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Original article

Preparation of a chlorophyll derivative and investigation of its photodynamic activities against cholangiocarcinoma

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ABSTRACT

Photodynamic therapy (PDT) is emerging as a promising method for the treatment of various cancer diseases. However, the clinical application of PDT is limited due to the lack of effective photosensitizers. In this study, a novel chlorophyll derivative, *N,N*-bis(2-carboxyethyl)pyropheophorbide a (BPPA), had been synthesized and characterized. BPPA had a characteristic long wavelength absorption peak at 669 nm and a singlet oxygen quantum yield of 0.54. To investigate the photodynamic ability of BPPA against cholangiocarcinoma (CCA), cellular uptake, subcellular location and bio-distribution, *in vitro* and *in vivo* PDT efficacy of BPPA were studied. The results showed that BPPA could rapidly accumulate in QBC-939 cells and localize in the cytoplasm. BPPA- PDT was effective in reducing the cell viability in a drug dose- and light dose-dependent manner *in vitro*. In CCA xenograft nude mouse model, the concentration of BPPA in the plasma lowered rapidly, and the fluorescence signal peaked at 0.5 h and 2 h after injection in the skin and tumor, respectively. Significant quantities could be observed in the tumor. BPPA followed by irradiation could significantly inhibit growth of tumors, and histological examination revealed necrotic damage in PDT-treated tumors. These results suggested that BPPA could be a promising drug candidate for photodynamic therapy in cholangiocarcinoma.

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1. Introduction

Cholangiocarcinoma (CCA) is a malignant tumor originating from biliary tract epithelial cells [1]. Due to its difficulty of diagnosis and high fatality rate, it is becoming the most common hepatic tumor-induced death [1,2]. As CCA is resistant to traditional chemotherapy and radiotherapy, surgery is the only therapeutic mode offering a cure. However, recurrence frequently occurs and the 5-year survival rate is only 5%–10% [3].

Photodynamic therapy (PDT) is a non-invasive treatment that involves the accumulation of a photosensitizer in malignant tissue followed by irradiation with laser light of an appropriate wavelength [4,5]. In the presence of oxygen, the activated photosensitizer can generate singlet oxygen ($^1O_2$) and reactive oxygen species (ROS) that ultimately leads to cell death through apoptosis or necrosis [6,7]. Up to date, many studies have been conducted on the use of PDT on CCA, which indicated that PDT has a promising trend toward improved survival as well as improvement in quality of life [8,9]. However, because clinically approved PDT drugs generally give many problems due to the prolonged cutaneous photo-sensitivity, poor water-solubility and inadequate selectivity, the clinical application of PDT is limited [10,11].

In general, an ideal photosensitizer for tumor PDT requires good tissue penetration, low dark toxicity but strong photocytotoxicity, high extinction coefficient, rapid removal from the body and multiple administration routes [12,13]. Recently, the chlorophyll a and its derivatives have been considered as having great promises and efficacy for treatment of some cancers due to their long-wavelength absorbance, and most efforts in our laboratory have been directed toward the synthesis of new potential photosensitizers related to chlorophyll [14].

As a degraded product of chlorophyll, pyropheophorbide-a is well known as an ideal synthetic precursor for the synthesis of photodynamic therapy drugs. In the present study, a novel chlorophyll derivative, *N,N*-bis(2-carboxyethyl)pyropheophorbide a (BPPA), had been synthesized and characterized. The cellular uptake, intracellular localization, *in vitro* and *in vivo* PDT efficacy

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were further investigated to evaluate the efficacy of PDT in treatment of CCA.

2. Results and discussion

2.1. Synthesis and photophysical properties of BPPA

The synthesis of *N,N*-bis(2-carboxyethyl)pyropheophorbide a amide (BPPA) as showed in Scheme 1. Pyropheophorbide-*a* is a derivative from plant chlorophyll. It was reacted with dimethyl 3,3′-azanediyldipropionate in the presence of HBTU and triethylamine to give *N,N*-dimethoxycarbonylethyl-pyropheophorbide-a amide (compound 2). After hydrolysis of compoud 2 with NaOH solution in THF, a novel chlorin (or dihydroporphin) derivative 1 was obtained with 95.3% yield. Compound 1 was purified by column chromatography and characterized by NMR spectrum, Mass spectrum, UV–vis spectrum and fluorescence spectra (shown in Fig.S1-S5).

The photophysical properties of BPPA were measured in DMF and the spectra were given in Fig. 1. Absorption spectrum of BPPA displayed an intense Soret band at 416 nm, and four less intense Q band at 510 nm, 540 nm, 612 nm and 669 nm, respectively. The Molar extinction coefficients were shown in Table 1. The ideal photosensitizer for use in PDT should have a strong absorbance with a high extinction coefficient in the long-wavelength (600–800 nm) region, where the maximum penetration of tissue by the penetrating light occurs [12,19]. BPPA showed a strong absorption at 669 nm with a high molar extinction coefficient (ε) of 38,800 M^{-1} cm^{-1}, suggesting that BPPA would be a potential photosensitizer for use in PDT.

