



Benzochlorophyrin derivative photosensitizer-mediated photodynamic therapy for Ewing sarcoma



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ABSTRACT

In this study, we evaluated the photodynamic efficacy of a new photosensitizer, benzochlorophyrin derivative 18 (BCPD-18), in Ewing sarcoma. We found that BCPD-18 decreased the viability of TC-71 cells irradiated by 670 nm laser in a concentration dependent manner. We also observed cells undergoing apoptosis as well as cell cycle arrest at the G2M phase after BCPD-18-mediated photodynamic therapy (BCPD-PDT). In addition, *in vivo* study (subcutaneous and orthotopic models) showed that BCPD-PDT reduced tumor size, tumor weight and tumor-bearing leg weight. After PDT, apoptosis was shown *in vivo*. Bax expression was increased, and Bcl-2 expression was decreased. This study provides evidence that BCPD-18 could probably be a useful photosensitizer in PDT for Ewing sarcoma.

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1. Introduction

Ewing sarcoma (EWS), first described by James Ewing in 1921 [1], is the second most common primary malignant bone tumor in children and young adolescents [2]. EWS cells are small, blue, and round and mainly develop in the metaphysis of long bones [2]. Although a rare cancer, EWS easily metastasizes to the lung [3]. Despite the significant progress made in diagnosing and treating EWS, the disease typically metastasizes by the time it is diagnosed in 25% of patients [4] and the 5-year survival rate is still very low [5]. Because current therapies produce long-term or short-term toxic effects [6], it is crucial to find a less toxic, more effective method for treating this kind of tumor.

Photodynamic therapy (PDT) is a minimally invasive therapeutic modality for neoplastic and nonmalignant diseases. PDT, which combines a drug (photosensitizer) and light, is a targeted cancer therapy that, compared with other cancer treatment modalities, produces less systemic cytotoxicity. Moreover, in the area of musculoskeletal oncology, it could preserve limb function when combined with intralesional tumor resection [7]. However, not much research about PDT has been done in EWS [8], which makes it crucial to focus on this area.

The photosensitizer is important in PDT. Currently, the most widely used photosensitizer is hematoporphyrin derivative (HpD). Although HpD has been playing an important role in the history of PDT, it may induce allergies [9] and long-lasting skin photosensitivity when used in the clinic. Moreover, the light wavelength for activating HpD is 630 nm, which penetrates tissue to only about 10 mm [10], although some satellite lesions can be deeper than 1 cm. It is necessary to find new drugs, which can be irradiated with a suitable wavelength of light that can penetrate deeper.

We synthesized a series of benzochlorophyrin derivatives (BCPDs) [11]. Compared with HpD and BPD-MA (clinically used photosensitizer), BCPDs showed low skin photosensitivity, rapid clearance from tissues, and strong absorption at a long wavelength (670 nm), which resulted in greater tissue penetration [11]. In this study, we investigated the antitumor effects of BCPD-18-mediated photodynamic therapy (BCPD-PDT) in EWS, both *in vitro* and *in vivo*, and provided evidence of the efficacy of a new photosensitizer in cancer PDT as well as a new treatment modality for EWS.

2. Materials and Methods

2.1. Reagents and PDT Irradiation Equipment

BCPD-18 (Fig. 1A) was synthesized [11] and it has an absorption peak at around 670 nm (Fig. 1B) when dissolved in the medium of dichloromethane. In this experiment, BCPD-18 was diluted in whole Dulbecco's modified Eagle's medium (DMEM) medium into 0.125,

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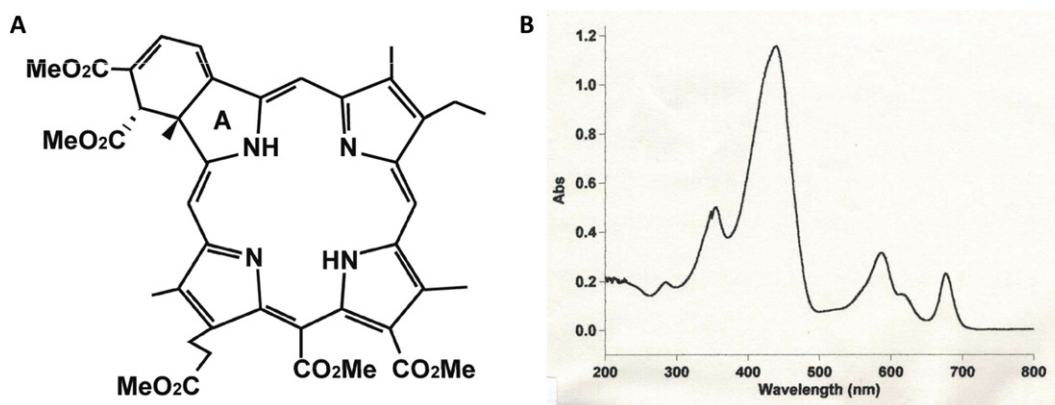


Fig. 1. A. Chemical structure of BCPD-18. B. UV-vis absorption patterns of BCPD-18 in dichloromethane, it has an absorption peak at around 670 nm.

