Published in final edited form as:

Nanoscale. 2017 October 05; 9(38): 14347–14356. doi:10.1039/c7nr03129g.

Carrier-free, Self-assembled Pure Drug Nanorods Composed of 10-Hydroxycamptothecin and Chlorin e6 for Combinatorial Chemo-Photodynamic Antitumor Therapy *in vivo*

Yan Wen^{a,b,†}, Wei Zhang^{a,†}, Ningqiang Gong^{a,b}, Yi-Feng Wang^{a,b}, Hong-Bo Guo^a, Weisheng Guo^a, Paul C. Wang^{c,d}, and Xing-Jie Liang^{a,b}

^aLaboratory of Controllable Nanopharmaceuticals, Chinese Academy of Sciences (CAS) Center for Excellence in Nanoscience and CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology of China, Beijing, 100190, P.R. China

^bUniversity of Chinese Academy of Sciences. Beijing 100049, P. R. China

^cLaboratory of Molecular Imaging, Department of Radiology, Howard University, Washington, DC 20060, United States

^dCollege of Science and Engineering, Fu Jen Catholic University, Taipei 24205, Taiwan

Abstract

Carrier-free nanodrugs formulated from supramolecular self-assembly of pure drug molecules have emerged as an innovative and promising strategy for tumor therapy. We report herein a new and simple method to directly assemble a small hydrophobic anticancer drug 10-Hydroxycamptothecin (HCPT) with a photosensitizer Chlorin e6 (Ce6) to form stable, discrete nanorods (NRs), which not only circumvent the extreme hydrophobicity of HCPT but also incorporate two different modalities into one delivery system for combination therapy. Different ratios of HCPT to Ce6 were evaluated to afford the optimal nanoformulation. The as-prepared HCPT/Ce6 NRs were fully characterized, indicating a relatively uniform size of about 360 nm in length and 135 nm in width, surface charge of about -33 mV. Efficient internalization of the NRs by cancer cells was observed by confocal microscope and the generation of singlet oxygen species arising from the NRs under 655 nm laser irradiation was detected by DCFH-DA. As a result, very potent *in vitro* efficacy against several kinds of cancer cell lines was achieved through chemo-photodynamic dual therapy. The *in vivo* tumor suppression effect of HCPT/Ce6 NRs was verified on a subcutaneous xenograft mouse model, achieving almost complete inhibition of the tumor growth, which may benefit from the superiority of nanomedicine and combination therapy. The rationale of this facile and green strategy for carrier-free nanodrug formulation via self-assembly approach might open up new opportunities for the development of combinatorial therapeutics for tumor.

Correspondence to: Wei Zhang; Xing-Jie Liang.

[†]These authors contributed equally to this work.

Electronic Supplementary Information (ESI) available: [DLS, Zeta potential, SOSG fluorescence, cytotoxicity to A549 and MCF-7 cells]. See DOI: 10.1039/x0xx00000x

Graphical abstract

Carrier-free nanodrug HCPT/Ce6 NRs was prepared through a facile and green supramolecular self-assembly approach for chemo-photodynamic combination therapy of tumor *in vivo*.

1. INTRODUCTION

Cancer is a leading cause of death all over the world, which makes it a major public health problem.^{1, 2} Up to now, the conventional therapeutic modalities (such as surgery, chemotherapy, radiotherapy) still dominate cancer treatment. However, the intrinsic limitations of the conventional cancer therapies (for example, poor water solubility of some drugs, lack of targeting ability, severe side effect, drug resistance) are always huge obstacles to satisfactory outcomes of cancer treatment.^{3–7} Recent years, nanomedicine has emerged as an innovative and promising strategy for more effective and safer cancer treatment.^{8–11} Tremendous attention has been paid to develop various kinds of nanoscale drug delivery systems (nDDS), such as liposomes,^{12–15} dendrimers,^{16–18} micelles,^{19–23} inorganic nanomaterials,^{24–30} and so on. Two main approaches have been employed to prepare nanoformulations of anticancer drugs, including directly encapsulating drugs with nanocarriers and conjugating drugs to macromolecular nanovectors. While these methods are effective, there are still some concerns regarding the potential issues, such as complicated manufacture process, low drug loading capacity (generally less than 10%), carrier-induced toxicity and inflammation to kidney and other organs.^{31–33} To address these limitations, carrier-free pure nanodrugs (PNDs) composed entirely of pharmacologically active molecules have attracted a lot of attention.^{32, 34–39} Generally, green and facile supramolecular self-assembly technology is applied to fabricate the nanoformulation of PNDs. Take advantage of this emerging strategy, a few chemotherapeutic agents (e.g., paclitaxel, doxorubicin, SN38, curcumin) have reported to be formulated as PNDs for enhanced anticancer therapy.^{40–45} There is no doubt that the rationale of PNDs has shown great promise to be regarded as next generation of anticancer drugs.

