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# Prodrug-embedded angiogenic vessel-targeting nanoparticle: A positive feedback amplifier in hypoxia-induced chemo-photo therapy

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ABSTRACT

Photodynamic therapy (PDT) induced hypoxia can significantly upregulate the expression of vascular endothelial growth factor (VEGF) at the tumor-stromal interface, resulting in a promoted angiogenesis. Thus, an angiogenesis vessel-targeting nanoparticle (AVT-NP) consisting of photosensitizer, angiogenic vessel-targeting peptide, and bioreductive prodrug is developed for a chemo-photo synergistic cancer therapy, with which anti-cancer effect is achieved first by PDT and immediately followed with hypoxia-activated cytotoxic free radicals. With targeting capability, the AVT-NPs can effectively accumulate at the tumor site due to the promoted angiogenesis in response to PDT-induced hypoxia. The more nanoparticles delivered to the tumor tissue, the higher efficacy of PDT can be achieved, resulting in a more severe hypoxia and increased angiogenesis. Therefore, the prodrug embedded AVT-NP functions as a positive feedback amplifier in the combinational chemo-photo treatment and indeed achieves an enhanced anti-tumor effect in both *in vitro* and *in vivo* studies.



## 1. Introduction

Photodynamic therapy is an emerging and noninvasive technique that has been employed for the treatment of various types of cancers [1]. However, several obstacles still exist and greatly reduce the efficacy of PDT, impairing its overall performance for the clinical translation. One important factor responsible for the poor responses in PDT is tumor-associated hypoxia, which has been recognized as a hallmark of solid tumors and plays key roles in the resistance to conventional anti-cancer therapies [2–4]. Specifically, PDT is an oxygen-dependent process and the photosensitizer-mediated consumption of $O_2$ would further exaggerate tumor hypoxia, which severely reduces its therapeutic efficacy [5–8].

At the meantime, the increased interstitial fluid pressure and the dense tumor stroma also significantly impede the transportation of photosensitizer-containing nanocarriers and reduce the overall performance of PDT treatments [9,10]. Therefore, rational design of effective drug delivery system (DDS) that takes this tumor-associated microenvironment into account is highly desirable for a better anti-cancer therapy. Indeed, various strategies have been developed to address above challenges. For instance, researchers have unveiled that the therapeutic effect of PDT could be remarkably improved by relieving tumor hypoxic microenvironment via the co-delivery of $O_2$ evolving materials during the PDT treatment [11–17]. However, these methods only exhibit marginal benefits since they could not efficiently change the tumor's entire hypoxia. To adapt to the hypoxic environment, less oxygen-dependent PDT has also been developed but its efficacy, thus far, is relatively low [18,19].

Contrary to being suffered from hypoxia, recent advances of cancer treatment utilize the hypoxic microenvironment for tumor-selective therapies, including prodrug activation by hypoxia [20,21], hypoxia-specific gene therapy [22], HIF-1α-specific therapy [23], hypoxia-sensitive groups [24], “active targeting” by signaling pathways, etc. [25,26]When combining with PDT, Shi et al. once loaded photosensitizer-modified upconversion nanoparticles with bioreductive prodrug (Tirapazamine, TPZ) for a combination anti-tumor treatment [27]. More recently, Liu's group [28] and Gu's group [29] also independently reported a nanoparticle-based combinational DDS that could utilize PDT-induced tumor hypoxia

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to activate the delivered chemotherapeutic agents, which resulted in greatly improved anti-cancer effect compared to conventional cancer PDT. Meanwhile, to overcome the increased interstitial fluid pressure and the dense tumor stroma, penetrating peptide was introduced to the combinational chemo-photo DDS to enhance the penetration of the nanoparticles [30].

In general, PDT achieves effective treatment by inducing the generation of intracellular reactive oxygen species (ROS) upon the irradiation with light, consequences of which include not only the exaggerated hypoxia, but also a series of cellular modulations when tumor and tumor-stromal interface encounter with such harsh stimulation. Unfortunately, previous investigations majorly focused on the hypoxia part and not much attention had been paid to utilize the cellular responses for the development of more effective chemo-photo DDS. It is well-known that the organism normally upregulates the expression of VEGF once it is exposed to a stimulated hypoxic condition. As a key mediator of angiogenesis, the upregulated VEGF at the tumor-stromal interface could promote the blood supply and reduce the hypoxic pressure of the tissue [31,32]. Based on the promoted angiogenesis accompanying with the PDT, herein we speculated that the PDT-induced angiogenic vessels may be employed as an ideal target for the delivery of hypoxia-activated prodrugs.

Therefore, a novel PDT-responsive DDS with capabilities of tumor homing, singlet oxygen ($^1O_2$) generation, and bioreductive drug releasing could be rationally designed for the chemo-photo combinational treatment (Fig. 1A). With angiogenic vessel targeting capability, one can imagine that the PDT treatment will enhance the nanodrug accumulation at the tumor-stromal interface due to the promoted angiogenesis in response to the PDT-induced hypoxia. The more nanoparticles delivered to the tumor tissue, the higher efficacy of PDT can be achieved, resulting in a more severe hypoxia and increased angiogenesis. In addition, hypoxia-activated prodrug TPZ would produce highly reactive TPZ radical under the hypoxia environment, which can cause the damage of nearby macromolecules [33,34]. Thus, TPZ should be an ideal bioreductive chemotherapeutic for further enhancing the antitumor efficacy. Overall, the new DDS would function as a positive feedback amplifier in the combinational chemo-photo treatment, which may dramatically enhance the anti-tumor efficacy.

