RESEARCH ARTICLE

Benzochloroporphyrin Derivative Induced Cytotoxicity and Inhibition of Tumor Recurrence During Photodynamic Therapy for Osteosarcoma

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Abstract

Photodynamic therapy (PDT) is a promising cancer treatment modality that uses dye-sensitized photooxidation of biologic matter in target tissue. This study explored effects of the photosensitizer BCPD-17 during PDT for osteosarcoma. LM-8 osteosarcoma cells were treated with BCPD-17 and cell viability after laser irradiation was assessed *in vitro* with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. The effects of BCPD-17 during PDT recurrence were then examined on tumor-bearing mice *in vivo*. BCPD-17 had dose-dependent cytotoxic effects on LM-8 osteosarcoma cells after laser irradiation which also had energy-dependent effects on the cells. The rate of local recurrence was reduced when marginal resection of mice tumors was followed by BCPD-17-mediated PDT. Our results indicated BCPD-17-mediated PDT in combination with marginal resection of tumors is a potentially new effective treatment for osteosarcoma.

Keywords: Inhibiting rate - photochemotherapy - apoptosis/drug effects - photosensitizing agents

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Introduction

Photodynamic light therapy (PDT) is a two-stage procedure, where a photosensitizer is injected into localized cancer lesions and then the tumor is irradiated (Moser, 1998; Ackroyd et al., 2001; Agostinis et al., 2011). This treatment is highly localized and is not affected by radioresistance or chemoresistance. Some photosensitizers are clinically approved, including the hematoporphyrin derivative HpD, trade named Photofrin; however, the application of HpD is limited by its weak selective tumor uptake (Detty et al., 2004) and possible skin photosensitivity for up to 30 to 90 days after treatment (Sternberg and Dolphin, 1998). New photosensitizers with more promising photophysical efficiency and fewer side effects are required for PDT. The novel benzochloroporphyrin derivative Benzochloroporphyrin Derivatives (BCPD-17) has low skin toxicity, strong absorption for greater tissue penetration, and promising activity against human hepatoma cell lines (Yao et al., 2008).

Osteosarcoma, a malignant tumor arising from primitive transformed cells of mesenchymal origin that exhibit osteoblastic differentiation and produce malignant osteoid, is difficult to completely remove by radical resection (Fagioli et al., 2008). The residual local tumor cells leave patients vulnerable to local recurrence and poor clinical outcomes. Despite aggressive therapies, long-term survival rates remain low (Wiromrat et al., 2012). Additionally, in many cases treatment consists of limb amputation which results in a marked reduction in quality of life (Han et al., 2012; Sun et al., 2012). There has been no previous report about effect of BCPD-17-mediated PDT to suppress local recurrence after tumor resection on osteosarcomas. We hypothesized that photosensitizer BCPD-17-mediated PDT could be a promising treatment to suppress local recurrence after tumor resection for osteosarcoma. In this study, we initially used the murine osteosarcoma LM-8 cell line to evaluate cell viability after treatment with BCPD-17-mediated PDT. Then, in an in vivo study we tested the ability of BCPD-17-mediated PDT to suppress local recurrence after tumor resection.

Materials and Methods

BCPD-17 and cell culture materials

The compound BCPD-17 was designed, synthesized, and characterized independently by Yao et al. (2008). The LM-8 cell line was a gift of professor Tatsuya Asai from University of Fukui (Japan) and was a highly metastatic

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murine osteosarcoma cell line, which that was maintained in MEM medium (Gibco, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Sigma Aldrich, USA) and grown at 37°C in a 5% CO_2 humidified environment.

MTT cell viability assay

The LM-8 cells were seeded into 96-well microplates with 2×10^3 cells per well. The cells were treated for 4 hours with 0, 0.5, 1, 2, 4, or 8 μg/ml of BCPD-17. Then, cells were washed twice with medium and irradiated with a 630 nm laser at an output of 0, 1.5, 3, 6, or 9 J/cm^2. The cells were then incubated for 24 hours, and then their viability was assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, which was quantitated based on optical density (OD) with the Bio-Rad Smart Spec 3000 multiwall scanning spectrophotometer (Hercules, CA, USA). Each of the above conditions was tested with five wells. The inhibition rates were calculated as: inhibition rate (%) = ([control well OD – experimental well OD]/control well OD) × 100%. All the experiments were repeated three times.