In order to achieve better therapeutic effect, combination therapy strategy is widely employed in the clinic. The multi-pronged assault to tumor through administering a cocktail of different anticancer drugs could exhibit synergetic anticancer efficacy and minimal side effects than respective monotherapeutic.^{46–49} Although PNDs mediated co-delivery of anticancer chemotherapeutic agents has been extensively explored, PNDs-based combination of chemotherapy with other modalities is still a burgeoning research area. Recent years, photodynamic therapy (PDT) is increasingly recognized as an alternative modality to treat various cancers in clinical practice due to its high therapeutic efficacy and

fewer side effects. PDT utilizes a light source to activate tumor localized photosensitizer, generating highly toxic reactive oxygen species (ROS), thus eradicating tumor tissues in an easy controlled and noninvasive manner.^{50, 51}

Based on these considerations, we report herein a simple and green method to fabricate a novel formulation of PNDs composed of a hydrophobic anticancer drug 10-Hydroxycamptothecin (HCPT) and a photosensitizer Chlorin e6 (Ce6) for combinatorial chemo-photodynamic dual therapy. 10-Hydroxycamptothecin (HCPT), an indole alkaloid isolated from the Chinese tree *Camptotheca acuminata*, has a wide spectrum of anticancer activity mainly through the pathway of topoisomerase I inhibition. Unfortunately, practical application of HCPT is hindered due to its poor solubility in water and in physiological acceptable organic solvents.^{52, 53} Previously, we demonstrated that self-assembled HCPT nanostructures can be constructed by reprecipitation method in aqueous solution.⁴² An amphiphilic photosensitizer Ce6 was introduced herein to enhance the stability of the nanostructures and at the same time to endow the system with combinatorial therapeutic effect. Ce6 is a porphyrin-based photosensitizer with strong absorbance around 655 nm, which is in the range of wavelengths with excellent tissue penetration ability for *in vivo* use. The mono-l-aspartyl chlorin e6 (NPe6) has already been approved for cancer treatment in the clinic.⁵⁴

Motivated by this rationale, in the present study HCPT and Ce6 were used as the building blocks to coassemble into discrete nanostructures. The preparation of HCPT/Ce6 NRs and their application for chemo-photodynamic dual antitumor therapy were illustrated in Scheme 1. Different ratios of HCPT to Ce6 were evaluated to afford the optimal nanoformulation. Owing to efficient cellular internalization of the NRs and laser triggered ROS generation, very potent *in vitro* efficacy against several kinds of cancer cell lines was achieved through chemo-photodynamic dual therapy. The *in vivo* tumor suppression effect of HCPT/Ce6 NRs was verified on a subcutaneous xenograft mouse model, achieving almost complete inhibition of the tumor growth, which may benefit from the superiority of nanomedicine and combination therapy.

2. RESULTS AND DISCUSSION

Reprecipitation method has been extensively explored as a green and simple approach for the fabrication of organic nanocrystals. Our previous work showed that pure HCPT structure could be easily obtained through this method, however, the as-prepared HCPT nanostructure exhibit irregular and non-uniform structures, and easily aggregate and precipitate in aqueous solution (Figure S1A&1F). To further improve the stability and confine the structure, Ce6 was introduced to coassemble with HCPT. Ce6 consists of a hydrophobic porphyrin ring and three pendant carboxyl groups. After deprotonated by NaOH, these carboxyl groups will turn into hydrophilic tails. The amphiphilicity of deprotonated Ce6 was confirmed by its formation of self-assembled nanoparticles as observed by TEM (Figure S1B). We hypothesized that hybrid HCPT/Ce6 NRs could be formulated through the porphyrin ring associating with HCPT through hydrophobic and π - π stacking interactions, while carboxyl terminals acting as the hydrophilic shell (Scheme 1). Different molar ratios (1:1, 2:1, 4:1 to 8:1) of HCPT to Ce6 were evaluated to get optimal formulation of the HCPT/Ce6 NRs. At

first, the formation of the nanostructures was monitored by DLS (Figure S2). Then, the morphology of the nanostructures was observed by TEM (As shown in Figure 1A–D), irregular needle-like nanostructures formed with the HCPT to Ce6 ratio of 1:1 and 2:1, while uniform rod-like nanostructures were obtained with the HCPT to Ce6 ratio of 4:1 and 8:1. Previous studies demonstrated that camptothecin derivatives molecules with π -electronic planar structure have a great tendency to pack to each other in one direction to form one-dimensional nanostructures through hydrophobic effect and π - π stacking interactions.^{34, 42, 55} It also has been revealed that the morphologies of the structures assembled from camptothecin or its derivatives are significantly relevant with the content of hydrophilic part in the system. High hydrophilic content always tends to afford filamentous structures, while lower hydrophilic content prefers rod-like or spherical structures.^{45, 56, 57} As a result, uniform nanorods were obtained at higher HCPT to Ce6 ratios through the confinement of the core HPCT nanostructure with an energy minimization process. It has been reported that cylindrical nanostructures exhibit very good pharmacokinetics and efficiency in drug delivery even better than spherical ones because of their multiple endocytotic mechanisms, enhanced internalization rates and effective adhesion to cell surface.^{58, 59} Meanwhile, in consideration of the following combinatorial therapy which also needs enough amount of photosensitizer Ce6, therefore HCPT/Ce6 NRs with the ratio of 4:1 were chosen as the optimal formulation for the subsequent investigations (all the entries of HCPT/Ce6 NRs or NRs in the following text refer to this formulation). The average size of this optimal HCPT/Ce6 NRs is calculated to be about 360 ± 28 nm in length and 135 ± 16 nm in width based on the TEM and SEM images (Figure 1C&E). It is also worth noting that coassembling with Ce6 greatly improved the water dispersity and colloid stability of HCPT (Figure 1F), and the concentration of HCPT of NRs can reach to 728 $\mu\text{g/mL}$ and even higher, which is at least 100 times of the solubility of free HCPT in water (20 $\mu\text{mol/L}$).⁶⁰ And the surface charge of the NRs was determined to be -33 mV (Figure S3), which may facilitate the elongation of blood circulation time for *in vivo* use. Because lots of studies suggested that negatively charged nanoparticles showed lower non-specific protein adsorption and displayed a slow or reduced opsonization by reticular endothelial system (RES).^{31, 61}

