Graphical Abstract

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Overcomes Spatiotemporal Limits of Singlet Oxygen

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Abstract

Photodynamic therapy (PDT) is a cancer treatment modality where photosensitizer (PS) is activated by visible and near IR light to produce singlet oxygen ($^1\text{O}_2$). However, $^1\text{O}_2$ has a short lifetime (< 40 ns) and cannot diffuse (< 20 nm) beyond the cell diameter (e.g., $\sim 1,800$ nm). Thus, $^1\text{O}_2$ damage is both spatially and temporally limited and does not produce bystander effect. In a heterogeneous tumor, cells escaping $^1\text{O}_2$ damage can regrow after PDT treatment. To overcome these limitations, we developed a prodrug concept (PS-L-D) composed of a photosensitizer (PS), an anti-cancer drug (D), and an $^1\text{O}_2$ -cleavable linker (L). Upon illumination of the prodrug, $^1\text{O}_2$ is generated, which damages the tumor and also releases anticancer drug. The locally released drug could cause spatially broader and temporally sustained damage, killing the surviving cancer cells after the PDT damage. In our previous report, we presented the superior activity of our prodrug of CA4 (combretastatin A-4), Pc-(L-CA4)₂, compared to its non-cleavable analog, Pc-(NCL-CA4)₂, that produced only PDT effects. Here, we provide clear evidence demonstrating that the released anticancer drug, CA4, indeed damages the surviving cancer cells over and beyond the spatial and temporal limits of $^1\text{O}_2$. In the limited light illumination experiment, cells in the entire well were killed due to the effect of released anticancer drug, whereas only a partial damage was observed in the pseudo-prodrug treated wells. A time-dependent cell survival study showed more cell death in the prodrug-treated cells due to the sustained damage by the released CA4. Cell cycle analysis and microscopic imaging data demonstrated the typical damage patterns by CA4 in the prodrug treated cells. A time-dependent histological study showed that prodrug-treated tumors lacked mitotic bodies, and the prodrug caused broader and sustained tumor size reduction compared to those seen in the tumors treated

with the pseudo-prodrug. This data consistently support that the released CA4 overcomes the spatiotemporal limitations of $^1\text{O}_2$, providing far superior antitumor effect.

1. Introduction

Photodynamic therapy (PDT) is a minimally invasive modality for the treatment of certain cancers. PDT works *via* the combined action of light and a photosensitizer (PS), to produce a toxic species called singlet oxygen ($^1\text{O}_2$), which causes cellular toxicity¹⁻⁵. Preferential accumulation of PS in the tumor and selective illumination of the tumor site to achieve minimal collateral damage set PDT apart from chemotherapy⁶. However, spatiotemporal barriers are significant limitations for PDT (Fig. 1).

The lifespan of $^1\text{O}_2$ is extremely short (< 40 ns)⁷. $^1\text{O}_2$ is only generated during the illumination. Hence, most direct damage by $^1\text{O}_2$ occurs during the illumination, that is, for 30 min in our PDT protocols. Due to the transient nature of $^1\text{O}_2$, the $^1\text{O}_2$ diffusion distance of 20 nm⁷ is far shorter than the cell diameter, which is $\sim 18,000$ nm for MCF-7 cells⁸, and even that of a single mitochondrion, which is, 530 nm for P388 cells⁹; (Fig. 1A). $^1\text{O}_2$ generation throughout the tumor is not homogeneous due to the heterogenous nature of the tumor itself, in addition to PDT variables, like $^3\text{O}_2$ concentration, light intensity, and PS concentration¹⁰⁻¹³.