Morphology analyzed by light microscopy and flow cytometry

A total of 1×10^5 LM-8 cells were seeded in each well of 6-well plates; treated with 0, 2, 4, or 8 μg/ml BCPD-17 for 4 hours, with three wells receiving each treatment; and then irradiated with 630 nm wavelength laser light with a spot diameter of 2.5 cm. The irradiation was applied vertically and equally, such that the energy of each hole was 6 J/cm^2 and the laser output power changes were controlled to < 5% amplitude. Then, the culture medium was changed and the cells were incubated for another 24 hours. The cells were either stained with conventional hematoxylin - eosin (HE) and observed under an optical microscope (Olympus, Tokyo, Japan) or stained with the Annexin -V-FITC apoptosis detection kit for flow cytometry (Becton-Dickinson, USA) at 488 nm to analyze apoptosis and with the Fluor-S MultiImager (Bio-Rad) to investigate the mechanism of cell death.

Marginal resection with PDT on C3H mice with LM-8 tumors

All experiments were performed in compliance with the relevant laws and approved by institutional animal care and use committee of Tongji University School of Medicine, Shanghai, China. We purchased 4-week-old female C3H nude mice from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, P.R. China) and kept them at 20 to 25°C, 40 to 60% relative humidity, with 12 hour day and night conditions. The LM-8 cells were diluted with sterile physiological saline to 10^6/ml so that 0.2 ml of the cell suspension could be inoculated subcutaneously into the left lumbosacral region of the mice. Tumor growth was observed every day, and when it reached 6 to 8 mm in diameter, after about a week, we performed photodynamic experiments on those mice with hemispherical, well-grown tumors and no skin ulcerations. Once the tumors of mice injected with LM-8 cells as described above reached 10 to 12 mm, 30 mice were divided into three treatment groups with 10 mice in each group: (1) marginal resection without PDT (control), which had their tumors resected without excising the surrounding normal tissue; (2) marginal resection, followed by intravenous injection of 5 mg/kg BCPD-17 and, after six hours, PDT with 240 J/cm^2 laser irradiation for 6 hours; and (3) marginal resection, followed by intravenous injection of 5 mg/kg BCPD-17 and, after 6 hours PDT with 360 J/cm^2 laser irradiation for 6 hours. The mice were observed for 4 weeks after the marginal resection of their tumors to compare tumor recurrence rates between these groups.

Statistical analysis

Experimental data from in vitro cell experiments were analyzed with SPSS 16.0 software (Chicago, IL, USA). Analysis of variance and t-test methods were used to test significance, with $P < 0.05$ considered significant. Experimental data on marginal resections using PDT on C3H mice bearing LM-8 tumors used Fisher's exact probability test to compare the rates of tumor recurrence, with $P < 0.05$ considered significant. Bonferroni correction was used for multiple comparisons in the test.

Results

Growth inhibition of LM-8 cells by BCPD-17-mediated PDT

First, we investigated growth inhibition rate of BCPD-17-mediated PDT on the LM-8 cell viability. The LM-8 cells were irradiated and incubated for 24 hours. Inhibition rates of LM-8 cell proliferation were positively proportional to the dose of BCPD-17 and energy of radiation (Figure 1). The growth inhibition rate exceeded 50% for ≥ 4 μg/ml with 6 J/cm^2 of radiation; thus that was considered an effective dose for the treatments.

