Acta Biomaterialia xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Zinc phthalocyanine conjugated with the amino-terminal fragment of urokinase for tumor-targeting photodynamic therapy

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ARTICLE INFO

Article history:
Received 1 March 2014
Received in revised form 10 June 2014
Accepted 17 June 2014
Available online xxxx

Keywords:
Tumor-targeting
Tumor-imaging
Photosensitizer
Zinc phthalocyanine

ABSTRACT

Photodynamic therapy (PDT) has attracted much interest for the treatment of cancer due to the increased incidence of multidrug resistance and systemic toxicity in conventional chemotherapy. Phthalocyanine (Pc) is one of main classes of photosensitizers for PDT and possesses optimal photophysical and photochemical properties. A higher specificity can ideally be achieved when Pcs are targeted towards tumor-specific receptors, which may also facilitate specific drug delivery. Herein, we develop a simple and unique strategy to prepare a hydrophilic tumor-targeting photosensitizer ATF-ZnPc by covalently coupling zinc phthalocyanine (ZnPc) to the amino-terminal fragment (ATF) of urokinase-type plasminogen activator (uPA), a fragment responsible for uPA receptor (uPAR, a biomarker overexpressed in cancer cells), through the carboxyl groups of ATF. We demonstrate the high efficacy of this tumor-targeting PDT agent for the inhibition of tumor growth both in vitro and in vivo. Our in vivo optical imaging results using H22 tumor-bearing mice show clearly the selective accumulation of ATF-ZnPc in tumor region, thereby revealing the great potential of ATF-ZnPc for clinical applications such as cancer detection and guidance of tumor resection in addition to photodynamic treatment.

1. Introduction

Cancer is one of the deadliest diseases of our time and throughout the world. Billions of dollars are spent annually on research in order to cure cancer or improve the quality of life of cancer patients. Traditional cancer treatments, including surgery, radiation therapy and chemotherapy, result in serious side effects caused by the loss of normal organ function. In contrast, photodynamic therapy (PDT) is more controllable and has the potential to selectively destroy malignant cells while sparing the normal tissues, and thus is recognized as a treatment strategy that is both minimally invasive and minimally toxic. It is a form of phototherapy using nontoxic light-sensitive compounds (i.e. photosensitizers) that are exposed selectively to light, whereupon they become toxic to targeted malignant and other diseased cells [1–3]. In addition, some photosensitizers are able to provide intense fluorescence signals in tumor tissues that allow their photodynamic imaging.

Among many types of photosensitizers for PDT, phthalocyanine (Pc) is one of the main classes of photosensitizers with advantageous photophysical properties [4]. Its stronger absorption at 670 nm where the depth of light penetration in tissue is twice that obtained at 630 nm with porfimer sodium (Photofrin), which has been used as an effective photosensitizer in clinical cancer treatment or in cancer clinical trials [5]. However, Pcs tend to have low tumor-targeting efficacy, which limits their use in clinical applications. A number of strategies have been used to enhance

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http://dx.doi.org/10.1016/j.actbio.2014.06.026
1742-7061/

the tumor-targeting specificity of Pc compounds [3,6,7]. In this study, we report a strategy to improve the tumor-targeting properties of zinc phthalocyanine (ZnPc) by coupling ZnPc to the amino-terminal fragment (ATF) of urokinase.

Urokinase, or urokinase-type plasminogen activator (uPA), is recommended as a prognostic marker for breast cancer by the American Society of Clinical Oncology (ASCO) [8] and the European Organization for Research and Treatment of Cancer (EORTC) [9]. Biochemical and structural studies from our laboratory [10,11] and others [12–14] have demonstrated that the ATF of uPA (uPA^{1-143}, molecular weight ~16 kDa) is solely responsible for uPA binding to the receptor of uPA (uPAR). uPAR has a low expression level in most quiescent cells, but greatly increases in several cancers including breast, colorectal and gastric cancers cells [15]. Several independent studies have correlated uPAR expression in vivo to various pathological conditions, especially cancer invasion and metastasis [16]. Moreover, a high level of soluble uPAR in plasma correlates with poor patient prognosis in many different human cancers [17]. Due to the importance of uPAR in cancer invasion and metastasis, several types of uPAR antagonists have been developed during the last decades, and used as targeting agents for imaging of uPAR via various imaging modalities such as magnetic resonance imaging, single-photon-emission computer tomography and positron emission topography [18]. A multifunctional nanoscale uPAR-targeted delivery vehicle was engineered to enhance the selective and specific delivery of the cargo (noscapine) to prostate cancer cells [19]. In another study, an uPAR-targeted dual-modality nanoparticle probe was generated and shown to selectively accumulate into primary and metastatic pancreatic cancer lesions in pancreatic cancer in mice by molecular imaging [20]. Recently, a ^{64}Cu-labeled peptidyl uPAR inhibitor was demonstrated to accumulate specifically on a xenografted tumor in mice 2- to 3-fold more than the non-inhibitory ^{64}Cu-labeled peptide [21]. Taken together, uPAR has attracted considerable attention as a promising molecular target for intervention and/or cytotoxin-based cancer therapies.

