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Application of a Novel Boronated Porphyrin (H₂OCP) as a Dual Sensitizer for Both PDT and BNCT

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Abstract

Background and Objective—Boronated porphyrins have emerged as promising dual sensitizers for use in both photo-dynamic therapy (PDT) and boron neutron capture therapy (BNCT), by virtue of their known tumor affinity, low cytotoxicity in dark conditions, and easy synthesis with high boron content. Octa-anionic 5,10,15,20-tetra[3,5-(nido-carboranyl)methyl]phenyl porphyrin (H₂OCP) is a boronated porphyrin having eight boron clusters linked to the porphyrin ring. To evaluate H₂OCP's applicability to both PDT and BNCT, we performed an in vitro and ex vivo study using F98 rat glioma cells.

Materials and Methods—We examined the time-dependent cellular uptake of H₂OCP by measuring the boron concentration over time, and compared the cellular uptake/clearance of boron after exposure to H₂OCP in conjunction with boronophenylalanine (BPA) and sodium borocaptate (BSH), both of which are currently used in clinical BNCT studies. We evaluated the cytotoxicity of H₂OCP-mediated PDT using a colony-forming assay and assessed the tumor-igenicity of the implantation of pre-treated cells using Kaplan–Meier survival curves. Fluorescence microscopy was also performed to evaluate the cellular uptake of H₂OCP.

Results—H₂OCP accumulated within cells to a greater extent than BPA/BSH, and H₂OCP was retained inside the cells to approximately the same extent as BSH. The cell-surviving fraction following laser irradiation (8 J/cm², 18 hours after exposure to 10 µg B/ml H₂OCP) was <0.05. The median survival times of the pre-treated cell-implanted rats were longer than those of the untreated group (*P* < 0.05). The fluorescence of H₂OCP was clearly demonstrated within the tumor cells by fluorescence microscopy.

Conclusions—H₂OCP has been proven to be a promising photosensitizer for PDT. H₂OCP has also been proposed as a potentially effective replacement of BPA or BSH, or as a replacement of both BPA/BSH. Our study provides more evidence that H₂OCP could be an effective novel dual sensitizing agent for use in both PDT and BNCT.

Keywords

boron neutron capture therapy; boronated porphyrin; F98 rat glioma cells; H₂OCP; photodynamic diagnosis; photodynamic therapy

INTRODUCTION

The prognosis of patients with malignant glioma, especially glioblastoma (GB), is poor. The median survival of GB patients is <2 years after the initial diagnosis [1], with most recurrence occurring at the site of the original tumor. Therefore, more aggressive local therapies are necessary to eradicate unresectable tumor cells that invade adjacent normal brain tissue. Two adjuvant therapies with the potential to destroy these cells are photodynamic therapy (PDT) [2–4] and boron neutron capture therapy (BNCT) [5–8]. Both are bimodal therapies, the individual components of which are non-toxic in isolation but tumoricidal in combination. Boronated porphyrins have emerged as promising dual sensitizers for both PDT and BNCT by virtue of the following characteristics: tumor affinity by the porphyrin ring; ease of synthesis with a high boron content; low cytotoxicity in dark conditions; and desirable photophysical properties, including strong light absorption in the visible and near infrared regions, the ability to generate singlet oxygen upon light activation, and fluorescence properties [9,10]. Several boronated porphyrins have been synthesized and evaluated in cellular and animal studies [9,10]. Among these, boronated porphyrins BOPP [11,12] and CuTCPH [13], each containing four boron clusters, have been extensively investigated. This type of boronated porphyrin was found to selectively deliver therapeutic concentrations of boron into tumor cells with low cytotoxicity in dark conditions and with long retention times within tumors. Boronated porphyrins having high boron content (up to 16 boron clusters) have been reported, [9,14] and it has been postulated that this type of compound could potentially deliver higher amounts of boron to tumors at the same dose. In particular, the synthesis and cellular evaluation of the octa-anionic 5,10,15,20-tetra[3,5-(nido-carboranylmethyl)phenyl] porphyrin (H₂OCP), containing eight boron clusters (38% boron by weight), have been reported previously by the authors[15]. In that study, H₂OCP was shown to deliver high amounts of boron to human glioma T98G cells with low cytotoxicity in dark conditions. In this study, we evaluated the potential of H₂OCP as a dual sensitizer for both PDT and BNCT using F98 rat glioma cells. Although several boronated porphyrins have been proposed as boron delivery agents for BNCT, only a few have been investigated as dual sensitizers for both PDT and BNCT of tumors [9,16].

