties, the prodrugs can self-assemble to form micelles, which could be advantageous as a drug-delivery system for PTX. Moreover, PTX can be replaced with other therapeutic agents as needed. We evaluated the cellular uptake of the prepared prodrugs in SKOV-3 cells in vitro. The results showed that the uptake was FR-mediated, which enables these conjugates to selectively target FR-positive tumors. In addition, we found that the optimal chain length for the FR-mediated uptake lies between 1k and 3.5k. By conjugating the bulky Pc group to the essential 2′-OH of PTX, we were able to reduce its cytotoxicity, as seen in the results from the dark toxicity study, where more than 90% of SKOV-3 cells survived after 72 h of incubation with folate PEG conjugates (7a−d). Furthermore, we tested the phototoxicity of these prodrugs, and the results were consistent with the results of the uptake study. Prodrugs 7a−c showed higher phototoxicity than 5 and 7d. From these findings, we can clearly conclude that phototoxicity was dependent upon the cellular uptake of these conjugates. Overall, prodrug 7b, with a PEG spacer of 2k, showed a better FR-mediated uptake and cell survival inhibition at a wide concentration range compared with prodrugs 7a and 7c. Therefore, we have selected prodrug 7b for further studies.

EXPERIMENTAL SECTION

Materials and Instruments. All reagents and solvents were of analytical grade and used without further purification. They were obtained from commercial sources (Sigma-Aldrich, USA, VWR, USA, and Fisher Scientific, USA). Starting materials (FA-conjugated PEG amines 6a−d) were obtained from Nanocs. The dialysis membrane (Spectra/Por 7 Standard RC) was purchased from Spectrum Laboratories, Inc. (cat # 132104 and 132108). High-performance liquid chromatography (HPLC) grade solvents were purchased from Pharmco-AAPER. An analytical thin-layer chromatography instrument was obtained from Sigma-Aldrich (silica gel matrix on an aluminum plate, cat # Z193291) and was used to monitor the reaction, either with UV or ninhydrin staining. Column chromatography was carried out in 40−63 μm (230−400 mesh) silica gel purchased from SiliCycle, Inc. (cat # R10030B). Gel permeation chromatography was performed on a Sephadex G15 (cat # 17-0020-01) or G25 (cat # 17-0032-01) medium obtained from GE Healthcare Life Sciences. NMR spectra were recorded on a Varian 400 MHz spectrometer. The
data were analyzed using iNMR (version 5.4.2), and the chemical shifts were calibrated according to the residual solvent peaks. Chemical shifts (δ) were recorded in ppm with coupling constants (J) in hertz. We used an Agilent 1260 series HPLC system (Agilent Technologies, USA) and a BDS Hypersil C18 column (250 × 4.6 mm, 5 μm particle size) with a Pinnacle DB C18 guard column (10 × 4 mm, 5 μm particle size) to evaluate the purity of compounds. We recorded the UV–vis absorption spectra using UV–vis LAMBDA 2S (PerkinElmer) and a 10 mm optical path length quartz cuvette. The fluorescence was recorded on a Jobin Yvon Fluorolog fluorometer from Horiba Scientific. High-resolution mass spectrometry (HRMS) analysis was done using an AB SCIEX QSTAR Elite hybrid quadrupole/time-of-flight mass spectrometer at the CORE facility of OHUSC. Human ovarian cancer cells (SKOV-3) were obtained from the American Type Culture Collection and used for all in vitro experiments. All reagents for cell culture were purchased from Invitrogen (Waltham, MA). Cells were maintained in the culture medium [McCoy's 5A medium supplemented with 10% fetal bovine serum, 50 units/mL penicillin G, 50 μg/mL streptomycin, and 1 μg/mL fungizone]. Cells were incubated at 37 °C in a 5% CO2 incubator (Sanyo MCO-18AIC-UV).