The UV spectra of the co-assembled HCPT/Ce6 NRs showed the typical absorbance peaks from both HCPT and Ce6 with somewhat elevated overall baseline (Figure 2A). This means that these two components indeed interacted with each other and successfully assembled into NRs at nanoscale. A noticeably broader and red-shifted Soret band of Ce6 was observed, implying the interaction between the porphyrin core of Ce6 with aromatic ring of HCPT by hydrophobic and π - π interactions.⁶² Moreover, the considerably strong absorbance around 655 nm endows the system with great promise for *in vivo* use, owing to its great tissue penetration ability (Figure 2A). Then, the fluorescence properties of the HCPT/Ce6 NRs were examined, exhibiting obvious green and red emission from HCPT and Ce6, respectively, which could facilitate the intracellular uptake and *in vivo* drug distribution tracking of the NRs (Figure 2B&C). As extensively revealed, the generation of highly cytotoxic ROS, especially singlet oxygen ($^1\text{O}_2$), arising from the photosensitizer under light activation, is the basic principle of PDT. So we evaluated the generation of singlet oxygen using the singlet oxygen sensor green (SOSG) as a probe.⁶³ The fluorescence intensity of

SOSG showed a time-dependent enhancement in the presence of NRs upon laser irradiation (Figure S4). The singlet oxygen generation capacity of HCPT/Ce6 NRs and free Ce6 was compared after plotting the normalized fluorescence intensity against irradiation time. It is worth noting that free Ce6 showed gradually reduced rate of $^1\text{O}_2$ generation, while the NRs maintained a higher and sustaining $^1\text{O}_2$ generation rate. Free Ce6 molecules may aggregate with each other and easily be degraded after laser irradiation, and higher dispersity and stability was achieved via assembling on the surface of the NRs, therefore exhibiting better $^1\text{O}_2$ generation capacity.

To assess the intracellular accumulation of ROS, we use DCFH-DA as a fluorogenic probe. DCFH-DA will be hydrolyzed to DCFH after permeating cell membrane. In the presence of ROS, DCFH is subsequently oxidized to DCF to emit bright green fluorescence. Cells were treated with PBS, free HCPT, free Ce6 or HCPT/Ce6 NRs, with or without 2 min continual 655 nm laser irradiation. Without laser irradiation, all the cells showed negligible fluorescence. After laser irradiation, cells treated with free Ce6 or NRs exhibited significant green fluorescence (Figure 3C), indicating the NRs could be efficiently internalized and exhibit great performance as ROS producer. The cellular uptake of the NRs was further confirmed by direct fluorescence observation through confocal laser scanning microscope owing to the intrinsic fluorescent properties of HCPT and Ce6, as evidenced by bright green and red fluorescence colocalized in cell cytoplasm after incubation with HCPT/Ce6 NRs (Figure 3A).

Subsequently, to further evaluate the *in vitro* anticancer efficiency of the NRs, the cytotoxicity of various formulations with or without laser irradiation was determined against three cancer cells lines (4T1, A549, MCF-7) by CCK-8 assay. As shown in Figure 3B, without laser irradiation the antiproliferation effect of HCPT/Ce6 NRs was similar to free HCPT, while significantly enhanced growth inhibition effect was achieved in the presence of laser irradiation. The considerable dark cytotoxicity of the NRs verified the sufficient cellular uptake of the hybrid nanodrug and subsequent drug release to exert anticancer effect, which is consistent with the CLSM results in Figure 3A. The enhancement of the cytotoxicity of free Ce6 and the NRs in the presence of laser irradiation arose from the generation of ROS, as demonstrated with ROS probe in Figure 3C. Similar cytotoxicity results were obtained against A549 and MCF-7 cells (Figure S5&S6). Taking together, these results indicated that the combination chemo-photodynamic dual therapy of HCPT/Ce6 NRs exhibited superior cytotoxicity to monochemotherapy (HCPT) and mono-PDT (Ce6) as evidenced by its significantly increased growth inhibition values in all the three cancer cell lines.

The excellent *in vitro* performance of HCPT/Ce6 NRs encouraged us to pursue their *in vivo* application of chemo-photodynamic combination therapy for tumor treatment. Firstly, Balb/c nude mice xenografts were established by subcutaneously transplanting 4T1 cells into the nude mice. As introduced above, activation of tumor localized photosensitizer by laser is the principle of PDT. In order to achieve the best therapeutic effect, laser irradiation should be carried out at the time point with highest photosensitizer accumulation at the tumor site. Take advantage of the intrinsic fluorescence of Ce6, the biodistribution of HCPT/Ce6 NRs was evaluated through whole body fluorescence imaging. Tumor bearing

mice were intravenously injected with HCPT/Ce6 NRs, then anesthetized and imaged by a Maestro EX *in vivo* fluorescence imaging system (CRi, Inc.) at predetermined time points. As shown in Figure 4, at the early time points the NRs were well distributed all over the body in mice, and it was found that the NRs tended to enrich at the tumor site over time, which could be attributed to the EPR effect of the nanorods. The maximum accumulation was observed at 4 h post-injection (Figure 4). Therefore, this time point was chosen as the best therapeutic window for 655 nm laser irradiation mediated photodynamic therapy.