MATERIALS AND METHODS

Boron Delivery Agents

The H₂OCP was prepared as previously described [15]. Boronophenylalanine (BPA) (L-isomer) was kindly supplied by the Stella Chemifa Corporation (Osaka, Japan) and was prepared as a fructose complex [17]. Sodium borocaptate (BSH) was purchased from Katchem Ltd. (Katchem, Prague, Czech Republic) and dissolved in sterile saline.

Cell Culture

F98 rat glioma cells produce infiltrating tumors in the brains of Fischer rats[18]. The tumors have been shown to be refractory to a number of treatment modalities, including radiation therapy [19]. Based on their in vivo histology, the F98 rat glioma cells have been characterized as anaplastic or undifferentiated glioma [20]. In the present study, F98 rat glioma cells were kindly obtained from Dr. Barth (Department of Pathology, the Ohio State University, Columbus, OH). They were routinely cultivated in our laboratory in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and penicillin at 37°C in an atmosphere of 5% CO₂. All the materials for the culture medium were purchased from Gibco Invitrogen (Grand Island, NY).

Cellular Uptake/Clearance of Boron

The F98 rat glioma cells were seeded in 100 mm dishes (BD Falcon™, Franklin Lakes, NJ), and the culture medium without H₂OCP was exchanged for the H₂OCP-containing culture medium just before confluence. In all cellular studies, three 100 mm dishes for each cellular study were used. H₂OCP was dissolved in DMSO prior to dilution into the culture medium; the final DMSO concentrations never exceeded 1%. After the completion of exposure, the H₂OCP-containing culture medium was removed and the cells were washed twice with 4°C phosphate-buffered saline (PBS). Finally, the cells were retrieved using trypsin and fed 60% nitric acid in the cellular solution to extract intracellular boron. In order to evaluate the time-dependent boron uptake, the cells were exposed to 20 µg B/ml of H₂OCP for 6, 12, or 18 hours. Cellular uptake/clearance experiments were conducted using culture media containing 20 µg B/ml boron from either the H₂OCP, BPA, or BSH stock solutions and were exposed to the cells for a 12-hour period, followed by clearance times of 0, 2, and 6 hours. The boron concentrations were analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES) using an iCAP6000 emission spectrometer (Hitachi High-Technologies, Tokyo, Japan). PBS and trypsin were purchased from Gibco Invitrogen, and the 60% nitric acid was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Colony-Forming Assay

F98 rat glioma cells were incubated in culture media with two different doses of H₂OCP (5 and 10 µg B/ml) and without H₂OCP (control) for 18 hours in 150 cm² flasks (TPP®; Zollstrasse, Trasadingen, Switzerland). Following incubation, the cells were retrieved from the flasks, seeded onto 60 mm dishes (BD Falcon™) with 10⁴ cells each and irradiated with visible light of 405 nm from a diode laser (Ball Semiconductor, Frisco, TX). The cells were evenly irradiated at powers of 0 (control), 2, 4, and 8 J/cm². Following laser irradiation, the cells were seeded onto dishes; each with the same predetermined number of cells, iteratively. After 7 days, all of the colonies (>50 cells) were counted and assessed by calculating the cell-surviving fraction.

Tumorigenesis of In Vitro Pre-Treated Tumor Cells

In the treated group, F98 cells were exposed to 20 µg B/ml H₂OCP for 12 hours at 37°C prior to laser irradiation (4 J/cm²), after which the tumor cells were implanted into 10 male Fischer rats. As a control study, untreated F98 cells were prepared and implanted in five male rats. Dead cells were stained with trypan blue just before implantation, counted under microscope, and expressed as a percentage of total cells perfield-of-view segment. Viable cells were counted and were implanted into the rat brains. All male Fischer rats (200–250 mg) were anesthetized with an intraperitoneal injection of Nembutal (50 mg/kg) and placed in a stereotactic frame (Model 900, David Kopf Instruments, Tujunga, CA). A mid-line scalp incision was made and the bregma was identified. A 1 mm burr hole was made in the right frontal region of the skull and a 22-gauge needle attached to a 25 µl syringe was inserted into the caudate nucleus using the same stereo-tactic coordinates, with the needle tip inserted 5 mm into the dura. An injection of 10⁵ cells in 10 µl of serum free medium was administered at a rate of 1 µl/minute. After the infusion, the needle was left in place for 3 minutes and the burr hole was then covered with bone wax. After implantation surgery, the body weight and neurological function of the rats were monitored daily. One day before death became imminent (defined by significant weight loss and a lack of activity or severe neurological deficits), the rats were euthanized and Kaplan–Meier survival curves were plotted and analyzed.