0.25, 0.5, and 1 $\mu\text{g/ml}$ before use. Benzoporphyrin derivative monoacid (BPD-MA) was also diluted in whole Dulbecco's modified Eagle's medium (DMEM) medium into 0.125, 0.25, 0.5, and 1 $\mu\text{g/ml}$. The 670-nm laser irradiation equipment was purchased from Guilin Xingda Photoelectric Medical Apparatus and Instruments Co., Ltd. (Guangxi, China). For the *in vitro* experiments, the laser (670 nm) was used at a fluence of 2.5 J/cm^2 (20.75 mW/cm^2 for 2 min), 5 J/cm^2 (20.75 mW/cm^2 for 4 min), 7.5 J/cm^2 (20.75 mW/cm^2 for 6 min), and 10 J/cm^2 (20.75 mW/cm^2 for 8 min). For the *in vivo* experiments, the fluence was 120 J/cm^2 (200 mW/cm^2 for 10 min).

2.2. Cell Culture

Human EWS cell line TC-71 and human osteoclast hFOB 1.19 were cultured in DMEM medium (Life Technologies, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. Cells were maintained at 37 $^{\circ}\text{C}$ (hFOB 1.19 cells were maintained at 35 $^{\circ}\text{C}$) in a humidified atmosphere of 5% CO_2 .

2.3. Cell Viability Assay

TC-71 cells (2×10^5) were cultured with various concentrations of BCPD-18 or BPD-MA (0, 0.125, 0.25, 0.5, and 1 $\mu\text{g/ml}$) for 4 h and then irradiated with a 670-nm laser at various densities (0, 2.5, 5, 7.5, and 10 J/cm^2). hFOB 1.19 cells (2×10^5) were cultured with various concentrations of BCPD-18 (0, 0.125, 0.25, 0.5, and 1 $\mu\text{g/ml}$) for 4 h and then irradiated with a 670-nm laser at various densities (0 and 10 J/cm^2). Here, the blank control group (without BCPD-18 or light irradiation), the BCPD-18-only group (BCPD-18 only) and the light-only group (light irradiation only) were set as the control groups. At 24 h after irradiation, the MTT assay was performed to evaluate cell viabilities.

2.4. Hoechst 33342 and PI Staining

TC-71 cells (2×10^5) were treated with various concentrations (0, 0.5, and 1 $\mu\text{g/ml}$) of BCPD-18 for 4 h and then irradiated with 670-nm visible light at a dose of 10 J/cm^2 . After 24 h, the cells were washed twice with cold phosphate-buffered saline and then stained with Hoechst 33342 (Beyotime Institute of Biotechnology, Shanghai, China) and propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at 37 $^{\circ}\text{C}$ in the dark and immediately observed under a fluorescence microscope (Olympus Inc., Japan).

2.5. Annexin V and PI Staining for Detecting Apoptosis

TC-71 cells (2×10^5) were treated with 0 or 1 $\mu\text{g/ml}$ of BCPD-18 for 4 h and then irradiated with or without 670-nm visible light at a dose of 10 J/cm^2 . After 24 h, cells were collected. Annexin V-FITC (5 μl , BD

Biosciences) and propidium iodide (5 μl , Sigma) were used according to the manufacturer's instructions. Apoptosis was detected by a FACSCalibur flow cytometer (BD Biosciences).

2.6. Cell Cycle

TC-71 cells (2×10^5) were treated with 0 or 1 $\mu\text{g/ml}$ of BCPD-18 for 4 h and then irradiated with or without 670 nm visible light at a dose of 10 J/cm^2 . After 24 h, cells were collected and fixed in 70% ethanol. Propidium iodide (100 $\mu\text{g/ml}$, Sigma) and RNase (200 $\mu\text{g/ml}$, Sigma) were added, and the solution was incubated in the dark at room temperature for 30 min. The cell cycle was then detected by a FACSCalibur flow cytometer (BD Biosciences).

2.7. Animal Experiments

TC-71 cells (1×10^6) were injected into the flank or tibia of 4-week-old BALB/C nude mice (Shanghai Slac Laboratory Animal Co., Ltd., Shanghai, China). Briefly, the mice were anesthetized with ketamine (80 mg/kg) and xylazine (7 mg/kg). Next the cell suspension (10 μl) was injected into the flank or medullary cavity of tibia with the use of a 25-gauge needle. At 13 days (for subcutaneous model) or 20 days (for tibia model) after the injection, mice were randomly selected into the control or PDT group, 10 mice in each group. Nothing was done with the control group while the PDT group was anesthetized with ketamine (80 mg/kg) and xylazine (7 mg/kg) and then received 1 mg/kg BCPD-18 plus 120 J/cm^2 light irradiation (the time between BCPD-18 injection and photoillumination was 4 h, and the laser light spot diameter was 1.5 cm). The size of the tumor was measured every 3 or 4 days, and the volume was calculated by using the following equations: subcutaneous model: volume = maximum diameter \times minimum diameter² / 2 [12]; tibia model: volume = (L + W)(L)(W)(0.2618) [13]. The width (W) was the average of the distance at the proximal tibia at the level the knee joint in the anterior–posterior and medial–lateral planes. The length (L) was the distance from the most distal extent of the calf musculature or distal tumor margin to the knee joint or proximal tumor margin [13]. At 12 days after PDT, the mice were euthanized, and tumor (subcutaneous model) and leg bearing the tumor (tibia model) were resected and weighed. The tumors were then fixed in 10% formalin, embedded in paraffin, and cut into 4- μm -thick sections. H&E staining was used to detect morphologic changes. The TUNEL assay was performed on tumor cells undergoing apoptosis. Immunohistochemical analysis was performed to detect Bax and Bcl-2 (Cell Signaling Technology Inc., Danvers, MA) expression in tibia model after treatment. All animal experiments were performed in the animal facility of Shanghai Tenth People's Hospital and the Animal Care and Use Committee of Shanghai Tenth People's Hospital approved all of the procedures.

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