The spatiotemporal limits of PDT, in conjunction with heterogeneous generation of $^1\text{O}_2$ in tumors, could produce incomplete ablation, which would eventually cause recurrence¹⁴. Cells with lower-than-lethal $^1\text{O}_2$ concentrations can survive PDT treatment (Fig. 1C). A combination of PDT and systemic chemotherapy seemed to be effective in killing residual cancer cells^{15, 16}. However, parenteral administration of anticancer drugs cannot circumvent the systemic side

effects of such a traditional combination therapy. Various liposomal-based strategies have been developed to achieve either temporal¹⁷ or spatial control.¹⁸

Figure 1. Light-activatable prodrug (PS-L-D) strategy overcoming the spatiotemporal barriers of PDT. A. Limited diffusion of 1O_2 can be overcome by unlimited diffusion of the released drug (D) after illumination. B. Transient damage from 1O_2 can be overcome by sustained damage from the released drug; [D] *in vivo* concentration is expected to decrease mainly due to dilution

1 by circulating blood; A.U.: arbitrary unit. C. Incomplete ablation from PDT can be overcome by
2 the sustained and expanded damage from the released drug.

3
4 In order to overcome the spatiotemporal limits of PDT and to avoid the systemic toxicity
5 of anticancer drugs that has been observed with a combination of PDT and chemotherapy, we
6 developed a light-activatable prodrug (PS-L-D) utilizing the concept of photo-unclick chemistry
7 (Fig. 1; ¹⁹⁻²³. The prodrug was prepared by conjugating a photosensitizer (PS) with a
8 chemotherapeutic drug (D) *via* an ¹O₂-cleavable aminoacrylate linker (L; Fig. 2A). Upon
9 excitation, the ¹O₂ generated from the PS cleaves the linker to release the chemotherapeutic drug
10 ¹⁹. We have further advanced this prodrug by making a fluorescent prodrug, Pc-(L-CA4)₂, using
11 a fluorescent PS phthalocyanine (Pc) and an anticancer drug (CA4, Fig. 2A; ²². Pc was detected
12 with *in vivo* optical imaging for real-time tracking of the prodrug, and was then used for a
13 combination of PDT and site-specific chemotherapy (Fig. 2B). The prodrug demonstrated far
14 superior activity to its pseudo-prodrug Pc-(NCL-CA4)₂, a structurally similar analog expressing
15 only PDT effects without releasing CA4 (NCL: non-cleavable linker), *in vitro* and *in vivo*,
16 without systemic side effects ²².

Figure 2. A. Structures of light-activatable prodrug of CA4, $\text{Pc}-(\text{L-CA4})_2$, and its pseudo-prodrug, $\text{Pc}-(\text{NCL-CA4})_2$. B. Their cytotoxic products after illumination.

Here, we present clear evidence supporting our hypothesis that the superior activity of the prodrug $\text{Pc}-(\text{L-CA4})_2$ over $\text{Pc}-(\text{NCL-CA4})_2$ is due to the released anticancer drug CA4, overcoming the spatial and temporal limits of $^1\text{O}_2$, a major toxic species of PDT. We used HPLC to quantify the CA4 released from the prodrug in the complete medium after *in vitro* illumination. We then acquired images of live cells after partial illumination of the prodrug-treated wells to examine the broader cell kill achieved with $\text{Pc}-(\text{L-CA4})_2$. To demonstrate the sustained cytotoxic effect of the released CA4, we determined the cell survival 30 min or 3 days after treatment, with or without replacing the culture medium. After treatment, we collected fluorescence images of the cells to observe the tubulin staining patterns. Finally, we generated histological images of the tumors to observe the time-dependent pattern of damage after treatment.

2. Materials and methods

2.1. Materials and animals

Pc-(NCL-CA4)₂ and Pc-(L-CA4)₂ were prepared based on our reported method²². Purity was confirmed at > 95% based on HPLC analysis (Figs. S1 and S2). Tubulin tracker green reagent was purchased from Life Technologies (cat # T34075). The prodrugs were prepared in 2 mM stock solutions with DMSO and were stored at -20°C. All other chemicals were either of the highest or analytical grades. Female BALB/c mice were purchased from Charles River Laboratories, Inc., through NCI (Frederick, MD). Mice were housed and handled in the animal facility of the College of Pharmacy or Rodent Barrier Facility in the Biomedical Research Center-West at the University of Oklahoma Health Sciences Center (OUHSC), Oklahoma City, OK. All animal experiments were approved by IACUC, OUHSC. We chose this mouse tumor model (colon 26 cells on BALB/c mice) instead of xenograft of MCF-7 cells because this tumor model has an intact immune system and has been commonly used in PDT studies. Detailed experimental procedures, such as HPLC methods, cell cycle analysis, microscopic imaging, and histological sample preparation, are found in the supporting information.