**Synthesis of Pc-L-PTX (3).** Compound 1 (319.5 mg, 0.40 mmol, 1.0 equiv) was equilibrated to be added to 150 mL of dry THF in a round-bottom flask and was stirred for 10 min in a N2 atmosphere. The reaction mixture was then dialyzed [molecular weight cut-off (MWCO) 1000] in DMF (24 h), followed by DCM (20 h). After dialysis in DCM, the solution was concentrated and further crashed out in cold ether to obtain compound 3 (33 mg, 52.9%) as a dark green solid.

1H NMR (300 MHz, CDCl3): δ 9.64–9.62 (m, 8H), 9.11 (d, J = 8.4 Hz, 1H), 8.63–8.42 (m, 8H), 7.95 (d, J = 5.2 Hz, 2H), 7.85 (d, J = 6.8 Hz, 2H), 7.63–7.44 (m, 12H), 7.17 (br s, 1H), 6.92 (br s, 1H), 6.90 (d, J = 12.8 Hz, 1H), 6.65 (d, J = 8.8 Hz, 2H), 6.28 (s, 1H), 5.79 (t, J = 8.4 Hz, 1H), 5.56 (t, J = 8.8 Hz, 1H), 5.40 (d, J = 6.8 Hz, 1H), 5.21 (d, J = 8.8 Hz, 1H), 4.93–4.90 (m, 2H), 4.57 (s, 1H), 4.48 (d, J = 5.2 Hz, 1H), 4.15 (d, J = 12.8 Hz, 1H), 4.13–4.09 (m, 1H), 4.01 (s, 1H), 3.58 (d, J = 6.4 Hz, 1H), 2.38–2.31 (m, 8H), 2.25 (br s, 1H), 2.24 (s, 3H), 2.08 (br s, 5H), 1.93 (t, J = 6.0 Hz, 2H), 1.84 (s, 1H), 1.79 (s, 3H), 1.66–1.60 (m, 1H), 1.49 (s, 3H), 1.21 (s, 3H), 0.97 (s, 3H), 0.16 (br s, 8H), 0.69 (br s, 2H), 0.74 (br s, 3H), −0.74 (br s, 2H), −2.04 (br s, 4H); HRMS-ESI: m/z calcd for [C113H111N20O22Si]+: 2127.7951 [M + H]+; found, 2120.6876; m/z calcd for [C134H132N210Si2]+: 2117.7951 [M + H]+; found, 2117.7971; m/z calcd for [C113H111N20O22Si]2+: 1064.4014 [M + 2H]+; found, 1064.4021. Purity = >95% (HPLC chromatogram, Figure S9).

**Preparation of Non-PEGylated Folate Conjugates of PTX.** Synthesis of FA-Pc-L-PTX (5). FA (19.4 mg, 0.044 mmol) was dissolved in anhydrous DMF (3 mL) under sonication. After complete dissolution, DCC (10.9 mg, 0.052 mmol) was added, followed by NHS (6.07 mg, 0.052 mmol) at rt. The reaction mixture was stirred for 16 h at rt. The white precipitate was removed by filtration through a 0.2 μm filter. To an activated solution of FA was added a solution of compound 3 (50.0 mg, 0.029 mmol) in anhydrous DMF (0.5 mL), followed by Et3N (12.2 μL, 0.088 mmol) slowly at rt. The reaction mixture was stirred at rt for 24 h. The compound was purified by passing through Sephadex G-15, using DMF as the eluent. It was then dialyzed [molecular weight cutoff (MWCO 1000)] in DMF (24 h), followed by DCM (20 h). After dialysis in DCM, the solution was concentrated and further crashed out in cold ether to obtain compound 5 (33 mg, 52.9%) as a dark green solid.