## 2. Materials and methods

### 2.1. Materials

All chemicals were of analytical grade and used without further purification if not indicated otherwise. *N*,*N*-diisopropylethylamine (DIEA), 5-(4-Carboxyphenyl)-10, 15, 20-tris(3-Hydroxyphenyl) chlorin (TPC), anhydrous dimethyl sulfoxide (DMSO), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), cisplatin, 1,3-diphenylisobenzofuran (DPBF), and TPZ were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). GX1 cyclopeptides were purchased from GL Biochem Co. Ltd (Shanghai, China). Alexa fluor® 488 annexin V/dead cell apoptosis assay kit was purchased from Invitrogen and used as received. 2′, 7′-Dichlorofluorescin diacetate (DCFH-DA) was purchased from Beyotime Ltd. ROS-ID® hypoxia/oxidative stress detection kit was purchased from Enzo Life Sciences Inc. CD31 and HIF-α antibodies were purchased from Abcam Inc. All secondary antibodies were obtained from Earthox LLC. (Earthox, USA).

### 2.2. Synthesis of TPC-GX1

The TPC-GX1 was prepared in a Schlenk flask under nitrogen atmosphere. Typically, hydrophobic photosensitizer TPC (65.9 mg, 0.10 mmol), EDC (21.1 mg, 0.11 mmol), NHS (12.7 mg, 0.11 mmol) under the $N_2$ atmosphere at 0 °C 8 h. Then, GX1 (97.7 mg, 0.11 mmol) was added into flask for another 24 h at room temperature. This was then dialyzed against DMSO for several days using a 1000 MWCO dialysis membrane, before being freeze dried for subsequent studies. Purple solid. Yield: 0.078 g (48%). HRMS (ESI) *m*/*z*: $[M + H]^+$ calcd for C78H82N16O15S2, 1547.56; found, 1547.7762. The same synthesis process was used to prepare TPC-PEG for targeted control.

### 2.3. AVT-NPs characterization

Critical micelle concentration (CMC) determination was chose the hydrophobic probe 1, 6-diphenyl-1, 3, 5-hexatriene (DPH). A DPH stock solution of 10 mM in THF was prepared. A designed volume of DPH solution was added to aqueous surfactant solutions of different concentrations. TPC-GX1 was first dissolved in PBS to designated concentration. Then 10 mM THF solution of DPH was added to a final concentration of $2 \times 10^{-4}$ mg/mL ($6 \times 10^{-7}$ M). The fluorescence of the solution was then measured by fluorescence spectrometer ($\lambda_{ex}$: 355 nm, $\lambda_{em}$: 428 nm). The CMC was determined by extending the linear fluorescence intensity of both the high and low concentration region.

The TPZ content in AVT-NPs was determined by the absorbance at 500 nm measured using a Shimadzu UV/Vis spectrophotometer (UV-3600). The mean particle diameter, morphology and size distribution of the micelles and TPZ-incorporated micelles were measured by transmission electronic microscopy (TEM, Tecnai G2spirit Biotwin) and dynamic light scattering (DLS, Malvern Nano-ZS). Drug loading content (DLC) was determined by subtracting dialyzed part from the initial addition of TPZ and calculated according to the following formula:

$$\text{DLC (wt\%)} = [\text{weight of loaded drug/weight of drug} - \text{incorporated micelle}] \times 100\% \quad (1)$$

*In vitro* drug release was determined as follows. The AVT-NPs were suspended in PBS (pH 7.4) or 10 mM GSH in microcentrifuge tubes, which were kept in a shaking incubator at 37 °C. At pre-set time points, aliquots were taken out and centrifuged at 10,000 rpm for 10 min. The supernatant was collected, and the TPZ concentration was measured by UV absorbance ($\lambda = 500$ nm) in the microplate reader.

### 2.4. $O_2$ depletion and $^1O_2$ detection test

50 mg of TPC-GX1 was dispersed in 25 mL of PBS. Then, 10 mL of liquid paraffin was added to isolate PBS and air. The solution was illuminated under a laser light (650 nm He-Ne laser, 1.2 W/cm$^2$). A DOG-3082 oxygen dissolving meter was used to measure the $O_2$ depletion in real-time.

DPBF was used as an indicator to investigate the generation of $^1O_2$, which could react irreversibly with $^1O_2$ to cause a decrease in the intensity of the DPBF absorption band at about 410 nm. DPBF in acetonitrile (20 μL, 8 mM) was added to a solution of TPC-GX1 (5 μg/mL) in 3 mL DMSO. Then, the solution was irradiated with 650 nm laser. The absorption spectra of the mixture were obtained on a UV–Vis spectrophotometer. For TPZ or PBS group, similar experiment was performed with the same weight as TPC-GX1. Experiments were performed in triplicate with results present as mean ± standard deviation.