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Organic Photodynamic Nanoinhibitor for Synergistic Cancer Therapy

Yuyan Jiang, Jingchao Li, Ziling Zeng, Chen Xie, Yan Lyu, and Kanyi Pu*

Abstract: Despite its great potential in cancer treatment, photodynamic therapy (PDT) often exacerbates hypoxia and subsequently compromises its therapeutic efficacy. To overcome this issue, an organic photodynamic nanoinhibitor (OPNi) is synthesized to have additional ability to counteract carbonic anhydrase IX (CA-IX), a molecular target in hypoxia-mediated signalling cascade. OPNi is composed of a metabolizable semiconducting polymer as the photosensitizer and a CA-IX antagonist-conjugated amphiphilic polymer as the matrix. Such a molecular structure allows OPNi not only to selectively bind CA-IX positive cancer cells to facilitate its tumor accumulation but also to regulate CA-IX-related pathway. The integration of CA-IX inhibition into targeted PDT process eventually has a synergistic effect, leading to the superior anti-tumor efficacy over sole PDT as well as the reduced probability of hypoxia-induced cancer metastasis. This study thus proposes a molecular strategy to devise simple yet amplified photosensitizers to conquer the pitfalls of traditional PDT.

Photodynamic therapy (PDT) that utilizes photosensitizers to transform oxygen (O_2) into cytotoxic reactive oxygen species (ROS) to trigger cellular apoptosis holds great promise for cancer therapy.^[1] Unlike other electromagnetic radiation therapy such as radiotherapy, PDT offers merits of non-invasiveness and minimal adverse effects.^[2] The crucial role of photosensitizer in PDT has stimulated the exploration of various optical agents for such application, which include porphyrin derivatives,^[3] small-molecule dyes,^[4] metallic oxides,^[5] quantum dots,^[6] upconversion nanoparticles,^[7] and scintillation nanomaterials.^[8] However, PDT efficacy is not only related to the ROS generation ability of photosensitizers, but also intrinsically limited by its reliance on O_2 . The consumption of O_2 in tumor microenvironment can exacerbate tumor hypoxia, trigger the signalling cascade mediated by hypoxia inducible factor (HIF) and subsequently activate a series of target genes (such as glucose transporters, and vascular endothelial growth factor) responsible for cancer cell survival, tumorigenesis, and distant metastases.^[9] Thereby, it is highly desired to bypass the side effect of PDT-exacerbated hypoxia so as to improve its therapeutic efficacy.

Although several approaches have been reported to conquer tumor hypoxia, each has its own pros and cons.^[10] For instance, direct O_2 supplement using MnO_2 or perfluorocarbon (PFC)

nanodroplets has been exploited to focally elevate oxygen level at tumor sites,^[7] which has poor temporospatial regulation of O_2 release. Moreover, effort to shift PDT from oxygen-dependent type II to H_2O -dependent type I pathway has been devoted, which often requires the use of inorganic photosensitizers containing heavy metal ions with high toxic potential.^[8] Another approach is to use bioreductive prodrugs with specific activation under hypoxic conditions,^[11] whose anti-hypoxia activity is largely constrained by the distribution of corresponding reductases. More recently, utilization of small molecule inhibitors against molecular targets in HIF-mediated pathway is emerging as a promising approach to mitigate the probability of hypoxia-induced metastasis, which however has been less exploited in PDT.

Figure 1. Schematic illustration of (a) nanoprecipitation of OPNi and (b) mechanism of OPNi mediated synergistic phototherapy. p300/CBP: p300 and cyclic adenosine monophosphate response element binding protein; HRE: hypoxia responsive element; NBC: sodium/bicarbonate cotransporter; MCT, monocarboxylate transporter.