**Preparation of PEGylated Folate Conjugates of PTX (7a–d).** Synthesis of FA-PEG1k-Pc-L-PTX (7a). Compound 4 (17.4 mg, 0.009 mmol) was dissolved in DCM (1 mL) and stirred for a few minutes. After complete dissolution, DCC (2.96 mg, 0.014 mmol) dissolved in anhydrous DMF (0.5 mL) was added slowly at rt under N2 gas. The reaction mixture was stirred for 30 min, and NHS dissolved in DMF (0.5 mL) was added slowly at rt under N2 gas. The reaction mixture was stirred at rt for 20 h. It was filtered off through a 0.2 μm poly(tetrafluoroethylene) filter. To the filtered reaction mixture was added compound 6a (15 mg, 0.011 mmol) dissolved in anhydrous DMSO (0.5 mL), followed by Et3N (4.8 μL, 0.034 mmol) at rt under N2 gas. The reaction mixture was further stirred at rt for 48 h. Purification was done by passing the sample through Sephadex G-15, using DMF as the eluent. It was then dialyzed (MWCO 1000) in DMF (48 h), followed by DCM (8 h). After dialysis in DCM, the solution was...
concentrated and further crashed out in cold ether to get compound 7a (9 mg, 29.3%) as a blue solid.

1H NMR (DMSO-d$_6$, 400 MHz): δ 9.67–9.64 (m, 8H), 9.11 (d, J = 8.4 Hz, 1H), 8.61 (s, 1H), 8.58–8.47 (m, 8H), 7.95 (d, J = 6.4 Hz, 2H), 7.85 (d, J = 7.6 Hz, 2H), 7.76–7.44 (m, 12H), 7.17 (br s, 1H), 6.92 (br s, 1H), 6.90 (d, J = 12.4 Hz, 1H), 6.65 (d, J = 8.8 Hz, 2H), 6.28 (s, 1H), 5.79 (t, J = 8.4 Hz, 1H), 5.56 (t, J = 8.8 Hz, 1H), 5.40 (d, J = 7.6 Hz, 1H), 5.21 (d, J = 8.8 Hz, 1H), 4.93–4.90 (m, 2H), 4.57 (s, 1H), 4.46 (br s, 2H), 4.15 (d, J = 13.2 Hz, 1H), 4.12–4.01 (m, 1H), 4.01 (s, 1H), 3.81 (s, 2H), 3.73 (2H), 3.58 (d, J = 6.8 Hz, 1H), 3.48–3.42 (br m, PEG), 3.22–3.16 (m, 4H), 2.34–2.31 (m, 8H), 2.24 (br s, 4H), 2.08 (br s, 5H), 1.99 (br s, 3H), 1.00 (s, 3H), 0.97 (s, 3H), 0.30 (br s, 4H), 0.13 (br s, 4H), −0.69 (br s, 2H), −0.75 (br s, 2H), −2.02 (br s, 4H); HRMS-ESI: m/z calcd for [C$_{165}$H$_{214}$N$_{22}$O$_{48}$Si]$^+$: 1649.7375 [M+2H]$^+$; found, 1649.6862.

Synthesis of FA-PEG$_{5k}$-Pc-L-PTX (7b). Compound 7b was prepared using a procedure similar to that used to obtain 7a. In brief, compound 4 (40 mg, 0.022 mmol) was activated using DCC (6.77 mg, 0.033 mmol) and NHS (3.78 mg, 0.033 mmol) for 20 h. It was then filtered off and reacted with 6b (53.0 mg, 0.0219 mmol) and Et$_3$N (9.1 µL, 0.065 mmol) for 48 h. Purification was done using Sephadex G-25, with DMF as the eluent, followed by dialysis (MWCO 2000) in DMF (24 h) and then DCN (24 h). After dialysis, it was crashed out in cold ether to obtain compound 7b (33 mg, 35.6%) as a blue sticky solid.