2.2. Photocleavage study of CA4 prodrug

We added 1 µM Pc-(L-CA4)₂ in 0.5 mL growth medium to a glass beaker (3 cm diameter) and either illuminated it with a 690 nm laser at 5.6 mW/cm² for 30 min or dark incubated it. The solution was then transferred into a 5-mL centrifuge glass tube. The beaker was rinsed with 3 mL of dichloromethane (DCM) and the contents were added to the centrifuge tube. To quantify CA4, the sample was processed as detailed in the HPLC conditions and Quantification of CA4 with HPLC sections in the SI.

2.3. Live cell imaging after treatment with the prodrugs

MCF-7 cells were seeded at 10,000 cells/ml/well in 24-well plates. Plates were then incubated for 24 h. Before seeding, the 24-well plates were prepared by physically removing each external plastic liner to avoid disturbing the horizontal illumination. Stock solutions [(2 mM in dimethyl sulfoxide (DMSO))] were diluted with medium. The diluted solutions (25 μ L) were added to the wells (1 mL) to get appropriate final concentrations. After 24 h incubation, the wells were horizontally illuminated with a 690 nm diode laser at 100 mW/cm² for 30 min (Fig. 3A). We used a higher intensity (100 mW/cm²) for this in vitro experiment due to the illumination angle. The illuminated plates were incubated for an additional 72 h. Then, a Calcein AM live cell staining assay was performed. Briefly, the cells were washed with Calcein AM wash buffer (1 x 1 mL). Then, fresh wash buffer (250 μ L) and the working reagent (250 μ L) were added to the wells. The cells were incubated for 30 min. Fluorescent images were captured using an Operetta microscope with a green fluorescence channel. All images were taken at 10 x magnification.

2.4. Phototoxic experiments

The time-dependent cytotoxicity of Pc-(L-CA4)₂ and Pc-(NCL-CA4)₂ was determined upon illumination. On day 0, MCF-7 cells (5,000 cells/well) were seeded on 96-well plates and were then incubated for 24 h at 37°C in 5% CO₂. Stock solutions (2 mM) were prepared in DMSO. The stocks were further diluted with the complete medium, of which 10 μ L was then added to each well (190 μ L) to achieve the necessary final concentrations. The plates were incubated for 30 min and were then removed from the incubator. The 96-well plates, without the lids, were placed on an orbital shaker (Lab-line, Barnstead International) and were illuminated vertically using a diode laser (690 nm) arranged vertically on the top of the well plate under light intensity 5.6 mW/cm² for 30 min to achieve a light dose of 10 J/cm². To ensure uniformity of the light,

each plate was shaken gently during the illumination. After illumination, the cells were either washed (EP III) or unwashed (EP II; Fig. 4A). An MTT assay was performed after 72 h. In another protocol (EP I), an MTT assay was performed 0.5 h after illumination, without washing the media. To perform the MTT assay, a 10- μ L solution of MTT (10 mg in 1 mL PBS buffer) was added to each well and the plate was incubated for 4 h. Then, the MTT solution was removed and the cells were dissolved in 200 μ L of DMSO. The absorbance of each well was measured at 570 nm, with background subtraction at 650 nm. Cell survival was then quantified by measuring the absorbance of the treated wells, which was compared with that of the untreated wells (controls), and expressed as a percentage.

2.5. Cell cycle analysis

On day 0 of the experiment, 2.0×10^7 MCF-7 cells/well were seeded in 24-well plates. On day 1, the 2 mM DMSO stock solutions of the respective compounds were diluted in the media and were added into each well to achieve the required final concentrations. The well plate was then illuminated from above with a 690 nm diode laser at 5.6 mW/cm² for 30 min, without removing the medium. After the illumination, the cells were incubated for another 72 h. Cell cycle analysis was performed according to the previous procedure²⁴ with a minor modification.