Semiconducting polymer nanoparticles (SPNs) comprising π -conjugated macromolecules have been validated as a versatile class of biophotonic nanoagents.^[12] By virtue of their high optical activity, chemical flexibility, and bio-inert nature, SPNs have been applied as phototheranostic agents for superficial/deep-tissue cancer treatment,^[13] as activatable probes for molecular imaging,^[14] and as light-sensitive transducers for bio-regulation.^[15] Particularly, SPNs has been designed to improve PDT by doping with nanoceria to afford pH-switchable self-regulating nano-photosensitizer with reduced damage to normal tissues^[16] or coating with MnO_2 nanosheet to improve the hypoxia condition in tumor.^[17] Additionally, we have demonstrated the

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utilization of SPNs as hypoxia-activatable nano-prodrug and as photodynamic pro-nanoenzyme for complete-remission cancer therapy.^[18] Nevertheless, synergistic cooperation of SPNs with small-molecule enzyme inhibitors to improve PDT efficacy has not been explored.

We herein report the first organic semiconducting photodynamic nanoinhibitor (OPNi) to overcome the hypoxia limitation of conventional PDT for synergistic cancer therapy (Figure 1a). Such a nanoinhibitor consists of a custom-designed NIR absorbing SP (PCVB) as the biodegradable photosensitizer and an acetazolamide (CAi)-terminated amphiphilic polymer (CAi-*b*-PEG-*b*-PPG-*b*-PEG-CAi) as the inhibitor to counteract carbonic anhydrase IX (CA-IX), a crucial downstream gene product in HIF-mediated signalling cascade. CA-IX is over-expressed on cell membranes of many aggressive breast cancer cells and responsible for the reversible hydration of carbon dioxide (CO₂) into proton (H⁺) and bicarbonate (HCO₃⁻), regulating both intra- (pHi) and extracellular (pHe) pH balance to promote cancer cell survival and invasive migration (Figure 1b).^[19] With CAIs on the surface, OPNi not only can specifically bind the cancer cells to exert targeted PDT, but also can inhibit the catalytic function of CA-IX and consequently dysregulate both pHe and pHi to suppress cancer metastasis. Furthermore, the biodegradability of OPNi enable its efficient metabolism and clearance in living animals.

Figure 2. (a) Synthetic routes of PCVB and CAi-PEG-*b*-PPG-*b*-PEG-CAi. (i) Pd₂(dba)₃, tri(*o*-tolyl)phosphine, toluene, 24 h, 100 °C; (ii) HCl, ethanol, 16 h, 95 °C; (iii) 4-(chlorosulfonyl)benzoic acid, triethylamine, dimethylformamide (DMF), r.t., 12 h; (iv) propargyl bromide, K₂CO₃, DMF, r.t., 5 h; (v) sodium hydride, *p*-toluenesulfonyl chloride, r.t., DMF, 24 h; (vi) sodium azide, r.t., DMF, 24 h; (vii) alkyne-CAi, CuSO₄·5H₂O, sodium ascorbate, r.t., DMF, 24 h. (b) DLS profiles of OPNi and OPNi after binding to CA-IX. Inset: TEM image of OPNi. (c) Normalized absorption (blue) and fluorescence emission (red) spectra of OPNi in 1 × PBS buffer. (d) Agarose gel electrophoresis of OPNi before (i) and after (ii) incubation with CA-IX. Error bars indicated standard deviations of triplicate independent experiments.

The NIR-absorbing polymer photosensitizer (PCVB) was synthesized via palladium catalysed Stille polycondensation of 2,6-dibromo-4,4-diethylhexyl-4H-cyclopenta[2,1-*b*;3,4-

b']dithiophene, 4,7-dibromo-2,1,3-benzothiadiazole, and trans-1,2-bis(tributylstannyl)ethene (Figure 2a). The vinylene linker of PVB was biodegradable because of its susceptibility to oxidation-induced cleavage.^[20] To ensure sufficient recognition and inhibition, CAIs were conjugated to the hydrophilic terminals of the amphiphilic polymer. Firstly, CAi was hydrolyzed and linked to 4-(chlorosulfonyl)benzoic acid, followed by alkylation by propargyl bromide to obtain alkyne-CAi. On the other hand, hydroxyl groups on both terminals of the amphiphilic polymer were transformed to azide groups (compound 6), enabling click reaction with alkyne-CAi to afford CAi-PEG-*b*-PPG-*b*-PEG-CAi. The chemical structures of the intermediates and final products were confirmed by proton nuclear magnetic resonance (¹H NMR) (Figure S1-S3, Supporting Information).