Subsequently, to evaluate the *in vivo* therapeutic potential, tumor bearing mice were randomly divided into five groups: PBS, free HCPT, free Ce6 (with laser) and HCPT/Ce6 NRs (with or without laser). Mice were intravenously injected with these different formulations at day 0, day 3 and day 6 during the entire 12-day treatment cycle. After various treatments, tumor volumes and body weights of each group were recorded every day (Figure 5A&B). Compared to the control group, monotherapy (free HCPT and the NRs in the absence of laser irradiation) and monophotodynamic therapy (free Ce6 plus laser irradiation) showed a moderate growth inhibition effect of the tumor growth. Remarkably, HCPT/Ce6 NRs mediated chemo-photodynamic dual therapy (the NRs in the presence of laser irradiation) showed outstanding therapeutic effect, as evidenced by almost complete inhibition of the tumor growth, which was further confirmed by the visual observation of the dissected tumor tissues of each group (Figure 5B&C, see more details in Figure S7). The excellent and synergistic therapeutic outcome of HCPT/Ce6 NRs could be attributed to the superiority of the nanomedicine and combination therapy, including prolonged circulation time, EPR effect for higher drug accumulation and simultaneously shuttling different drugs to tumor site, multi-pronged assault, and so on.^{8,9} To further understand the therapeutic effects, hematoxylin and eosin (H&E) staining assays were introduced to study the morphology of tumor cells after various treatments. The slices of tumor tissues from Figure 5C were compared (Figure 5D). As expected, severe damages were observed in the tumor from the chemo-photodynamic combination therapy group, while much less or no notable damages could be found in other control groups.

The potential *in vivo* toxicity has always been a problem in the development of nanomedicine.⁶⁴ In our study, the body weights of mice in each group were recorded and showed no significant changes in all mice during the whole experimental period (Figure 5A). Furthermore, in the collected capital organs, there were no obvious cell damages and morphology changes for all treatment groups compared to the vehicle control group (Figure 6). Therefore, the as-designed nanodrug HCPT/Ce6 NRs mediated combination therapy was considered to be safe without any activation of inflammation or immune response, which holds great promise to be further developed for clinical use.

3. CONCLUSION

In summary, a kind of carrier-free nanodrug HCPT/Ce6 NRs was formulated with pure drug molecules (HCPT and Ce6) through a simple and green supramolecular self-assembly approach. The asprepared HCPT/Ce6 NRs showed uniform structures with high stability. This hybrid nanodrug not only circumvents the extreme hydrophobicity of HCPT (with the solubility at least 100-fold higher than free HCPT in water), but also integrates two tumor

treatment modalities into one system. Efficient cell uptake of the NRs and subsequent intracellular ROS generation in the presence of laser irradiation were monitored by CLSM. The *in vitro* and *in vivo* antitumor studies revealed that HCPT/Ce6 NRs mediated chemophotodynamic combination therapy exhibited great superior antitumor efficacy than monotherapy or monophotodynamic therapy, achieving complete inhibition of tumor growth. We attribute this enhanced therapeutic effect of HCPT/Ce6 NRs to the superiority of nanomedicine and combination therapy, including higher drug accumulation at tumor site, multi-pronged assault, and so on. In conclusion, our study provided a simple and green solution to construct carrier-free PNDs that combine two treatment modalities, chemotherapy and photodynamic therapy, into one single platform to circumvent the drawbacks of traditional small molecules and to achieve very potent antitumor capacity, which could be easily expanded to other drugs and modalities. The rationale of this facile and green strategy for carrier-free PNDs might open up new opportunities for the development of combinatorial therapeutics for tumor.

4. EXPERIMENTAL SECTION

4.1. Materials

10-Hydroxycamptothecin (HCPT) was purchased from Knowshine (Shanghai, China). Cholrin e6 (Ce6) was obtained from Frontier Scientific. Fetal bovine serum (FBS), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, Dulbecco modified Eagle medium (DMEM) were purchased from WISENT (Beijing, China). Penicillin–streptomycin solution and Singlet Oxygen Sensor Green (SOSG) were purchased from life technologies (USA). CCK-8 was purchased from ZOMANBIO (Beijing, China). 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were obtained from Solarbio (Beijing, China). ACS grade dimethyl sulfoxide (DMSO) was supplied by AMRESCO (Solon, OH, USA). Water was purified using a Milli-Q system (Millipore, Millford, MA, USA). Other chemicals were provided by Beijing Chemical Reagents Institute (Beijing, China). All of the chemicals were used without further purification unless otherwise stated.

4.2. Preparation of HCPT/Ce6 Nanstructures

The HCPT/Ce6 nanostructures were prepared by a reprecipitation method. Stock solution of HCPT (100 mM) and Ce6 (100 mM) in DMSO was first prepared. Five microliter Ce6 solution and 20 μ L HCPT solution was mixed together, and then injected rapidly into 1 mL of water containing 10 μ L NaOH solution (150 mM) under ultrasonication. The sample was further ultrasonicated for 10 min. Other formulations with different molar ratios of HCPT to Ce6 were prepared in the same way with the constant NaOH to Ce6 molar ratio of 3:1.

4.3. Characterization of HCPT/Ce6 Nanorods

The morphology of the as-prepared nanorods was investigated using a transmission electron microscope (TEM, Hitachi HT7700) and a scanning electron microscope (SEM, Hitachi S4800). The size distribution and surface charge of HCPT/Ce6 NRs were measured by dynamic light scattering (DLS, Nano ZS90, Malvern, UK). The fluorescence intensity was detected using a Perkin-Elmer LS55 fluorescence spectrophotometer. The absorption spectra

were measured on a ultra-violet and visible spectrophotometer (UV-Vis, Lambda 950, Perkin-Elmer, USA).