2.6. Microscopic imaging

MCF-7 cells were seeded at cell densities of 1.0 - 1.5 $\times 10^5$ cells/well in 24-well plates, each containing a 12-mm diameter cover slip. Cells were then incubated for 24 h. The **prodrug (2 mM, DMSO)** stock solutions were diluted to the appropriate concentrations, were added to the plates, and were incubated for 24 h. The cells were illuminated from above with a 690 nm diode laser at

5.6 mW/cm² for 30 min. After 72 h of incubation, cells were stained with **Tubulin Tracker Green** for the microtubule staining.

2.7 Animal study after the treatment with the prodrugs

Four-to-six-week-old BALB/c mice (18–20 g) were used for the murine tumor model. Mice were subcutaneously (S.C.) implanted with 2×10^6 colon 26 cells in PBS (100 μ L) on the lower backs of their necks. Tumor growth was monitored using digital calipers. The longest axis of the tumor (l) and the axis perpendicular to l (w) were used to calculate tumor volume ($lw^2/2$). Mice with tumors 4–6 mm in diameter were used for the experiments. Stock solutions of Pc-(L-CA4)₂ and Pc-(NCL-CA4)₂ were prepared in 2 mM DMSO. Further dilution (**0.1 x**) was made in 5% Cremophore EL/PBS solution to achieve final doses of 1 μ mol/kg each. To each mouse, ~**100** μ L of the final solution (**10 % DMSO**) was injected *via* one intravenous administration. Twenty-four hours later, mice were anesthetized by intraperitoneal injection of 80 mg/kg ketamine and 6 mg/kg xylazine. Tumors were illuminated with a 690 nm diode laser at 200 mW/cm² for 30 min (dose: 360 J/cm²). The illuminating beam diameter (10 mm) was larger than the tumor diameter (4–6 mm), indicating that the beam covered the whole tumor. Tumor size was measured daily. Mice were photographed before they were euthanized.

2.8. H&E staining and imaging of the histology sample

To evaluate the effects of the prodrug, mice were euthanized 1, 24, 48, 72, and 96 h after the illumination. Tumors were collected. Hematoxylin and eosin (H&E) staining was performed on the tumor specimens.

2.9. Statistical analysis

Student's t-test was used for statistical analysis. *P* values less than 0.05 were considered significant.

3. Results and discussion

3.1. HPLC determination of CA4 from Pc-(L-CA4)₂ in media after illumination

Yield of CA4 released by the illumination was determined using HPLC. Briefly, 1 μ M Pc-(L-CA4)₂ in the complete culture medium was illuminated at 5.6 mW/cm² for 30 min (using the same illumination conditions as the phototoxic study). Then, CA4 was extracted using DCM. After DCM was dried, the extract was reconstituted in an HPLC injection solution [100% Acetonitrile (ACN)]. To examine the contents of the medium itself and to determine the extraction yield, CA4 amounts were determined in three additional samples: 1) DCM extracts from the complete medium, 2) 2 μ M CA4 in ACN, and 3) extracted CA4 from the complete medium (Fig. S4a-c). The CA4 peak was distinctively isolated from the other small peaks, shown at 11.7 min. The extraction efficiency of CA4 from the medium was calculated to be 82.1 $\pm$ 5.6% for our method. The HPLC from the illuminated sample with Pc-(L-CA4)₂ showed the CA4 peak as a single major peak after the illumination, with 61.8 $\pm$ 2.6% of cleavage yield in the complete medium (Fig. S4d). The photo-unclick chemistry of Pc-(L-CA4)₂ released the parent CA4 in a reasonable yield as a single major product. Similarly, HPLC analysis of the unilluminated Pc-(L-CA4)₂ sample in the media was performed with the same procedures. The percentage of recovered Pc-(L-CA4)₂ from complete media after 30 min in dark (without irradiation) was 98.2 $\pm$ 5.9% (Fig. S3). The linker was stable during the procedures.