Cytotoxicity of H₂OCP in Dark Conditions

We examined the cytotoxicity of H₂OCP in the dark with a viable cell-counting method and a colony-forming assay. F98 rat glioma cells were seeded in 100 mm dishes and were incubated in culture media containing two different concentrations of H₂OCP (0, 20 µg B/ml) for cell counting. After exposure to H₂OCP for 24 hours, the cells were counted using the trypan blue dye exclusion method. This assay was performed in triplicate. The cytotoxicity in the cell count was assessed by the percentage of viable of cells. For the colony-forming assay, F98 rat glioma cells were seeded in 100 mm dishes and were incubated in culture media containing five different concentrations of H₂OCP (0, 5, 10, 20, 40 µg B/ml). After exposure to H₂OCP for 24 hours, the cells were retrieved from the dishes and were seeded onto 100 mm dishes, each with the same predetermined number of cells. This assay was also performed in triplicate. After 7 days, all of the colonies (>50 cells) were counted and assessed by calculation for the cell-surviving fraction.

Fluorescence Microscopy

F98 rat glioma cells were seeded in a two-well chamber mounted on glass slides with a cover (Nalge Nunc International, Rochester, NY) and the culture medium without H₂OCP was exchanged for the H₂OCP-containing culture medium just before confluence. The cells were exposed to 20 µg B/ml H₂OCP for 24 hours. After exposure, the glass slides were washed with 4°C PBS and the two-well chamber was removed. The nucleus-specific hoechst dye (Hoechst 33342, Lonza, Maryland, MD) was added (10 µg/ml) and the glass slides mounted onto cover glasses using DPX Mountant for histology (44581, Fluka Biochemika, Darmstadt, Germany). The two-well chamber slides were observed using an inverted fluorescence microscope system (BZ-8000, Keyence, Tokyo, Japan).

RESULTS

Cellular Uptake/Clearance of Boron

The measured cellular boron concentrations obtained by in vitro cellular delivery using H₂OCP were 158.2 ± 3.8 , 272.2 ± 15.3 , and 405.1 ± 22.6 ng B/ml 10^6 cells after 6, 12, and 18 hours of exposure, respectively. Nearly three times more boron was found within cells after 18 hours of exposure than after 6 hours of exposure (Fig. 1). The determined cellular boron concentrations for in vitro cellular uptake/clearance of boron in response to exposure to H₂OCP, BPA, and BSH for 12 hours were 272.2 ± 15.3 , 239.7 ± 12.3 , and 85.2 ± 2.0 ng B/ml 10^6 cells, respectively. In contrast, at 6 hours after exposure the cellular boron concentrations were 246.7 ± 14.6 , 84.9 ± 0.7 , and 67.0 ± 4.6 ng B/ml 10^6 cells, respectively. At the same boron dose, H₂OCP delivered significantly higher amounts of boron to cells than did BPA or BSH (log-rank test, $P < 0.05$). Furthermore, while BPA cleared rapidly from cells, both H₂OCP and BSH showed high cellular retention of boron for up to 6 hours (Fig. 2).

Colony-Forming Assay

The cytotoxicity of H₂OCP determined by laser irradiation using a colony-forming assay showed that the surviving fraction of cells following exposure to H₂OCP (10 µg B/ml for 18 hours) and laser irradiation was 0.326 ± 0.031 , 0.246 ± 0.037 , and 0.045 ± 0.001 using 2, 4, and 8 J/cm² light dose, respectively. Under the same conditions, the surviving fractions of the laser-only control (without H₂OCP) were 0.861 ± 0.182 , 0.776 ± 0.035 , and 0.299 ± 0.023 , respectively. The most efficient PDT-induced tumoricidal effect was achieved when the cells were irradiated with 8 J/cm², 18 hours after exposure to H₂OCP (<0.05) (Fig. 3).

Tumorigenesis of In Vitro Pre-Treated Tumor Cells

The observed tumorigenicity of the implanted pre-treated cells using Kaplan–Meier survival curves revealed median survival times of 12 and 14 days in the untreated and the treated groups, respectively, and mean survival times of 11.8 and 14.6 days after implantation, respectively. In Kaplan–Meier survival curve analysis, these survival times demonstrated a significant difference (log-rank test, $P < 0.05$) (Fig. 4).