4.4. Cytotoxicity Studies

4T1 cells and A549 cells were incubated in RPMI 1640 medium, MCF-7 cells were incubated in DMEM medium, which were all supplemented with 10% FBS, 100U/mL penicillin-streptomycin. The cells were seeded with a density of 5×10^3 per well into a 96-well plate for 24 h, respectively, then replaced with 100 μ L medium containing various formulations. After 48 h, the medium was replaced with fresh medium containing CCK (0.5 mg/mL, 100 μ L). The cells were further incubated for 2 h at 37 °C. Then the plates were analyzed with a microplate reader (Tecan Infinite M200, USA) with the absorbance set at 450 nm. For PDT treatment, the plate was irradiated with 655 nm laser (150 mW cm⁻², 2 min) after 12 h incubation with various formulations. All the experiments were performed in triplicate.

4.5. Uptake of HCPT/Ce6 NRs Measured by Confocal Microscopy Imaging

4T1 cells were seeded into 8-well glass bottom plate with a density of 2×10^4 per well. After 24 h, the cells were incubated with free HCPT, free Ce6 and NRs (20 μ M HCPT and 5 μ M Ce6) for 6 h. The cells were examined by confocal microscopy (Zeiss LSM 760 fluorescence spectrophotometer, Germany). Ce6 and HCPT were both excited at 405 nm and monitored at 655 ± 30 nm (Red) and 540 ± 30 nm (Green), respectively.

4.6. Detection of Singlet Oxygen

Singlet oxygen sensor green (SOSG), is a commercially available luminescent probe for singlet oxygen detection. To evaluate the singlet oxygen generation, SOSG was dissolved in water containing 2% methanol with the final concentration of 2 μ M, free Ce6, HCPT/Ce6 NRs or free HCPT (80 μ M HCPT and 20 μ M Ce6) was added. After the mixed solutions were irradiated with 655 nm laser (10 mW/cm²) for different time periods, the oxidized SOSG was monitored by measuring the fluorescence intensity (excited at 494 nm).

For cellular ROS generation detection, 4T1 cells were seeded into 8-well glass bottom plate with a density of 2×10^4 per well. After 24 h incubation, the medium was replaced with fresh medium containing free HCPT, free Ce6 or HCPT/Ce6 NRs (20 μ M HCPT and 5 μ M Ce6) and further incubated for 6 h. The cells were washed with serum-free DMEM and incubated with DCFH-DA (10 μ M) in serum-free DMEM for 20 min. After washing twice with PBS, cells were irradiated with 655 nm laser (150 mW cm⁻², 2min). Then the cells were immediately examined under a confocal microscope (LSM 760) with excitation at 488 nm and emission at 530 ± 20 nm.

4.7. *In vivo* Fluorescence Imaging

Female Balb/c mice (6 weeks old) were purchased from Beijing Vital River Company (Beijing, China) and housed under standard conditions with free access to food and water. All animal experiments were performed in accordance with the principles of care and use of laboratory animals. A Tumor-bearing mice model was established by subcutaneous injection 4T1 cells in the right flank of the mice. Two weeks after inoculation, the tumor-bearing mice

were intravenously injected with HCPT/Ce6 NRs solution. At pre-determined time points, the mice were anesthetized and scanned by a Maestro 2 multi-spectral imaging system (Cambridge Research & Instrumentation, USA).

4.8. *In vivo* Therapeutic Efficacy

The tumor-bearing Balb/c mice were randomly divided into 5 groups: (1) PBS as a control, (2) pure HCPT, (3) HCPT/Ce6 NRs without irradiation, (4) free Ce6 with irradiation, (5) HCPT/Ce6 NRs with irradiation. Therapy was continued through tail vein injection for 12 days. Mice were administrated (at day 0, day 3, day 6) with the corresponding drug formulations at the dose of HCPT 4.8 mg/kg and Ce6 1.2 mg/kg. For PDT treatment, the mice were locally irradiated with 655 nm laser (150 mW cm^{-2} , 15 min) 4 h after injection. The body weight and the tumor size of each mouse were recorded to evaluate the therapy efficiency and toxicity: the tumor volume (V) was calculated: $V = (\text{the major axes of the tumor} \times \text{the minor axes of the tumor}^2)/2$. Finally, normal organs of the mice, including heart, livers, spleen, lung, kidney, and tumors were harvested and collected immediately for H&E histology analysis. All of the animal experiments were conducted under approved protocols of the Institutional Animal Care and Use Committee at the Institute of Tumor in the Chinese Academy of Medical Science.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Natural Science Foundation key project (31630027 and 31430031), National Distinguished Young Scholars grant (31225009). P. W. was supported by a NIH/NIMHHD grant G12MD007597. The authors also appreciate the support by the “Strategic Priority Research Program” of the Chinese Academy of Sciences, Grant No. XDA09030301 and support by the external cooperation program of BIC, Chinese Academy of Science, Grant No. 121D11KYSB20130006.