The shape of excitation spectrum was similar to that of absorption spectrum, suggesting that the molecules did not show any degradation during excitation in DMF (shown in Fig. 1b). Upon exciting at 416 nm, the fluorescence emission spectrum of BPPA showed a strong emission peak at 676 nm and a weak fluorescence at 720 nm (Fig. 1c). The excitation–emission matrix fluorescence spectra were shown in Fig. 1d.

2.2. Singlet oxygen quantum yield

Since the singlet oxygen is a high energy form of oxygen and is responsible for destruction of cancer cells during PDT [20,21], the singlet oxygen generation ability was investigated for determination of possibility of BPPA for photodynamic therapy of cancer. The generation of singlet oxygen by BPPA was measured after exposure to 560 nm light at the laser intensity of 5 mW/cm^2 in the presence of DPBF. As shown in Fig. 2a, the absorbance of DPBF at 414 nm continuously decreased with the increasing time after irradiation, which demonstrated the PDT efficiency of BPPA. The $^1O_2$ quantum yield (Φ_D) of BPPA in DMF was determined to be 0.54 using Rose Bengal as the standard photosensitizer ($\Phi_\Delta = 0.47$) (shown in Fig. 2b.), suggesting that BPPA might be an effective photosensitizer.

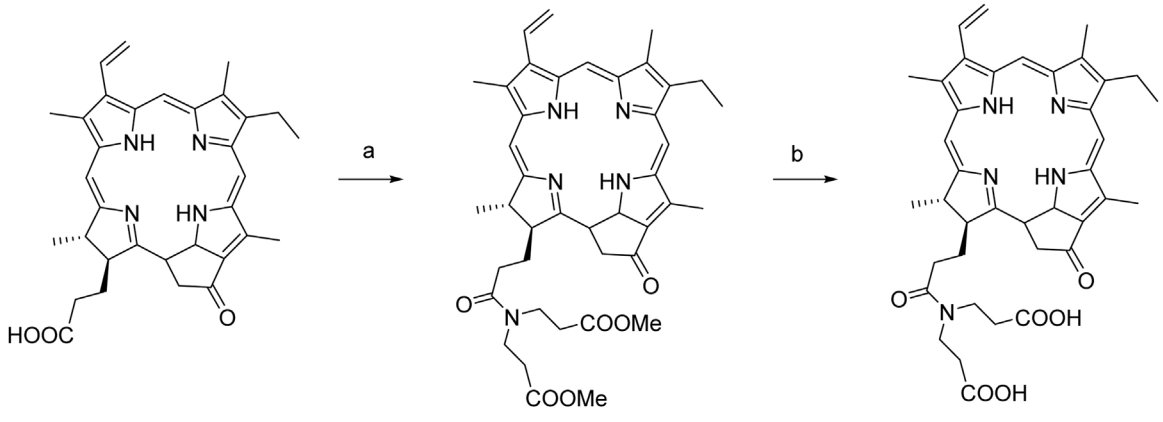
2.3. Cellular uptake and subcellular localization

Uptake and subcellular localization of BPPA in QBC-939 cells was examined to evaluate BPPA as a potential photosensitizer for PDT. The intracellular concentration changes of BPPA at different time points after addition to QBC-939 cells were determined using a fluorescence spectrometry. As shown in Fig. 3, the intracellular concentration of BPPA increased quickly for the first 3 h after administration, and then slightly increased.

To investigate subcellular localization of BPPA in QBC-939 cells, the cells loaded with BPPA were incubated with Lyso Tracker Blue and Hoechst 33342 to label lysosomes and nucleus, respectively. The confocal laser scanning microscopy images showed that the red fluorescence of BPPA overlapped with the blue fluorescence from the probe for lysosomes, indicating that BPPA was mainly localized in the lysosomes (Fig. 4).

2.4. Dark and light-dependent cytotoxicity

The dark and light-dependent cytotoxicities of BPPA against QBC-939 cells were investigated using the MTT assay. Without the photosensitizer, the exposure of QBC-939 cells to 665 nm light did not affect cell survival (Data not given). As shown in Fig. 5, BPPA at concentrations up to 1 μM did not show any significant dark cytotoxicity. The TC_{50} (half inhibitory concentration without irradiation) was 252.158 μM. After irradiation, the cell viability decreased in drug concentration- dependent and light dose-dependent manner. The IC_{50} (half inhibitory concentration under light) values corresponding to 4–20 J/cm^2 were found to be 11.872, 4.271, 2.641, 0.698 and 0.574 μM, respectively. The "therapeutic ratio" values for BPPA irradiated with different light dose obtained by comparing TC_{50} and IC_{50} values were all larger than 20. The



Scheme 1. Synthesis of *N,N*-bis(2-carboxyethyl)pyropheophorbide a amide (BPPA). Reagents and reaction conditions: (a) HBTU, Et_3N, CH_2Cl_2, R.T., 6 h, 56.5%; (b) NaOH, THF, 3 h, 95.3%.