3.2. Live cell imaging *in vitro* after treatment with Pc-(L-CA4)₂ or Pc-(NCL-CA4)₂ – spatial effects of the released CA4

Because the diffusion distance of ¹O₂ is < 0.02 μm, the ¹O₂ damage in PDT is spatially limited to the area where ¹O₂ is formed above the lethal dose (Fig. 1). In contrast, anticancer drug that is released from the illuminated prodrug can diffuse to other cells in cultured cell medium, causing spatially extended damage *via* bystander effects. To evaluate the damaged area of the horizontally illuminated wells, whole wells were stained with calcein AM to visualize live cells after treatment with [Pc-(NCL-CA4)₂ + *hν*] or [Pc-(L-CA4)₂ + *hν*]. Wells were illuminated from the side to mimic the vertical section of an illuminated tumor (Fig. 3A). From the point of incidence, light intensity gradually diminishes as light travels from the left to the right side of the well, due to absorption and scattering. These conditions are similar to the lessened penetration of light into deeper tumors. Upon treatment with Pc-(NCL-CA4)₂, cells disappeared to an approximate distance of 5 mm. Cells in the wells treated with Pc-(CL-CA4)₂ disappeared entirely.

Partial damage was observed in the Pc-(NCL-CA4)₂-treated wells, in which most of cells in the incident light side died. More cells were alive in the opposite side of the well, where less SO was formed due to the limited light (Fig. 3C). Since ¹O₂ cannot diffuse, cells with a low ¹O₂ concentration could survive. In stark contrast, almost all of the Pc-(CL-CA4)₂-treated cells in the opposite side from the incident light died (Fig. 3D). It is not hard to imagine that the CA4 released in the left side of the well diffused throughout the well and killed the cells in the right side *via* bystander effects. Photodynamic oxidative stress products, such as H₂O₂, have been suggested to induce bystander effects in PDT^{25, 26}. However, the bystander effects that we

observe from our prodrug are due to the effects of the released anti-cancer drug, which seem to be more effective than the bystander effects of PDT (Fig. 3C vs. 3D).

Figure 3. (A) Illustration of experimental setup for the horizontal illumination of a well. Live cell images 72 h after illumination [690 nm diode laser, 100 mW/cm² (at the incident point of the well), 30 min] (B) no prodrug treatment, (C) 25 nM Pc-(NCL-CA4)₂, and (D) 25 nM Pc-(L-CA4)₂. Data are representative of at least two independent experiments.

3.3. Phototoxicity of Pc-(NCL-CA4)₂ and Pc-(L-CA4)₂ at 0.5 h or 72 h after illumination – sustained killing from the released CA4

Direct damage from ¹O₂ occurs during the illumination in PDT, because the lifetime of ¹O₂ is extremely short (< 40 ns). For example, when the Pc-(NCL-CA4)₂-treated cells are illuminated for 30 min, the direct damage to the cells occurs for 30 min (Fig. 1B). When the Pc-(L-CA4)₂-treated cells are illuminated for 30 min, the direct damage from ¹O₂ occurs for 30 min (PDT effect). However, additional cell death from the released CA4 is expected for the entire

incubation time (72 h) because the released CA4 concentration in the medium remains the same, unless the medium is replaced. To demonstrate the sustained cell kill effect of the released CA4, three experimental procedures (EP) were performed: 1) EP I: Shortly (0.5 h) after the illumination, cell survival was determined; 2) EP II: Three days after the illumination, cell survival was determined, and; 3) EP III: The cell culture medium was replaced 0.5 h after the illumination to remove the released CA4 and then cell survival was determined 3 days after the illumination (Fig. 4).

Figure 4. A. Schematic diagram of three experimental procedures. B. Hypothetical time-dependent concentration of 1O_2 and the released drug [D] in the medium; A.U.; arbitrary unit. C & D. Concentration-dependent cell survival curves after treatment with (C) $Pc-(NCL-CA4)_2 + h\nu$ and (D) $Pc-(L-CA4)_2 + h\nu$. Each data point represents the average from three separate experiments.