References

1. Siegel RL, Miller KD, Jemal A. CA Cancer J Clin. 2016; 66:7–30. [PubMed: 26742998]
2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Int J Cancer. 2015; 136:E359–386. [PubMed: 25220842]
3. Ries F, Klastersky J. Am J Kidney Dis. 1986; 8:368–379. [PubMed: 3538860]
4. Gottesman MM, Fojo T, Bates SE. Nat Rev Cancer. 2002; 2:48–58. [PubMed: 11902585]
5. Green MR, Manikhas GM, Orlov S, Afanasyev B, Makhson AM, Bhar P, Hawkins MJ. Ann Oncol. 2006; 17:1263–1268. [PubMed: 16740598]
6. Wei T, Chen C, Liu J, Liu C, Posocco P, Liu X, Cheng Q, Huo S, Liang Z, Fermeglia M, Priol S, Liang XJ, Rocchi P, Peng L. Proc Nat Acad Sci. 2015; 112:2978–2983. [PubMed: 25713374]
7. Zhang W, Shen J, Su H, Mu G, Sun JH, Tan CP, Liang XJ, Ji LN, Mao ZW. ACS Appl Mater Interfaces. 2016; 8:13332–13340. [PubMed: 27164222]
8. Shi J, Kantoff PW, Wooster R, Farokhzad OC. Nat Rev Cancer. 2017; 17:20–37. [PubMed: 27834398]
9. Blanco E, Shen H, Ferrari M. Nat Biotechnol. 2015; 33:941–951. [PubMed: 26348965]
10. Ma X, Zhao Y, Liang XJ. Acc Chem Res. 2011; 44:1114–1122. [PubMed: 21732606]
11. Pelaz B, Alexiou C, Alvarez-Puebla RA, Alves F, Andrews AM, Ashraf S, Balogh LP, Ballerini L, Bestetti A, Brendel C, Bosi S, Carril M, Chan WCW, Chen C, Chen X, Cheng Z, Cui D, Du J,

Dullin C, Escudero A, Feliu N, Gao M, George M, Gogotsi Y, Grünweller A, Gu Z, Halas NJ, Hampf N, Hartmann RK, Hersam MC, Hunziker P, Jian J, Jiang X, Jungebluth P, Kadhireshan P, Kataoka K, Khademhosseini A, Kopeček J, Kotov NA, Krug HF, Lee DS, Lehr C-M, Leong KW, Liang X-J, Ling Lim M, Liz-Marzán LM, Ma X, Macchiarini P, Meng H, Möhwald H, Mulvaney P, Nel AE, Nie S, Nordlander P, Okano T, Oliveira J, Park TH, Penner RM, Prato M, Punties V, Rotello VM, Samarakoon A, Schaak RE, Shen Y, Sjöqvist S, Skirtach AG, Soliman MG, Stevens MM, Sung H-W, Tang BZ, Tietze R, Udugama BN, VanEpps JS, Weil T, Weiss PS, Willner I, Wu Y, Yang L, Yue Z, Zhang Q, Zhang Q, Zhang X-E, Zhao Y, Zhou X, Parak WJ. *ACS Nano*. 2017; 11:2313–2381. [PubMed: 28290206]

12. O'Brien MER, Wigler N, Inbar M, Rosso R, Grischke E, Santoro A, Catane R, Kieback DG, Tomczak P, Ackland SP, Orlandi F, Mellars L, Alland L, Tendler C. *Ann Oncol*. 2004; 15:440–449. [PubMed: 14998846]
13. Stathopoulos GP, Antoniou D, Dimitroulis J, Michalopoulou P, Bastas A, Marosis K, Stathopoulos J, Provata A, Yiamboudakis P, Veldekis D, Lolis N, Georgatou N, Toubis M, Pappas C, Tsoukalas G. *Ann Oncol*. 2010; 21:2227–2232. [PubMed: 20439345]
14. Ko AH, Tempero MA, Shan YS, Su WC, Lin YL, Dito E, Ong A, Wang YW, Yeh CG, Chen LT. *Br J Cancer*. 2013; 109:920–925. [PubMed: 23880820]
15. Bhunia S, Radha V, Chaudhuri A. *Nanoscale*. 2017; 9:1201–1212. [PubMed: 28045167]
16. Hu W, Qiu L, Cheng L, Hu Q, Liu Y, Hu Z, Chen D, Cheng L. *Acta Biomater*. 2016; 36:241–253. [PubMed: 26995505]
17. Li HJ, Du JZ, Du XJ, Xu CF, Sun CY, Wang HX, Cao ZT, Yang XZ, Zhu YH, Nie S, Wang J. *Proc Nat Acad Sci*. 2016; 113:4164–4169. [PubMed: 27035960]
18. Kesharwani P, Jain K, Jain NK. *Prog Polym Sci*. 2014; 39:268–307.
19. Kataoka K, Harada A, Nagasaki Y. *Adv Drug Deliv Rev*. 2001; 47:113–131. [PubMed: 11251249]
20. Kamaly N, Yameen B, Wu J, Farokhzad OC. *Chem Rev*. 2016; 116:2602–2663. [PubMed: 26854975]
21. Dai J, Lin S, Cheng D, Zou S, Shuai X. *Angew Chem Int Ed*. 2011; 50:9404–9408.
22. Cai K, He X, Song Z, Yin Q, Zhang Y, Uckun FM, Jiang C, Cheng J. *J Am Chem Soc*. 2015; 137:3458–3461. [PubMed: 25741752]
23. Zhang F, Zhang S, Pollack SF, Li R, Gonzalez AM, Fan J, Zou J, Leininger SE, Pavía-Sanders A, Johnson R, Nelson LD, Raymond JE, Elsbahy M, Hughes DMP, Lenox MW, Gustafson TP, Wooley KL. *J Am Chem Soc*. 2015; 137:2056–2066. [PubMed: 25629952]
24. Yang X, Yang M, Pang B, Vara M, Xia Y. *Chem Rev*. 2015; 115:10410–10488. [PubMed: 26293344]
25. Li Z, Barnes JC, Bosoy A, Stoddart JF, Zink JJ. *Chem Soc Rev*. 2012; 41:2590–2605. [PubMed: 22216418]
26. Yu L, Chen Y, Wu M, Cai X, Yao H, Zhang L, Chen H, Shi J. *J Am Chem Soc*. 2016; 138:9881–9894. [PubMed: 27441571]
27. Kumar A, Huo S, Zhang X, Liu J, Tan A, Li S, Jin S, Xue X, Zhao Y, Ji T, Han L, Liu H, Zhang X, Zhang J, Zou G, Wang T, Tang S, Liang XJ. *ACS Nano*. 2014; 8:4205–4220. [PubMed: 24730557]
28. Liu T, Wang C, Gu X, Gong H, Cheng L, Shi X, Feng L, Sun B, Liu Z. *Adv Mater*. 2014; 26:3433–3440. [PubMed: 24677423]
29. Min Y, Li J, Liu F, Yeow EKL, Xing B. *Angew Chem Int Ed*. 2014; 53:1012–1016.
30. Li JM, Wang YY, Zhao MX, Tan CP, Li YQ, Le XY, Ji LN, Mao ZW. *Biomaterials*. 2012; 33:2780–2790. [PubMed: 22243797]
31. Zhu M, Nie G, Meng H, Xia T, Nel A, Zhao Y. *Acc Chem Res*. 2013; 46:622–631. [PubMed: 22891796]
32. Huang P, Wang D, Su Y, Huang W, Zhou Y, Cui D, Zhu X, Yan D. *J Am Chem Soc*. 2014; 136:11748–11756. [PubMed: 25078892]
33. Min Y, Caster JM, Eblan MJ, Wang AZ. *Chem Rev*. 2015; 115:11147–11190. [PubMed: 26088284]
34. Kasai H, Murakami T, Ikuta Y, Koseki Y, Baba K, Oikawa H, Nakanishi H, Okada M, Shoji M, Ueda M, Imahori H, Hashida M. *Angew Chem Int Ed*. 2012; 51:10315–10318.