1H NMR (DMSO-d$_6$, 400 MHz): δ 9.67–9.64 (m, 8H), 9.11 (d, J = 8.8 Hz, 1H), 8.61 (s, 1H), 8.58–8.47 (m, 8H), 7.95 (d, J = 6.4 Hz, 2H), 7.85 (d, J = 7.6 Hz, 2H), 7.76–7.44 (m, 12H), 7.18 (br s, 1H), 6.92 (br s, 1H), 6.90 (d, J = 13.2 Hz, 1H), 6.65–6.57 (m, 2H), 6.28 (s, 1H), 5.79 (t, J = 8.4 Hz, 1H), 5.56 (t, J = 8.8 Hz, 1H), 5.40 (d, J = 6.8 Hz, 1H), 5.21 (d, J = 8.8 Hz, 1H), 4.94–4.89 (m, 2H), 4.57 (s, 1H), 4.46 (d, J = 4.8 Hz, 2H), 4.15 (d, J = 13.2 Hz, 1H), 4.12–4.09 (m, 1H), 4.01 (s, 1H), 3.81 (s, 2H), 3.73 (s, 2H), 3.58 (s, 1H), 3.48 (br s, PEG), 3.21–3.15 (m, 4H), 2.35–2.31 (m, 8H), 2.24 (br s, 4H), 2.13–2.10 (m, 2H), 2.08 (br s, 3H), 1.99–1.92 (m, 2H), 1.86 (s, 1H), 1.79 (s, 3H), 1.66–1.60 (m, 1H), 1.49 (s, 3H), 1.21 (s, 3H), 1.00 (s, 3H), 0.97 (s, 3H), 0.30 (br s, 4H), 0.14 (br s, 4H), −0.69 (br s, 2H), −0.75 (br s, 2H), −2.02 (br s, 4H); UV–Vis and Fluorescence Measurement. Concentrations of all prodrugs were adjusted with the UV–vis spectrum based upon the extinction coefficient of compound 4 (Figure S22). UV–vis and fluorescence spectra in DMSO were measured at a 2 µM concentration. Fluorescence was measured using 0.6 mL of the sample. The sample was excited at 605 nm with a slit width of 8 nm. Emission was recorded from 640–680 nm with a slit width of 5 nm. Both UV–vis and fluorescence spectra were normalized at 800 nm. Concentration-dependent fluorescence was measured using a plate reader (SpectraMax Gemini EM, Molecular Devices) with an excitation wavelength of 605 nm and emission at 680 nm. The sample volume used was 200 µL.

log $D_{7A}$ Determination. log $D_{7A}$ for all of the folate conjugates (5 and 7a–d) was determined using the previously reported method.19 Briefly, DMSO stocks of prodrugs were prepared in 5 mM concentrations. n-Octanol and PBS were presaturated with PBS and n-octanol, respectively. Ten microliters of DMSO stock was added to presaturated n-octanol and PBS (1 mL each) in 2 mL Eppendorf tubes. The mixture was then vortexed for 30 min. After that, it was centrifuged at 3000 rpm for 10 min (5, 7a, and 7b), 15 min (7c), or 30 min (7d) and was kept in the dark to stand for phase separation. Once there was a clear phase separation, both layers were collected in different vials. Fifty microliters of the sample from each layer was diluted to 3 mL with DMSO, and UV–vis spectrum was measured. log D was measured as the log ratio of the absorbance in octanol to absorbance in PBS: log $D_{7A} = \log_{10}$(octanol Absmax/PBS Absmax).

Cellular Uptake of Folate Conjugates on SKOV-3 Cells. SKOV-3 cells were seeded in 96-well plates at a density of 10 000 cells/well in 180 µL of the complete medium and were incubated at 37 °C for 24 h. The 4 mM stock solutions of folate...
conjugates in DMSO were diluted to 50 μM with the complete medium before the solutions were added to the wells. The diluted solutions (20 μL) were added to each well to achieve a final concentration of 5 μM per well.