1 There were no significant differences between Pc-(NCL-CA4)₂-treated cells in EP II and
2 EP I/EP III except at one concentration, (t-test **at each concentration**, $p > 0.05$, Fig. 4C). At 2 μ M
3 of Pc-(NCL-CA4)₂, longer incubation yielded lower cell survival (11%, EP II) than did shorter
4 incubation (40%, EP I). However, when the media was replaced (EP III), cell survival increased
5 32%. We noted a much greater difference between Pc-(L-CA4)₂-treated cells in EP II and EP
6 I/EP III, at a wide range of drug concentrations (Fig. 4D). [Without illumination, Pc-(L-CA4)₂
7 did not show the potentiation of cytotoxicity that was observed in EP III.] An almost 100-fold
8 left shift in the curve was noted from EP I to EP II. Again, an almost 100-fold right shift was
9 seen from EP II to EP III. The higher cytotoxicity at a longer incubation time is understandable
10 because CA4 is a cell cycle-specific cytotoxic drug inhibiting tubulin polymerization^{27, 28}. In
11 addition, the IC₅₀ of EP II (12 nM Pc-(L-CA4)₂ $\rightarrow$ 12 nM x 2 CA4 x 0.618 (photo-release yield)
12 = ~14.8 nM CA4, after the illumination) is close to the IC₅₀ of CA4 itself (9 nM). The right shift
13 of the curve from EP II to EP III should be due to the removal of CA4 from the medium,
14 canceling the sustained cytotoxic effect of the released CA4.

15 We generally incubate the cells with prodrugs (or photosensitizers) for 24 hr in our
16 standard protocol. However, we did not see much difference between the two prodrugs with 24
17 hr incubation. We assumed that it was because of high potency of these prodrugs and rapid
18 uptake of these prodrugs to the cells. To limit the uptake of these prodrugs, we chose the shorter
19 incubation time, 30 min, to demonstrate the difference in the phototoxic study.

3.4. Cell cycle analysis and imaging after staining with tubulin tracker – mechanistic evidence of CA4 damage

The above two experiments demonstrated that the prodrug therapy with $\text{Pc}-(\text{L-CA4})_2$ overcomes the spatiotemporal limits of PDT with $\text{PC}-(\text{NCL-CA4})_2$, most likely due to the released CA4 that remains in the medium after the illumination. To find more direct evidence for the contribution of CA4 to the $\text{Pc}-(\text{L-CA4})_2$ treatment, we performed two additional experiments. Since CA4 inhibits tubulin formation, CA4-treated cells are arrested in G2 phase²⁹. Therefore, we used FACS to perform cell cycle analysis after treatment with CA4, $\text{Pc}-(\text{NCL-CA4})_2 + h\nu$, or $\text{Pc}-(\text{L-CA4})_2 + h\nu$, expecting a high population of the cells treated with CA4 or $\text{Pc}-(\text{L-CA4})_2 + h\nu$ to be in G2 phase. Cells were analyzed by FACS 72 h after the treatment to give enough time for the treated cells to accumulate at a specific phase. Second, since the CA4 inhibits the tubulin polymerization, CA4-damaged cells show a specific cell-staining pattern when stained with tubulin-staining dye. Thus, we performed fluorescence microscopic imaging of the treated cells using tubulin tracker, expecting to find a similar staining pattern in CA4 and $\text{Pc}-(\text{L-CA4})_2 + h\nu$ -treated cells.

While only 9% of the untreated control cells were in G2 phase, more CA4-treated cells were in G2 phase (72%, Figs. 5A and B). As expected, the $\text{Pc}-(\text{NCL-CA4})_2$ -treated cells did not show any enrichment in G2 phase (7%, Fig. 5C). However, more $\text{Pc}-(\text{L-CA4})_2$ -treated cells were in G2 phase (52%, Fig. 5D). Interestingly, more of the $\text{Pc}-(\text{L-CA4})_2$ -treated cells were in G2 phase as time after the illumination increased: 1 to 72 h $\rightarrow$ 15 to 50% (Fig. 5F). These data support the idea that the released CA4 in the $\text{Pc}-(\text{L-CA4})_2$ -treated wells arrested the cells in G2 phase in a time-dependent manner.

Figure 5. Cell cycle analysis with FACS 72 h after treatment with (A) control (no treatment), (B) 1 μ M CA4, (C) 1 μ M Pc-(NCL-CA4)₂ + *h* ν , or (D) 125 nM Pc-(L-CA4)₂ + *h* ν . E. Chart from data A-D. F. Cell cycle analysis of the cells treated with 200 nM Pc-(L-CA4)₂ + *h* ν after various time points. *h* ν : 690 nm 5.6 mW/cm² for 30 min (Additional cell cycle data at 150 nM are shown in Fig. S5). Data are representative of at least two independent experiments.