Cell morphology and apoptosis

Untreated LM-8 cells had normal polygonal growth; large, deeply-stained nuclei; homogeneous, red-stained cytoplasm; and observable mitotic figures (Figure 2A). Cells treated with 2 μg/ml BCPD-17 and 6 J/cm^2 irradiation appeared similar to the control group (Figure

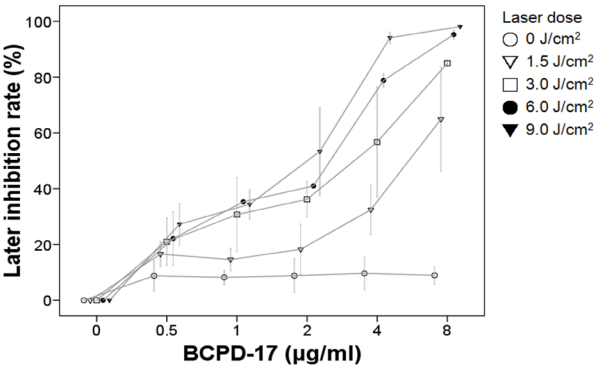

Figure 1. Effects of BCPD-17-mediated PDT on LM-8 Cell Growth. The LM-8 cells were treated with 0 (control), 0.5, 1, 2, 4, or 8 μg/ml BCPD-17. Four hours later, the cells were irradiated with 0 (control), 1.5, 3.0, 6.0, or 9.0 J/cm^2 630 nm laser. After further incubation for 24 hours, the MTT assay measured growth inhibition rates

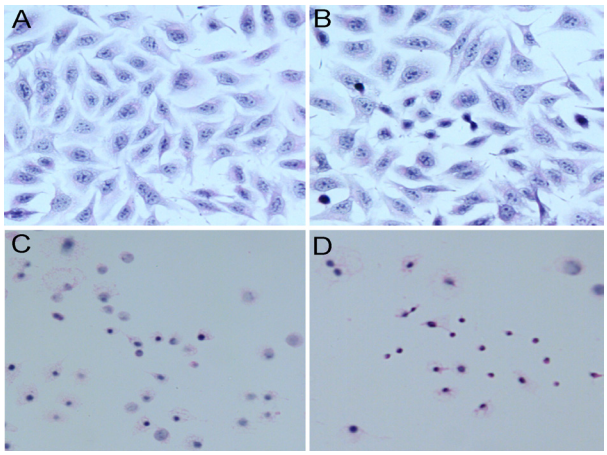

Figure 2. The Morphology of LM-8 Cells Treated with (A) control, (B) 2 μg/ml BCPD-17 and 6 J/cm^2 irradiation, (C) 4 μg/ml BCPD-17 and 6 J/cm^2 irradiation, and (D) 8 μg/ml BCPD-17 and 6 J/cm^2 irradiation