35. Li W, Yang Y, Wang C, Liu Z, Zhang X, An F, Diao X, Hao X, Zhang X. *Chem Commun.* 2012; 48:8120–8122.
36. Ma W, Cheetham AG, Cui H. *Nano Today.* 2016; 11:13–30. [PubMed: 27066106]
37. Qin SY, Zhang AQ, Cheng SX, Rong L, Zhang XZ. *Biomaterials.* 2017; 112:234–247. [PubMed: 27768976]
38. Zhang J, Li Y, An FF, Zhang X, Chen X, Lee CS. *Nano Lett.* 2015; 15:313–318. [PubMed: 25514014]
39. Zhang J, Liang YC, Lin X, Zhu X, Yan L, Li S, Yang X, Zhu G, Rogach AL, Yu PKN, Shi P, Tu LC, Chang CC, Zhang X, Chen X, Zhang W, Lee CS. *ACS Nano.* 2015; 9:9741–9756. [PubMed: 26390118]
40. Zhang J, Li S, An FF, Liu J, Jin S, Zhang JC, Wang PC, Zhang X, Lee CS, Liang XJ. *Nanoscale.* 2015; 7:13503–13510. [PubMed: 26199064]
41. Zhou M, Zhang X, Yang Y, Liu Z, Tian B, Jie J, Zhang X. *Biomaterials.* 2013; 34:8960–8967. [PubMed: 23958027]
42. Chen F, Zhao Y, Pan Y, Xue X, Zhang X, Kumar A, Liang XJ. *Mol Pharm.* 2015; 12:2237–2244. [PubMed: 25996761]
43. Yu C, Zhou M, Zhang X, Wei W, Chen X, Zhang X. *Nanoscale.* 2015; 7:5683–5690. [PubMed: 25740312]
44. Hu M, Huang P, Wang Y, Su Y, Zhou L, Zhu X, Yan D. *Bioconjugate Chem.* 2015; 26:2497–2506.
45. Zhang C, Jin S, Xue X, Zhang T, Jiang Y, Wang PC, Liang XJ. *J Mater Chem B.* 2016; 4:3286–3291.
46. Jia J, Zhu F, Ma X, Cao ZW, Li YX, Chen YZ. *Nat Rev Drug Discov.* 2009; 8:111–128. [PubMed: 19180105]
47. Kolishetti N, Dhar S, Valencia PM, Lin LQ, Karnik R, Lippard SJ, Langer R, Farokhzad OC. *Proc Nat Acad Sci.* 2010; 107:17939–17944. [PubMed: 20921363]
48. Kemp JA, Shim MS, Heo CY, Kwon YJ. *Adv Drug Deliv Rev.* 2016; 98:3–18. [PubMed: 26546465]
49. Liao L, Liu J, Dreaden EC, Morton SW, Shopsowitz KE, Hammond PT, Johnson JA. *J Am Chem Soc.* 2014; 136:5896–5899. [PubMed: 24724706]
50. 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. *CA Cancer J Clin.* 2011; 61:250–281. [PubMed: 21617154]
51. Dolmans DEJGJ, Fukumura D, Jain RK. *Nat Rev Cancer.* 2003; 3:380–387. [PubMed: 12724736]
52. Wang J, Wang R, Li LB. *J Colloid Interface Sci.* 2009; 336:808–813. [PubMed: 19520376]
53. Yang J, Ni B, Liu J, Zhu L, Zhou W. *Spine J.* 2011; 11:218–223. [PubMed: 21377604]
54. Usuda J, Tsutsui H, Honda H, Ichinose S, Ishizumi T, Hirata T, Inoue T, Ohtani K, Maehara S, Imai K, Tsunoda Y, Kubota M, Ikeda N, Furukawa K, Okunaka T, Kato H. *Lung Cancer.* 2007; 58:317–323. [PubMed: 17698240]
55. He D, Zhang W, Deng H, Huo S, Wang YF, Gong N, Deng L, Liang XJ, Dong A. *Chem Commun.* 2016; 52:14145–14148.
56. Cheetham AG, Zhang P, Lin Y-a, Lock LL, Cui H. *J Am Chem Soc.* 2013; 135:2907–2910. [PubMed: 23379791]
57. Lin YA, Cheetham AG, Zhang P, Ou YC, Li Y, Liu G, Hermida-Merino D, Hamley IW, Cui H. *ACS Nano.* 2014; 8:12690–12700. [PubMed: 25415538]
58. Chauhan VP, Popovid Z, Chen O, Cui J, Fukumura D, Bawendi MG, Jain RK. *Angew Chem Int Ed.* 2011; 50:11417–11420.
59. Gratton SEA, Ropp PA, Pohlhaus PD, Luft JC, Madden VJ, Napier ME, DeSimone JM. *Proc Nat Acad Sci.* 2008; 105:11613–11618. [PubMed: 18697944]
60. Morgan MT, Nakanishi Y, Kroll DJ, Griset AP, Carnahan MA, Wathier M, Oberlies NH, Manikumar G, Wani MC, Grinstaff MW. *Cancer Res.* 2006; 66:11913. [PubMed: 17178889]
61. Xiao K, Li Y, Luo J, Lee JS, Xiao W, Gonik AM, Agarwal RG, Lam KS. *Biomaterials.* 2011; 32:3435–3446. [PubMed: 21295849]