The fluorescence microscopic images also exhibited a consistent pattern (Fig. S6). The Pc-(L-CA4)₂-treated cells displayed a staining pattern similar to that of CA4-treated cells, but Pc-(NCL-CA4)₂-treated cells did not. The CA4- and Pc-(L-CA4)₂-treated cells exhibited a somewhat distinct tubulin imaging pattern compared with the control cells. Most treated cells were egg-shaped, and no tubulin tracker was found inside the nuclei, an imaging pattern that is typical of CA4-treated cells ²⁴. The Pc-(NCL-CA4)₂-treated cells showed a unique imaging pattern. The cells were circular (rounded), and evenly stained with tubulin tracker throughout the cells. These imaging data also support our hypothesis that the released CA4 damaged cells in the Pc-(L-CA4)₂-treated wells.

3.5. Histological study of the tumors after treatment with Pc-(L-CA4)₂ or Pc-(NCL-CA4)₂

Based on the above *in vitro* data supporting our hypothesis that the released CA4 overcomes the spatiotemporal limits of ¹O₂, we examined whether such superior spatiotemporal antitumor effects would be observed in the tumors treated with Pc-(L-CA4)₂ + *hν* over tumors treated with Pc-(NCL-CA4)₂ + *hν*. Balb/c mice with S.C. colon 26 tumors were treated once with either prodrug + *hν* or pseudo-prodrug + *hν* (6 mice per group). The tumors were photographed and were collected for histological staining at various time points (1-96 h and 15 days; Fig. 6). The centers of the tumors were vertically sliced for H&E staining.

A number of differences were evident (Fig. 6). Skin damage was observed in both groups, but seemed slightly less in the tumors treated with Pc-(L-CA4)₂, particularly at 24 h (left column of A and B in Fig. 6). While the surviving cancer cells developed into donut-shaped tumors from 48 h in Pc-(NCL-CA4)₂-treated tumors, no visible tumors were observed in the Pc-(L-CA4)₂-treated tumors. Fifteen days after the treatment, we observed a clear difference in the

1 sizes of tumors in mice, based on treatment type. The superior antitumor effects of the light-
 2 activatable prodrug over its pseudo-prodrug are consistent with our previous reports²². Tumor
 3 volumes calculated from the lengths and widths noted from the histology images are found in the
 4 SI (Fig. S7).

5
 6 **Figure 6.** Photographic and H&E stained images of the tumors after treatment with (A) 1
 7 $\mu\text{mole/kg}$ Pc-(NCL-CA4)₂ + hv or (B) 1 $\mu\text{mole/kg}$ Pc-(L-CA4)₂ + hv (hv: 690 nm, 200 mW/cm²,

30 min). The images were made at 1, 24, 48, 72, 96 h, and 15 days after the illumination. The photographic images were taken immediately before the collection of the tumors. Average tumor diameter at the time of treatment: 4-6 mm. Light beam diameter: 10 mm. Red stars indicate donut-shaped tumors and the corresponding area in the section. Histology images: magnification 20 X and scale bar 1 mm.

We examined four components of the histological samples: 1) damaged (necrotic) area; 2) vascular damage (hemorrhage); 3) immune responses, and; 4) mitotic bodies. We observed broader necrotic damage in the Pc-(L-CA4)₂-treated tumors than in the Pc-(NCL-CA4)₂-treated tumors (24-96 h, Fig. 6A and B, right column). While the major necrotic damage was limited to the centers of the tumors in the Pc-(NCL-CA4)₂-treated group, almost the entire tumors became necrotic in the Pc-(L-CA4)₂-treated group. While the tumor sizes increased in the Pc-(NCL-CA4)₂ group, confirming donut-shaped tumor growth after the illumination, the tumors were smaller and eventually disappeared in the Pc-(L-CA4)₂-treated group. In both groups, there was less damage to the deeper outsides of the tumor than to the centers near the skin (Figs. 1C and 6). This finding might be due to the decreased light intensity by light attenuation. Interestingly, while the lower sides of the surviving tumors regrew in the Pc-(NCL-CA4)₂-treated group, the bottom layers of less damaged cancer cells eventually disappeared in the Pc-(L-CA4)₂-treated group. The damage in the prodrug group was broader and more sustained than the damage in the pseudo-prodrug group.