To determine whether the uptake of the conjugates was mediated through FRs, additional uptake assays were performed under the same conditions with 0.5 mM FA. Ten thousand SKOV-3 cells in 96-well plates were preincubated with 0.5 mM FA. After 1 h, the diluted conjugate solutions were added to each well to achieve a final concentration of 5 μM per well. After incubation for 0, 1, 3, 6, 9, 16, and 24 h, the medium was collected to quantify the extracellular concentration of conjugates with 10x dilution with DMSO. DMSO solution (200 μL) containing 10% of the medium was added to each well to lyse the cells. The cell lysate was then used to determine the intracellular concentration of conjugates. The extracellular and intracellular prodrug concentrations were determined by fluorescence measurement of the diluted medium and cell lysate. The prodrug concentration was quantified by its fluorescence intensity using a fluorescence plate reader (SpectraMax Gemini EM, Molecular Devices) with excitation at 605 nm and emission at 680 nm and a bottom reading option. Data were analyzed using SoftMax Pro software version 5.4.1.

**Size Measurement and Stability of Folate Conjugates (5 and 7a–d) in PBS and Complete Medium.** Self-assembled aggregates or micelles of folate conjugates (5 and 7a–d) in the aqueous solution were characterized by measuring their hydrodynamic diameter, zp, and PDI via the dynamic light scattering (DLS) method. The size and zp were measured at a concentration of 5 μM at 37 °C. The measurements were carried out on days 0, 1, 2, 3, 5, and 7.

**Dark and Phototoxicity Study.** The cytotoxicity of all conjugates was evaluated under both dark and illuminated conditions. For the dark toxicity assay, SKOV-3 cells (5000 cells/well) were seeded on 96-well plates and then incubated for 24 h. Four millimolar stock solutions of conjugates in DMSO were diluted to 0.5, 1.0, 2.0, 5.0, and 10 μM in the complete medium. Twenty microliters of diluted solutions were added to each well (180 μL), and the plates were gently shaken using an orbital shaker for 30 min in the dark. The plates were incubated for another 3 days in the dark at 37 °C.

For phototoxicity assays, cells were prepared in the same way as cells used in the dark toxicity assays. After adding the conjugates and incubating for 24 h, the medium containing drugs that were not taken up into cells was removed. The cell monolayers were washed three times with PBS, and 200 μL of fresh medium was added to each well. The plates, without lids, were placed on an orbital shaker (Lab-Line, Barnstead International) and illuminated using a diode laser (690 nm) at 5.6 mW/cm² for 30 min to achieve a light dose of 10 J/cm². Then, the plates were placed back into the incubator for another 72 h. After the incubation, the MTT assay was performed as described previously.20

The concentration–cytotoxicity relationships for targeted conjugates were analyzed with the sigmoidal Hill Equation using the nonlinear least squares regression (Prism, Version 7.02, GraphPad Software, Inc. CA). The analysis provided relevant pharmacodynamic parameters (Emax, IC₅₀, and Hill slope), where Emax is the maximum cytotoxicity, IC₅₀ is the drug concentration producing 50% Emax, and Hill slope represents the steepness of the curves.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01105.

Spectral data (1H, mass, UV–vis, and fluorescence) of the selected compounds and HPLC chromatograms of 4 and 5 (PDF)

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**Author Contributions**

P.T., R.K., M.B., and G.N. designed the prodrug structures and developed the synthetic scheme for these prodrugs. P.T. obtained the spectroscopy data (UV–vis and fluorescence) and log D₄₋₅ values for the prepared folate conjugates. M.L. and P.R. performed the cellular uptake, dark and phototoxicity. M.L. evaluated the stability of aggregates or micelles using DLS. M.L. and M.B. performed in vivo optical imaging. Y.Y. and S.W. designed the overall project and were involved in the interpretation of data. All authors participated in manuscript preparation and revision.

**Notes**

The authors declare no competing financial interest.

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### ABBREVIATIONS

PTX, paclitaxel; PEG, polyethylene glycol; PDT, photodynamic therapy; FA, folic acid; FR, folate receptor; CA4, combeartatin A-4; Pc, silicon phthalocyanine; Zp, zeta potential; DLS, dynamic light scattering; PET, photoinduced electron transfer; L, singlet oxygen cleavable aminoacyliner link; TLC, thin layer chromatography; NMR, nuclear magnetic resonance

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