Nanoprecipitation of PCVB and CAi-*b*-PEG-*b*-PPG-*b*-PEG-CAi led to OPNi, whose aqueous solution was translucent in navy color (Figure S4, Supporting Information). Dynamic light scattering (DLS) indicated the average hydrodynamic diameter of OPNi as 47 nm and transmission electron microscope (TEM) revealed the uniform spherical morphology of OPNi (Figure 2b). Additionally, no significant change in diameter could be observed after storage of OPNi solutions for 8 weeks (Figure S4, Supporting Information), demonstrating its excellent colloidal stability. In phosphate buffered saline (PBS), OPNi had peak absorption at 630 nm and peak emission at 820 nm (Figure 2c), showing the capability of NIR excitation and a large Stokes shift (~190 nm) for fluorescence imaging. The binding between OPNi and CA-IX was examined by the increased hydrodynamic diameter (82 nm) after incubation of OPNi with CA-IX (Figure 2b). Besides, the surface potential of OPNi was neutralized from -19.1 to -10.13 mV (Figure S5, Supporting Information). Similarly, agarose gel electrophoresis revealed that OPNi showed remarkably retarded migration after treatment with CA-IX (Figure 2d).

The CA-IX-targeting ability of OPNi was studied against MDA-MB-231 cells, an aggressive human breast cancer cell line with a high expression level of CA-IX.^[19] After incubation of 231 cells with OPNi for 1 h, intense NIR fluorescence of OPNi was clearly observed from both membrane and cytoplasm of 231 cells (Figure 3a), which was not possible for the non-targeting control, OPNc (PCVB encapsulated by an amphiphilic polymer without CAi conjugation). Furthermore, flow cytometry analysis indicated that CA-IX-negative cell lines (HeLa, MCF-7, and NIH/3T3) had 5.8–12.4 times lower fluorescence intensity than CA-IX-positive MDA-MB-231 cells. The high cellular uptake of OPNi by MDA-MB-231 was attributed to the receptor-mediated endocytosis driven by the efficient binding between OPNi and CA-IX on the membrane of MDA-MB-231.

NIR photodynamic property of OPNi was measured by a ¹O₂ indicator Singlet Oxygen Sensor Green (SOSG). To minimize laser-induced cellular damage, a low power density (0.2 W/cm²) within the maximum permissible exposure limit of 680 nm laser was used. After continuous irradiation for 30 min, the fluorescence enhancement of OPNi was 10-fold higher than that of the control PBS solution, validating the production of ¹O₂ mediated by OPNi. In vitro synergistic therapeutic effect of OPNi was substantially studied on 231 cells, and cell viabilities were quantified by [3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-

sulfophenyl)-2H-tetrazolium] (MTS) assay. Without laser irradiation, OPNi induced higher cytotoxicity than OPNc due to the catalytic inhibition of CA-IX by CAIs. With laser irradiation, OPNi had the highest cytotoxicity among all groups. In contrast, single treatment of either nanoinhibitor or PDT (indicated by OPNc treatment with laser irradiation) failed to achieve so. These data thus validated the superior therapeutic efficacy of OPNi over monotherapy owing to the cooperative effect of PDT and CA-IX inhibition.