Figure 7. H&E stained images of the tumors showing vascular damage (left two panels, A and B; magnification 40 X; scale bar 20 μ m) and mitotic bodies (indicated by yellow circles; right two panels, A and B; magnification 60 X; scale bar 40 μ m) after 1, 24, and 48 h of treatment with (A) Pc-(NCL-CA4)₂ + *h* ν or (B) Pc-(L-CA4)₂ + *h* ν . (*h* ν : 690 nm, 200 mW/cm², 30 min); *The locations of these images in the whole tumor sections are shown at higher resolution in the SI (Fig. S9-11 & S14-16).

Vascular damage (hemorrhage), which is one of the well-known effects of PDT³⁰, was observed as early as 1 h post-illumination in both groups (Fig. 7, left two panels A and B). This finding was noted well before necrotic cellular damage was evident at 24 h post-illumination. Interestingly, hemorrhage was observed in most tumors in both groups at 1 h, even in the deepest tumor sites (Fig. S9.3 and S14.3). Leaked RBCs were visible in the necrotic areas in both groups until 24 h post-illumination, but were not visible 48 h post-illumination (Fig. 6). In the non-necrotic areas of the Pc-(NCL-CA4)₂-treated tumors, the vasculature became normal after 48 h

(Fig. S11.3). Vascular damage from the illumination was faster than observable necrotic cell death in both groups, but recovery from vascular damage was also rapid in the non-necrotic regions of the Pc-(NCL-CA4)₂-treated tumors.

The population of mitotic bodies was extremely low in the tumors treated with Pc-(L-CA4)₂ after 24 h post-illumination (Figs. 7 right panel B and S15). CA4-treated cells are known to have a lower mitotic cell population, due to CA4's inhibitory effect on microtubule formation²⁹. We assume that the reduction of the mitotic cell population in the prodrug-treated tumors might be due to the impact of the CA4 that is released by the illumination.

In both groups, flux of immune cells under the tumors was rapidly observed 1 h after the illumination (Fig. S9.2 & S14.2). However, while the immune cells did not appear after 24 h in the Pc-(NCL-CA4)₂-treated tumors, immune cells continued to appear in the Pc-(L-CA4)₂-treated tumors until 72 h after the illumination (Fig. S17.2). The immune response was also sustained in the Pc-(L-CA4)₂ treated tumors.

4. Conclusion

We provided clear evidence supporting the mechanism of our near IR-activatable prodrug for a combination of PDT and site-specific chemotherapy, using prodrug Pc-(L-CA4)₂ and its pseudo-prodrug Pc-(NCL-CA4)₂. Pc-(L-CA4)₂ overcame the spatiotemporal limits of Pc-(NCL-CA4)₂ due to the CA4 released by the near IR (690 nm laser) illumination. The locally released CA4 with high photo-release yield (62% in the media) produced broad and sustained killing of the cells surviving after initial PDT damage. The histological data also showed the spatiotemporally superior effects of Pc-(L-CA4)₂ + $h\nu$ over Pc-(NCL-CA4)₂ + $h\nu$ in terms of necrosis, hemorrhage, mitotic cells, and immune response. A detailed pharmacokinetic study of released

CA4 is underway to establish physiologically based pharmacokinetic modeling (PBPK) models of the locally released anticancer drug from the light-activatable prodrug, and to determine the significance of the immune response. Due to the straightforward preparation of the prodrug, clean and efficient release of the parent drug using tissue penetrable near-IR light *via* photoclick chemistry, and a combination of PDT and localized drug effects, we envision that this strategy has great potential not only to overcome the limits of PDT in the clinic setting, but also for broader applications using other functional molecules.

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CHECKLIST FOR COMPOUND CHARACTERIZATION

only for new/unknown compounds

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Corresponding Author		
Title		

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