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Novel theranostic nanoporphyrins for photodynamic diagnosis and trimodal therapy for bladder cancer

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ABSTRACT

The overall prognosis of bladder cancer has not been improved over the last 30 years and therefore, there is a great medical need to develop novel diagnosis and therapy approaches for bladder cancer. We developed a multifunctional nanoporphyrin platform that was coated with a bladder cancer-specific ligand named PLZ4. PLZ4-nanoporphyrin (PNP) integrates photodynamic diagnosis, image-guided photodynamic therapy, photothermal therapy and targeted chemotherapy in a single procedure. PNPs are spherical, relatively small (around 23 nm), and have the ability to preferably emit fluorescence/heat/reactive oxygen species upon illumination with near infrared light. Doxorubicin (DOX) loaded PNPs possess slower drug release and dramatically longer systemic circulation time compared to free DOX. The fluorescence signal of PNPs efficiently and selectively increased in bladder cancer cells but not normal urothelial cells *in vitro* and in an orthotopic patient derived bladder cancer xenograft (PDX) models, indicating their great potential for photodynamic diagnosis. Photodynamic therapy with PNPs was significantly more potent than 5-aminolevulinic acid, and eliminated orthotopic PDX bladder cancers after intravesical treatment. Image-guided photodynamic and photothermal therapies synergized with targeted chemotherapy of DOX and significantly prolonged overall survival of mice carrying PDXs. In conclusion, this uniquely engineered targeting PNP selectively targeted tumor cells for photodynamic diagnosis, and served as effective triple-modality (photodynamic/photothermal/chemo) therapeutic agents against bladder cancers. This platform can be easily adapted to individualized medicine in a clinical setting and has tremendous potential to improve the management of bladder cancer in the clinic.

1. Introduction

Bladder cancer is the fourth and eleventh most common cancer among men and women, respectively [1]. Approximately 80% of patients have non-myoinvasive bladder cancer at diagnosis that is treated with transurethral resection followed by intravesical instillation of therapeutic agents, such as Bacillus Calmette–Guérin, in high-risk patients. Transurethral resection is associated with microscopic residue tumor in at least a third of the cases regardless of the experience of the surgeon [2]. This treatment is associated with a recurrence rate of approximately 60% at two years [3], and disease progression to invasive cancer in around 25% of cases. Because of the high recurrence rate, surveillance with intrusive, uncomfortable and costly cystoscopy is performed once every few

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months during the first two years and at longer intervals for life. These surveillance procedures make bladder cancer the most costly cancer per case among all cancer types [4]. The overall prognosis of bladder cancer has not changed over the last three decades [5]. Therefore, there is a great unmet medical need for the diagnosis and therapy for bladder cancer.

Photodynamic diagnosis and therapy have been an attractive alternative modality in the management of bladder cancer [6–9], as it is minimally invasive, relatively tumor selective, and has low risk for development of resistance [10]. Compared to traditional white light transurethral resection, Photodynamic diagnosis assisted transurethral resection significantly improved the detection of bladder cancer and lowered the risk for recurrence [8,11–14] Photosensitizers, Photofrin® and Hexaminolevulinate, had been approved in Canada and USA, respectively, for bladder cancer, while others, such as 5-aminolevulinic acid (5-ALA), 3-(1′-hexyloxyethyl) pyropheophorbide-a (HPPH), Hematoporphyrin derivate, and chlorin E6, are at the different stages of clinical development [15–17].

However, current photosensitizers have poor selectivity, a low absorption band, poor bioavailability, low efficiency [18], and no photothermal effect or ability to co-deliver chemotherapeutic drugs and thus are limited in their clinical utility. To address these limitations, we introduce a small (~25 nm), multi-functional, highly water soluble micelle combining photodynamic therapy with imaging, cancer-specific drug delivery and extended drug retention. This enhanced functionality results from the self-assembly of micelles combining two species of cholic acid-polymer conjugates: 1) a porphyrin-cholic acid (CA)-polyethelene glycol (PEG) conjugate, and 2) a molecularly-targeted, cholic acid-polyethelene glycol conjugate [19] (Fig. 1A). We have previously reported the discovery of a bladder cancer-specific cyclic peptide named PLZ4 (amino acid sequence: cQDGRMGFc). PLZ4 specifically binds to the αvβ3 integrin on bladder cancer cells even in the presence of bladder inflammation [20,21]. We previously demonstrated that PLZ4-coated micelles (PMs, a mixture of PLZ4-PEG^{5k}-CA$_8$ and PEG^{5k}-CA$_8$ telodendrimers) specifically delivered the drug paclitaxel to canine and human bladder tumor cells *in vitro* and *in vivo,* resulting in superior anti-cancer efficacy in comparison to drug-loaded non-targeted micelles and free drug [22]. Thus, we mixed original PLZ4-PEG^{5k}-CA$_8$ (providing molecular targeting) and newly introduced PEG^{5k}-Por$_4$-CA$_4$ (providing photodynamic diagnosis/therapy) to form PLZ4-nanoporphyrin (PNPs) which address current clinical challenges in treating bladder cancers.