Figure 3. (a) Confocal microscopic images of MDA-MB-231 cells incubated with OPNi, OPNc ([PCVB] = 50 μg/mL), or PBS buffer at 37 °C for 1 h. Nuclei were indicated in blue. (b) Flow cytometry analysis of different cell lines after incubation with OPNi ([PCVB] = 50 μg/mL) at 37 °C for 1 h. (i) 231 cells with OPNi; (ii) 231 cells without OPNi; (iii) MCF-7 cells with OPNi; (iv) MCF-7 cells without OPNi; (v) HeLa cells with OPNi; (vi) HeLa cells without OPNi; (vii) NIH/3T3 cells with OPNi; (viii) NIH/3T3 cells without OPNi. (c) Fluorescence enhancement (F/F₀) of SOSG at 520 nm in OPNi (25 μg/mL) or PBS solutions under 680 nm laser irradiation (0.2 W/cm²). (d) Relative cell viabilities of 231 cells after incubation with different concentrations of OPNc or OPNi with or without 680 nm laser irradiation (0.2 W/cm², 30 min). (e) Absorption spectra of OPNi (5 μg/mL) after respective incubation with H₂O₂ (300 mM), or H₂O₂ (300 mM) and MPO (50 μg/mL) for 24 h. Inset: confocal microscopic images of RAW264.7 macrophages at 0 and 24 h after pre-activation by LPS and pre-incubation with OPNi (25 μg/mL). Error bars indicated standard deviations of triplicate independent experiments.

Biodegradation of OPNi was evaluated in both phagocyte-mimicking myeloperoxidase (MPO) solution and macrophages, because nanoparticles administered in vivo are often sequestered by mononuclear phagocyte system (MPS).^[21] MPO is abundant in phagocytes, which catalyses the conversion of hydrogen peroxide to hypochlorous acid to exert digestion of foreign substances. After incubation of OPNi with MPO and H₂O₂, the absorbance of OPNi dramatically declined concomitant with a bathochromic shift (Figure 3e). This was caused by the cleavage of vinylene bonds in PCVB, which was further confirmed by gel permeation

chromatography showing the significantly decreased molecular weight after MPO treatment (Figure S6, Supporting Information). The biodegradability of OPNi was further examined on macrophages RAW264.7 activated by lipopolysaccharide (LPS). At 24 h after pre-incubation of OPNi with stimulated macrophages, NIR fluorescence from intact OPNi declined to undetectable level in cytoplasm (Figure 3e, Figure S7, Supporting Information), validating its efficient biodegradation in phagocytes.

The in vivo targeting ability of OPNi was tested in MDA-MB-231 xenograft mouse model. NIR fluorescence imaging were longitudinally conducted after intravenous injection of OPNi or the non-targeting OPNc into living mice. Fluorescence intensity in tumor regions from OPNi or OPNc injected group continuously increased until reaching a plateau at 48 h post-injection of nanoparticles (Figure 4a). At this time point, the fluorescence signal in tumor region from OPNi-treated group was 1.3-fold higher than that for OPNc-treated mice. This was consistent with in vitro results and attributed to the specific binding between OPNi and CA-IX on the cell membrane of MDA-MB-231. At 72 h post-injection of nanoparticles, mice were euthanized, and organs were harvested for biodistribution studies (Figure S8, Supporting Information). Liver had the highest uptake of OPNi or OPNc, followed by tumor and the other organs. Notably, OPNi-treated mice showed 1.6-fold higher tumor-to-liver uptake ratio (TLR) than OPNc, which coincided with imaging outcome and further validated the superior tumor accumulation of OPNi due to the targeting ability of CA-IX.

Figure 4. In vivo NIR fluorescence imaging guided synergistic cancer therapy. (n = 3) (a) Fluorescence images of 231 xenograft tumor bearing living mice after intravenous administration of OPNi and OPNc ([PCVB] = 250 μg/mL, 200 μL), respectively. Fluorescence images were acquired with excitation and emission at 680 and 820 nm, respectively. Tumor regions were indicated with white circles. (b) Quantification of fluorescence signals in tumor regions in (a). (c) Tumor growth curves of mice after

intravenous administration of OPNi, OPNc, or PBS ([PCVB] = 250 µg/mL, 200 µL) with or without 680 nm laser irradiation (0.2 W/cm², 30 min) at 48 h post-injection. (d) H&E staining of tumors of mice after different treatments. (e) Immunofluorescent staining of caspase-3 (green fluorescence) of tumors from mice after different treatments. Blue fluorescence indicated staining of nuclei by Hoechst. (f) Immunofluorescent staining of VCAM-1 and HGF (green fluorescence) in tumors from mice administered with PBS, OPNc, or OPNi after laser irradiation. *Statistically significant difference in relative tumor volume between OPNc and OPNi injected mice after laser irradiation ($p < 0.001$, $n = 3$). Error bars indicated standard deviations of triplicate independent experiments.