62. Liu K, Xing R, Zou Q, Ma G, Möhwald H, Yan X. *Angew Chem Int Ed*. 2016; 55:3036–3039.
63. Huang P, Lin J, Wang X, Wang Z, Zhang C, He M, Wang K, Chen F, Li Z, Shen G, Cui D, Chen X. *Adv Mater*. 2012; 24:5104–5110. [PubMed: 22718562]
64. Wicki A, Witzigmann D, Balasubramanian V, Huwyler J. *J Control Release*. 2015; 200:138–157. [PubMed: 25545217]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.
 (A–D) TEM images of HCPT/Ce6 hybrid nanostructures with different ratios (HCPT:Ce6=1:1, 2:1, 4:1, 8:1), (E) SEM image of the as-prepared HCPT/Ce6 NRs (HCPT:Ce6=4:1), (F) The representative digital photos of pure HCPT (left) and HCPT/Ce6 NRs (right) aqueous solution after 24 h storage.

Figure 2.

(A) The UV-vis spectra and (B) the fluorescence spectra of HCPT, Ce6, HCPT/Ce6 NRs. (C) Fluorescence image of HCPT, Ce6 and HCPT/Ce6 NRs aqueous solutions. (D) Plots of the changes of fluorescence intensity at the characteristic peak of SOSG (530 nm) against laser irradiation time. The SOSG was dissolved in water containing 2% methanol with the final concentration of 2 μ M.

Figure 3.

(A) Confocal laser scanning microscope (CLSM) images of 4T1 cells after incubation with free HCPT (20 μM), free Ce6 (5 μM) or HCPT/Ce6 NRs (20 μM HCPT and 5 μM Ce6) for 6h. Green and red channels are corresponding to HCPT and Ce6, respectively. Scale bar, 20 μm . (B) Viability of 4T1 cells after treating with various formulations with (+Laser) or without laser irradiation ($\lambda=655$ nm, 150 mW/cm², 2 min). (C) Intracellular ROS levels evaluation by DCFH-DA. CLSM images of 4T1 cells after incubation with different formulations (HCPT 20 μM , Ce6 5 μM) for 6h, then added DCFH-DA (5 μM) for 20 min, and followed with (+Laser) or without laser irradiation ($\lambda=655$ nm, 150 mW/cm², 2 min). BF, bright field. Scale bar, 20 μm .

Figure 4. Whole body fluorescence images of 4T1 tumor-bearing mice at different time points after treated with HCPT/Ce6 NRs via intravenous injection.

Figure 5.

(A) Body weight changes of tumor-bearing mice during one treatment cycle. (B) Suppression of tumor growth after various treatments: intravenous injection of different formulations (dose of HCPT: 4.8 mg/kg and/or dose of Ce6: 1.2 mg/kg) with or without laser irradiation. Arrows indicate the injection and laser irradiation treatment. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance is assessed by t-test: *, $p < 0.05$, Ce6 + L or HCPT versus NRs + L; ***, $P < 0.001$, PBS versus NRs + L. (C) Digital photos of the excised 4T1 tumor tissues after various treatment for 12 days. (D) H&E histology of tumor tissues from different groups of mice after various treatments. The bar is 100 μ m.

Figure 6. Hematoxylin and eosin (H&E) staining images of capital organs from different groups of mice after treating with various formulations. The bar is 200 μm.

Scheme 1.

Schematic representation of the preparation of HCPT/Ce6 nanorods through self-assembling and the application for chemo-photodynamic combinatorial antitumor therapy.