The ATF of uPA is an often used targeting agent for uPAR and has been used to conjugate to different nanoparticles [20,22]. However, recent studies have raised concern that chemical modification of ATF may alter its uPAR binding capability. Small molecular antagonists were developed in the 1990s by several pharmaceutical companies to intervene in the uPAR–uPA interaction. These inhibitors were reported to have high potencies, and some of them had IC_{50}s in the nanomolar range. However, two inhibitors were later resynthesized by an independent group and found to have much weaker potencies (IC_{50}s were of the order of micromoles) [23]. This discrepancy was traced to a problem in the first competitive assay using ATF with ^{125}I labeling, which modified the ATF residues important for uPAR binding and greatly reduced the affinity for uPAR of the labeled fragment [24], thus making the labeled fragment an invalid probe to measure uPAR binding. This example highlights that caution should be taken in the modification of ATF protein in order to preserve its uPAR-binding capability. In this study, we conjugate ZnPc to the carboxyl groups of ATF, which was shown not to interfere with uPAR binding [10], and thus preserve the binding activity of conjugated ATF to uPAR. Besides the increased tumor-targeting efficacy, this conjugation of ATF with ZnPc has an added benefit of enhancing the water solubility of ZnPc. ZnPc is not soluble in aqueous solution, and various formulation approaches have been used in order to solve this solubility issue, which include the use of detergents (castor oil) or nanoparticles [25,26]. Derivatization of the Pc ring is also commonly used to enhance the solubility [27].

Both in vitro and in vivo PDT studies demonstrate that ATF-ZnPc preferentially accumulates onto uPAR-positive cells, internalizes into cells and localizes in the lysosomes, and thus exhibits selective PDT effects on tumor cells (as shown in Fig. 1). The selectivity of ATF-ZnPc towards uPAR-positive cells is further identified by fluorescent imaging of a tumor model in mice.

2. Experimental

2.1. Materials and cell lines

All the starting materials were obtained from commercial suppliers and used as received without further purification. Common chemicals were purchased from Shanghai Chemical, Inc. Sepharose fast flow (SPFF) and Superdex75 HR 10/30 size exclusion columns were obtained from GE Life Sciences. All enzymes were from Takara Bio Inc., except that Pfu DNA polymerase was from Sangon Biotech. Synthetic DNA oligonucleotides were from Beijing Sunbiotech Co. Ltd. Zeocin, Top10F' and eukaryotic *Pichia pastoris* cells were purchased from Invitrogen. Deionized water was used throughout the experiments.

The mammalian cell lines used in the studies were histiocytic lymphoma cell line U937, non-small cell lung carcinoma cell line H1299, mouse hepatocellular carcinoma cell line H22, and human embryo lung fibroblast cell line HELF. All these cell lines were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences and grown in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics. Cells were kept at 37 °C in a humidified incubator with 5% CO_2 atmosphere. The viability of cells was determined by the dye Trypan blue. Cells were maintained in logarithmic phase with viability >95%.

2.2. Preparation of ATF protein

The cloning, expression and purification of human ATF (residues 1–143) were carried out according to our previously published protocols [28]. ATF was expressed in eukaryotic *P. pastoris* to ensure proper protein folding and disulfide bond formation, and was captured from expression medium by an SPFF cation exchange column. The protein was further purified by a preparative C4 reversed-phase column (VYDAC®, 250 × 10 mm, 5 μm) on a high-performance liquid chromatography (HPLC) system (Dalian Elite Analytical Instruments Co. Ltd., Dalian, China), eluted with a linear gradient of 20–70% acetonitrile. The final product ATF was

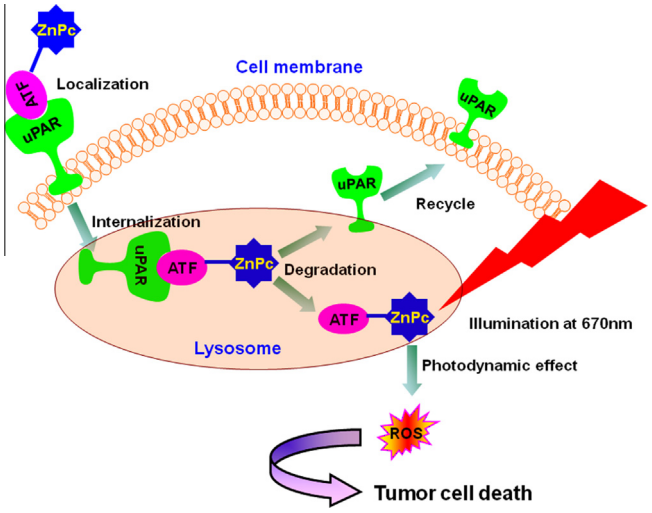

Fig. 1. Conjugation of ATF with zinc phthalocyanine (ZnPc) renders ZnPc soluble in water and generates a high degree of specificity to uPAR-abundant tumor cells. ATF-ZnPc preferentially accumulates in lysosome organelle, mainly through uPA receptor-dependent endocytosis, and induces cell death upon light illumination.

Please cite this article in press as: Chen Z et al. Zinc phthalocyanine conjugated with the amino-terminal fragment of urokinase for tumor-targeting photodynamic therapy. Acta Biomater (2014), http://dx.doi.org/10.1016/j.actbio.2014.06.026