The potential of OPNi for synergistic cancer therapy was evaluated. OPNi, OPNc, or saline was intravenously administered into 231 xenograft tumor bearing mice, and NIR laser irradiation (0.2 W/cm², 30 min) was applied to mice at 48 h post administration according to the in vivo imaging data. During the irradiation process, the tumor temperature was monitored and controlled below the threshold (43 °C) to avoid hyperthermia-induced ablation (Figure S9, Supporting Information). The tumor volumes of mice were monitored to quantitatively evaluate the therapeutic effect after treatment. Without laser irradiation, OPNi-treated mice showed a slower growth rate than OPNc- or PBS-treated mice, implying the partial inhibition of tumor growth attributed to the presence of CAIs in OPNi. With laser irradiation, OPNc-treated mice showed reduced tumor size for the first 10 days, followed by a gradual increase as an indication of tumor recurrence. In contrast, tumor growth for OPNi-treated mice was totally inhibited and no recurrence was observed for 18 days. Such superior therapeutic performance was further confirmed by hematoxylin and eosin (H&E) staining. Tumor after OPNi-mediated PDT showed more obvious cellular necrosis and apoptosis than those after monotherapy. Furthermore, immunofluorescent staining of caspase-3 indicated that massive apoptosis was only detected in tumor tissues after OPNi-mediated PDT. These data confirmed the superior therapeutic efficacy of OPNi-mediated cancer therapy over monotherapy.

To examine the potential ability of OPNi to inhibit cancer metastasis, expression of metastasis-related proteins vascular cell adhesion molecule-1 (VCAM-1) and hepatocyte growth factor (HGF) in tumor tissues after treatments was evaluated by immunofluorescent staining. Negligible green fluorescence from staining of either VCAM-1 or HGF was observed in tumors after OPNi-mediated PDT (Figure 4f). In contrast, high expression levels of both proteins were found in the other groups. These data further confirmed the advantage of OPNi for synergistic cancer therapy.

In conclusion, we synthesized organic photodynamic nanoinhibitors (OPNi) that can specifically interfere with CA-IX for synergistic cancer therapy to conquer hypoxia pitfall of conventional PDT. By virtue of the binding between CAIs and CA-IX, OPNi selectively targeted to CA-IX positive cancer cells, devoted to enhanced accumulation of OPNi in tumor with a 1.6-fold increase of TLR than the counterpart nanoparticle (OPNc); more importantly, the catalytic function of CA-IX was inhibited to cause pH dysregulation. Thereby, the synergistic PDT of OPNi led to the superior therapeutic effect over monotherapy, which was indicated by the inhibited tumor growth and downregulated metastasis-related proteins. Furthermore, OPNi could be

gradually metabolized by phagocytes in MPS, highlighting its translation potential for cancer therapy. To the best of our knowledge, this study not only presents the first biodegradable photodynamic nanoinhibitor, but also sheds light on molecular design of photosensitizers to overcome the drawbacks of PDT in cancer therapy.

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Keywords: photodynamic therapy • nanoinhibitor • hypoxia • photosensitizer • polymer nanoparticles

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COMMUNICATION

Entry for the Table of Contents
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An organic photodynamic nanoinhibitor with specific antagonism to carbonic anhydrase IX is developed for synergistic cancer therapy, aiming to conquer hypoxia pitfall of traditional photodynamic therapy. The synchronous integration of CA-IX inhibition mediated pH dysregulation with the near-infrared light induced phototoxicity permits effective cancer therapy with a high potential of minimized cancer metastasis.

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