To the best of our knowledge, this is the first report of a targeted nanoparticle platform that is able to integrate such a broad range of clinically relevant functionalities in a single nanoformulation specifically for bladder cancer. It has the great potential to significantly change the clinical management paradigm of bladder cancer.

2. Materials and methods

2.1. Synthesis and characterization of PLZ4-Nanoporphyrins (PNPs)

The pyropheophorbide a containing telodendrimer (PEG^{5k}-Por$_4$-CA$_4$, Fig. 1A) was synthesized via solution-phase condensation reactions according to our published method [23]. Our previously reported PLZ4-PEG^{5k}-CA$_8$ telodendrimer was synthesized by the conjugation of alkyne-derivatized bladder cancer targeting ligand PLZ4 (CPC scientific, Sunyvale, CA) [24,25] to PEG^{5k}-CA$_8$ telodendrimer via click chemistry [26–28].

PNPs were obtained via a mixed micelle strategy. Briefly, 10 mg of PLZ4-PEG^{5k}-CA$_8$ and PEG^{5k}-Por$_4$-CA$_4$ (Fig. 1A) were dissolved in the chloroform, and evaporated on a rotavapor to obtain a homogeneous dry polymer film. The film was reconstituted in 1 ml PBS, followed by sonication for 30 min, allowing the sample film to disperse into PNP solution. DOX was loaded into PNPs by following the same solvent evaporation method after mixing neutralized DOX with telodendrimers [29]. PNP-DOX stock (20 mg of PNP/ml) contains 2 mg/ml Pyropheophorbide a and 1 mg/ml DOX. Finally, the nanoparticle solution was filtered with 0.22 μm filter to sterilize the sample. Similarly, a PLZ4-micelle (PM) formed from a mix of PLZ4-PEG^{5k}-CA$_8$ and PEG^{5k}-CA$_8$, while nanoporphyrin (NP) was formed from a mix of PEG^{5k}-CA$_8$ and PEG^{5k}-Por$_4$-CA$_4$.

The particle size and morphology were analyzed by dynamic light scattering (Microtrac, Montgomeryville, PA) and transmission electron microscopy (Philips, CM-120, Andover, MA), respectively. The drug release profiles of the DOX-loaded nanoparticles was investigated using dialysis method in the presence of 10% FBS as described previously [22].

2.2. Pharmacokinetic study

Four Jugular vein cannulated rats (Harlan Laboratories, Livermore, CA) were employed for the pharmacokinetic study. Each rat received 5 mg/kg DOX or PNP-DOX (5 mg/kg DOX and 100 mg/kg PNPs (10 mg/kg Pyropheophorbide a). Fifty microliters of blood were collected at different time points and fluorescence was measured.

2.3. Cellular uptake and ROS production

Human bladder cancer 5637 cells (ATCC®, Manassas, VA) were seeded into 96-well plates overnight. After treatment with various concentrations of PNPs and NPs for 4 h, free drugs were washed and cells were lysed with 100 μl of lysis buffer for 30 min with shake. Fluorescence was measured by ELISA reader (Molecular devices, Sunnyvale, CA).

For intracellular reactive oxygen species (ROS) productions, 5637 cells were treated with 10 μg/ml PNPs (Pyropheophorbide a: 2 μg/ml) for 2 h and washed by PBS for 3 times in suspension. Cells were then loaded with 10 μM 2′,7'-dichlorofluorescein (DCF) (Sigma) for 30 min followed by 4.2 J/cm^2 light treatment (Omnilux New-U LED panel with 635 nm light, Clifton Park, NY). ROS production was then analyzed by flow cytometry. Methods for assessment of the tissue level of ROS production were described previously [19].

To specifically confirm the signet oxygen generation *in vitro*, we incubated singlet oxygen sensor green (SOSG, Sigma) with different concentrations of PNPs with or without SDS. SOSG and porphyrin fluorescence were measured by ELISA reader.

2.4. Confocal microscope – selective uptake and cellular bio-distribution

Primary normal dog urothelial cells [22] were co-cultured with DiO (Sigma) dye labeled 5637 cells. Plate was treated with 10 μg/ml PNPs for 2 h and imaging was acquired under fluorescence microscope without wash. For subcellular bio-distribution study, 5637 cells were treated with DOX-loaded PNPs. Images were then obtained through confocal microscope (Leica, Buffalo Grove, IL). To detect intracellular Glutathione changes, Thiol Tracker™ Violet Glutathione Detection Reagent (Molecular Probes, Eugene, OR) was incubated for 30 min after treatment and slides were analyzed by confocal microscope.