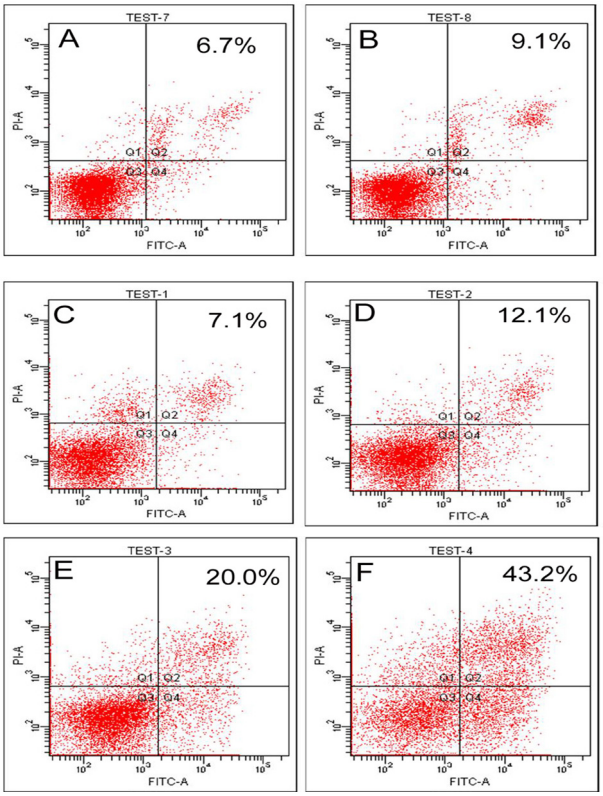

Figure 3. Flow Cytometry Analyses of Apoptosis of LM-8 Cells Treated with (A) control, (B) 4 μg/ml BCPD-17 only, (C) 6 J/cm^2 irradiation only, (D) 1 μg/ml BCPD-17 and 6 J/cm2 irradiation, (E) 2 μg/ml BCPD-17 and 6 J/cm^2 irradiation, and (F) 4 μg/ml BCPD-17 and 6 J/cm^2 irradiation. The values indicate percentage of cells undergoing apoptosis, which appear in Q2

2B), while those treated with 4 μg/ml BCPD-17 (Figure 2C) and 8 μg/ml BCPD-17 (Figure 2D) had severe necrosis and apoptosis. Then, we detected apoptosis rates of LM-8 cells treated with BCPD-17 with the Annexin V staining assay. For the controls, 4 μg/ml BCPD-17 treated only or 6 J/cm^2 irradiation treated only cells, the apoptosis rates were about 6.7%, 9.1%, or 7.1%, respectively (Figure 3A-C). Under 6 J/cm^2 irradiation, with increasing BCPD-17 concentrations of 1 μg/ml, 2 μg/ml, and 4 μg/ml, the apoptosis rates of the treated LM-8 cells at 24 hours after

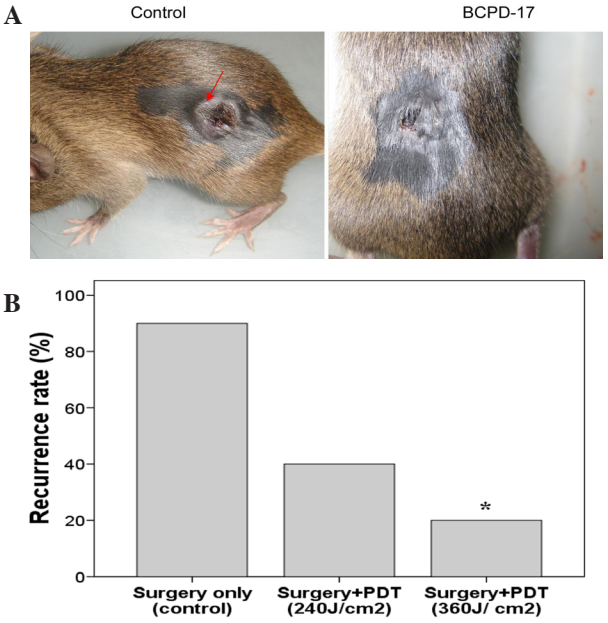

Figure 4. The C3H Nude Mice with Tumors from LM-8 Cells were Treated by Resection Only (A, left) or 5 mg/kg BCPD-17 and 240 J/cm^2 irradiation for 6 hours (A, right), with a recurrent tumor in the control group indicated by the red arrow. (B) Recurrence rates are compared for mice treated with resection (surgery only, control), surgery with PDT consisting of 5 mg/kg BCPD-17 and 240 J/cm^2 irradiation, or surgery with PDT consisting of 5 mg/kg BCPD-17 and 360 J/cm^2

irradiation were 12.1%, 20.0%, and 43.2%, respectively (Figure 3 D-F).

Local tumor recurrence after marginal resection with or without BCPD-17-mediated PDT

We examined local tumor recurrence after marginal resection with or without BCPD-17-mediated PDT. Local recurrence was found after marginal resection of the tumor in mice without BCPD-17-mediated PDT, while local recurrence was obviously suppressed by BCPD-17-mediated PDT (Figure 4A). Local recurrence occurred within 4 weeks for 9 of 10 of the control mice. Of the mice treated with resection and 5 mg/kg BCPD-17 with 240 J/cm^2 irradiation, four of the 10 had recurrence, while recurrence occurred in two of the 10 mice treated with 5 mg/kg BCPD-17 and 360 J/cm^2 irradiation (Figure 4B). Treatment with BCPD-17-mediated PDT in combination with marginal resection did not produce side effects like delayed wound healing or skin defects.