Cytotoxicity of H₂OCP in Dark Conditions

The viable cell-counting method revealed the following results. The percentage of cell viability with exposure of 20 $\mu\text{g B/ml}$ H₂OCP was $98.0 \pm 1.4\%$ (mean $\pm$ SD), while that of cell viability without H₂OCP was $98.0 \pm 0.9\%$. The colony-forming assay showed the following: the surviving fractions with each boron concentration (0, 5, 10, 20, 40 $\mu\text{g B/ml}$ H₂OCP) were 1, 0.99 ± 0.04 (mean $\pm$ SD), 0.98 ± 0.05 , 0.98 ± 0.01 , and 0.98 ± 0.03 , respectively. These results showed no significant differences (Welch's t -test, $P > 0.05$).

Fluorescence Microscopy

The fluorescence microscopy showed the intracellular porphyrin fluorescence and images from the co-localization experiment using the nucleus-specific Hoechst dye. These results showed that H₂OCP was taken up into the cells and also localized in the nuclei (Fig. 5B–D). Although the cells showed evidence of cytotoxic damage, the cytotoxicity of H₂OCP in dark conditions was not found at twice the concentration of H₂OCP used in this fluorescence microscopy experiment. (Fig. 5A).

DISCUSSION

BNCT is a targeted chemo-radiation therapy that significantly increases the therapeutic ratio relative to conventional radiotherapeutic modalities. In BNCT, a ¹⁰B-labeled compound delivers therapeutic concentrations of ¹⁰B ($\sim 30 \mu\text{g } ^{10}\text{B/g}$ tumor) to the target tumor, with high tumor-to-blood and tumor-to-normal-tissue ratios and low cytotoxicity [5,6]. Subsequently the tumor is irradiated with epithermal neutrons that become thermalized at a certain depth within the tissues. The short range ($<10 \mu\text{m}$) of the α and ⁷Li high linear energy transfer (high-LET) particles released from the ¹⁰B(n, α) ⁷Li neutron capture reaction makes the tumor microdistribution of ¹⁰B critically important in BNCT [21]. Since the high-LET particles are highly cytotoxic, their killing effect depends on the site of generation. These characteristics contribute to the tumor selectivity and strong tumoricidal activity of BNCT, with negligible damage to normal tissue. Therefore, if sufficient quantities of boron can be selectively delivered to tumor tissues, BNCT could be an ideal tumor-selective particle beam irradiation local therapy for malignant gliomas. Clinically, BPA and BSH are currently available for BNCT as boron delivery agents. BPA is a boronated derivative of an essential amino acid (L-phenylalanine) that is actively taken up by tumor cells, presumably via the amino acid transport mechanism [22]. BSH, on the other hand, is believed to preferentially accumulate within tumor tissue via a partially destroyed or leaky blood brain barrier, and is thought to be retained longer than BPA due to its higher hydrophobic character [23]. We have used both of these boron delivery agents in combination in clinical BNCT studies, and have previously been reported on the survival benefit from BNCT for newly diagnosed GB patients [7] as well as for recurrent malignant glioma patients [8]. However, the present results using BPA and BSH are far from satisfactory, and the use of more effective boron delivery agents should provide enhanced clinical outcomes for BNCT. The so-called third-generation of boron delivery agents [6], including boronated porphyrin derivatives, molecular-targeted agents (e.g., to EGFR), and liposome-linked boron delivery agents could potentially greatly increase the efficacy of BNCT in the clinical setting. Among these boron delivery agents, boronated porphyrins are particularly promising because they

contain a porphyrin ring with tumor affinity, and they are also excellent photosensitizers for PDT [9]. In a similar fashion to BNCT, PDT is a localized therapy that relies on the specific uptake of a photosensitizer in the tumor relative to the surrounding normal tissue, followed by laser irradiation for activation of the photosensitizer [2,24]. The photoactivation of the sensitizer causes oxidative damage to a variety of cellular targets via the release of singlet oxygen and other reactive oxygen species, with subsequent tumor necrosis. To date, the clinical trials with PDT employed as an adjuvant treatment for human gliomas have used the poorly defined heterogeneous porphyrin mixture hematoporphyrin derivative (HpD) or its more enriched commercial preparation, Photofrin [2], which does not contain boron. This photosensitizer has been shown to localize preferentially in glioma relative to normal brain tissue. Moreover, reports of PDT as a treatment for animal and human glioma have been encouraging, and a photosensitizer that is more tumor selective than HpD or Photofrin would have great clinical benefits.