Discussion

The treatment of cultured LM-8 osteosarcoma cells with BCPD-17-mediated PDT inhibited cell growth in a dose-dependent manner. Photodynamic therapy mediated by BCPD-17 is potentially useful for the treatment of osteosarcomas.

In recent years, much research has been directed at methods of sensitizing tumor cells to the effects of radiation (Yu et al., 2012). BCPD-17 as a secondary generation light sensitizer has greater purity, lower toxicity, and greater efficacy. Therapy with BCPD-17-mediated PDT induces cytotoxic substances that lead to

apoptosis and necrosis in the lesion (Plaetzer et al., 2003; Burch et al., 2009; Jeong et al., 2011). The most vulnerable parts of cells are the membranes, particularly those of the mitochondria and the lysosomes (Lavi et al., 2002; Mak et al., 2004; Liu et al., 2011). Damage to the mitochondria results in the release of cytochrome c, which begins the cascade that leads to apoptosis. Also, PDT may induce the disintegration of the cell structure and alter genetic information, which directly causes cell death (Rancan et al., 2005; Pogue et al., 2011). This is consistent with the rapid apoptosis (after 15 minutes) we observed (Figure 1), while the experimental doses exhibited no differences at 60 to 90 minutes. High doses of PDT may cause cell death without obvious early apoptosis. Our results are consistent with previous reports (Das et al., 2000; Woods et al., 2004).

When tumors were marginally resected and subsequently treated with PDT, the rate of local recurrence was reduced, and less of a surgical margin was required which preserved critical anatomic structures adjacent to the tumor as muscles, nerves, and blood vessels. The reduced recurrence rate and preservation of function that we observed with high irradiation doses agree with reports that recurrence rates were reduced to 23% with PDT treatment compared with 80% with control treatment, and that function was preserved (Kusuzaki et al., 2000). All of the recurrences after high-dose PDT treatment that we observed were near the surgical margins, which suggests that selecting a high dose of irradiation to more fully clear the deep tumor cells may reduce the chance of recurrence from residual tumor tissue.

Angiogenesis has been shown to be an important factor in the growth and metastasis of osteosarcoma (Chen et al., 2012), and the destruction of tumor blood vessels is an important effect of PDT. The capillary chamber is penetrated by BCPD-17 in the early stage of treatment (Henderson and Fingar, 1989), and PDT may injure the capillary endothelial cells of the target tissue so that microcirculation stops and vasculature closes. We observed vascular injuries in the tissue treated with PDT mediated by BCPD-17, including a large number of small artery occlusions. Also, the deeper necrotic foci had many inflammatory cells, which is an obvious difference from spontaneous tumor necrosis and suggests the need for further study of the immune response.

This study has several limitations. As the mechanisms of cell death caused by PDT are complex (Berkovitch-Luria et al., 2011; O'Connor et al., 2012), it is difficult to predict if necrosis or apoptosis will dominate. Although tumor necrosis was observed, we failed to obtain a complete cure of the tumor, which may be due to the doses of photosensitizer and laser irradiation that we used. Determining the proper doses requires further study. Additionally, the effects of BCPD-17 should be examined in more tumor cell lines, such as non-osteosarcoma tumor cell lines.

In conclusion, photodynamic therapy with BCPD-17 exhibited an in vitro cytotoxic effect on cultured LM-8 osteosarcoma cells that depended on the dose of BCPD-17 and of irradiation. When PDT with BCPD-17 was performed after tumors were marginally resected, the rate of local recurrence and the surgical margins were reduced, which preserved critical anatomic structures as muscles, nerves, and vessels adjacent to the tumor. Our results indicate that BCPD-17-mediated PDT in combination with marginal resection of tumors may be a potentially useful treatment for osteosarcoma.

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