In this study, our results showed the positive efficacy of PDT using H₂OCP in a colony-forming assay (Fig. 3) and in tumorigenesis of in vitro pre-treated cells in Fisher rats (Fig. 4). Additionally, H₂OCP accumulated within the glioma cells to a significantly higher extent than BPA or BSH ($P < 0.05$), and was retained inside the cell to approximately the same extent as BSH (Fig. 2). Based on these findings, we postulated that H₂OCP could be applied to both PDT and BNCT for treatment of glioma tumors. In the fluorescence microscopy experiment, although the cells in the bright field image showed evidence of cytotoxic damage (Fig. 5A), the cytotoxicity of H₂OCP in dark conditions was not observed, even at double the concentration of H₂OCP used in the fluorescence microscopy experiment. Therefore, we considered that the damage to the cells in the bright field image was most probably due to technical complications related to irradiation with the laser during imaging, rather than to the cytotoxic effects of H₂OCP itself. Furthermore, H₂OCP was shown to be taken up by F98 rat glioma cells (Fig. 5B–D). Therefore, our results suggest that H₂OCP can be used intraoperatively for photodynamic diagnosis (PDD) and fluorescence-guided resection of brain tumors.

Pre-operative administration of a boronated porphyrin has a number of advantages in the clinical setting. As noted previously, boronated porphyrins are useful in PDD and in fluorescence-guided resection of brain tumors during surgery. Using fluorescence-guided resection of such tumors during surgery, the resection rate can be augmented, with expected further improvements in patient prognosis [25]. In addition, boronated porphyrins can be used with intra-operative PDT and post-operative BNCT. Although the initial results with commonly used photosensitizers for PDT such as Photofrin (or its unpurified form HpD) were very encouraging, treatment failures did occur, mainly due to the limited penetration of light into the brain. In cases with deep lesions, PDT alone may be inadequate to achieve complete tumor treatment, and it would be preferable in such cases to use BNCT as a supplementary treatment, with boron-containing porphyrin as a photosensitizer. Fairchild et al. [26] reported that thermal and epithermal neutrons are transported to a depth of approximately 10 cm in fact, BNCT has been shown to treat deep lesions. Since boronated porphyrins can be effective for BNCT as boron delivery agents while retaining their photosensitizer ability, the limited penetration of light can be overcome using a combination of BNCT and PDT for the treatment of human gliomas.

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reviewed the manuscript; TK reviewed the manuscript; MWE synthesized H₂OCP and reviewed the manuscript; MGHV synthesized H₂OCP, interpreted data, and reviewed the manuscript. All authors have read and approved the final manuscript.

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Fig. 1. Cellular uptake of boron (ng B/ml 10^6 cells) after 6, 12, and 18 hours of exposure to 20 μ g B/ml H_2OCP .

Fig. 2.

Comparison of cellular uptake/clearance of boron (ng B/ml 10^6 cells) after exposure to $20 \mu\text{g B/ml}$ of either H_2OCP , BPA, or BSH under identical conditions. Left and right Y-axes show the measured value (ng B/ml 10^6 cells) and percentage of boron concentration, respectively. The cellular uptake of boron using H_2OCP showed values higher than those for BPA and BSH ($P < 0.05$), and the cellular retention of boron using H_2OCP showed values similar to those obtained using BSH.

Fig. 3. Colony-forming assay using F98 rat glioma cells exposed to 0 (control), 5, and 10 µg B/ml of H₂OCP and irradiated with light doses of 0, 2, 4, and 8 J/cm², respectively. The cell-surviving fraction following laser irradiation (8 J/cm², 18 hours after exposure to 10 µg B/ml H₂OCP) was < 0.05.

Fig. 4.

Kaplan–Meier survival curves following in vitro pre-treated F98 cells using H₂OCP-mediated PDT. Rats were implanted i.c. with F98 cells and were either untreated (dotted line) or treated with PDT (continuous line). Cells were exposed to 20 µg B/ml of H₂OCP for 24 hours at 37°C prior to laser irradiation. After laser irradiation (4 J/cm²), the tumor cells were implanted into the rats. Cell viability was determined by trypan blue exclusion staining and 10⁵ viable cells were implanted stereotactically into the caudate nucleus. The median survival times of the untreated control and the treated group were 12 and 14 days, and the mean survival times were 11.8 and 14.6 days after implantation, respectively ($P < 0.05$).

Fig. 5.

Images obtained using an inverted fluorescence microscope. **A:** Bright field image. Although the cells showed evidence of cytotoxic damage, the cytotoxicity of H₂OCP in dark conditions was not found at twice the concentration of H₂OCP used in this fluorescence microscopy experiment. **B:** Fluorescence of porphyrin H₂OCP. **C:** Nuclear fluorescence by Hoechst dye (excitation wavelength was 340–380 nm). **D:** Merged image. (Magnification of all images: $\times 600$).