Selective tumor kill of cerebral glioma by photodynamic therapy using a boronated porphyrin photosensitizer

(brain tumor/hematoporphyrin/boron/neutron capture/phototherapy)

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ABSTRACT   The prognosis for patients with the high-grade cerebral glioma glioblastoma multiforme is poor. The median survival for primary tumors is <12 months, with most recurring at the site of the original tumor, indicating that a more aggressive local therapy is required to eradicate the unresectable "nests" of tumor cells invading into adjacent brain. Two adjuvant therapies with the potential to destroy these cells are porphyrin-sensitized photodynamic therapy (PDT) and boron-sensitized boron neutron capture therapy (BNCT). The ability of a boronated porphyrin, 2,4-(α,β-dihydroxyethyl)deuteroporphyrin IX tetrakiscarborane carboxylate ester (BOPP), to act as a photosensitizing agent was investigated in vitro with the C6 rat glioma cell line and in vivo with C6 cells grown as an intracerebral tumor after implantation into Wistar rats. These studies determined the doses of BOPP and light required to achieve maximal cell kill in vitro and selective tumor kill in vivo. The data show that BOPP is more dose effective in vivo by a factor of 10 than the current clinically used photosensitizer hematoporphyrin derivative and suggest that BOPP may have potential as a dual PDT/BNCT sensitizer.

Cerebral tumors are responsible for ≈2% of all cancer deaths, although they are the fourth most important tumor in terms of life years lost, since they disproportionately affect younger people (1). The majority of these deaths are due to high-grade gliomas—anaplastic astrocytoma and glioblastoma multiforme. At present there is no curative treatment for these tumors. Surgery provides a definitive histological diagnosis and relief of raised intracranial pressure, and radiotherapy and adjuvant chemotherapy are of limited benefit. Most studies utilizing these treatments report median survival times of <1 year (1–3). Treatment failures are due to recurrence of the tumor at the original site, suggesting that more aggressive local therapies may be beneficial in controlling the tumor. Many locally targeted experimental therapies have been investigated, but none have demonstrated definite clinical benefit. These include interstitial brachytherapy (4), retroviral gene therapy (5), locally directed immunotherapy (6), and hyperosmolar disruption of the blood–brain barrier prior to chemotherapy (7).

Two therapies with the potential to control local recurrence are photodynamic therapy (PDT) and boron neutron capture therapy (BNCT). Both are bimodal therapies, the individual components of which are nontoxic in isolation, but which are tumoricidal in combination. PDT relies on the selective uptake of a photosensitizing chemical in tumor relative to the surrounding normal tissue, followed by irradiation with light of an appropriate wavelength to activate the photosensitizer (8). The

photoactivation of the sensitizer causes oxidative damage to a variety of cellular targets and subsequent tumor necrosis (8). To date, the clinical trials of PDT 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 (for review, see ref. 9).

These sensitizers have been shown to localize in glioma relative to normal brain (9–11), and while the reports of PDT as a treatment of animal (9, 11–15) and human (9, 13, 16, 17) glioma have been encouraging, the use of a purified sensitizer that is more tumor selective than HpD or Photofrin would have great clinical benefit.

BNCT, like PDT, is a bimodal therapy that relies on selective localization in tumors of a compound containing the ^{10}B isotope of boron followed by activation by epithermal neutrons, resulting in production of the high linear energy particles ^4He and ^7Li, which can cause extremely localized tumor cell death (18). Currently, few of the potential BNCT sensitizers exhibit highly selective tumor uptake, and the neutron sources that are available are suboptimal as they do not produce an epithermal beam free from a contaminating fast neutron component that can cause severe normal brain toxicity. Nevertheless, the capacity for epithermal neutrons to deeply penetrate brain and reach the invasive nests of tumor cells suggests that if the two components of the therapy are optimized, then BNCT may be of great benefit.

We have previously investigated the uptake of 2,4-(α,β-dihydroxyethyl)deuteroporphyrin IX tetrakiscarborane carboxylate ester (BOPP) (19) into the intracerebral C6 glioma xenograft grown in CBA mice (20). BOPP exhibits spectral absorbance properties similar to HpD (19, 20) and is a potential sensitizer for PDT and/or BNCT. Pharmacological studies have shown that BOPP is extremely selectively taken up by the glioma relative to the surrounding normal brain, with tumor/brain ratios as high as 400:1 (20), which are much greater than those observed for HpD in the same animal model or in human gliomas, where ratios between 10:1 and 50:1 have been noted (10). Importantly, there was also retention of BOPP in the tumor, with high selective ratios being maintained for a number of days postsensitization (20). In addition the tumor/blood ratios were high, indicating that the BOPP probably partitioned into the lipid-rich membranes within the tumor and was not present in the more aqueous environments such as the tumor stroma or blood vessels (20). However, the most critical finding of that study was that BOPP was detected by confocal laser scanning microscopy in the isolated "nests"

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of tumor cells invading into the edematous brain adjacent to the tumor (20, 21). It is these cells that are responsible for tumor recurrence (1, 2, 9) and that must be the target of any adjuvant therapy aiming to improve the treatment of cerebral glioma (9). We report in this paper on the use of BOPP as a photosensitizer of rat C6 glioma cells *in vitro* and of the comparison of BOPP vs. HpD as PDT photosensitizers in the C6 intracranial glioma model in Wistar rats.

MATERIALS AND METHODS

Materials. All reagents used were of analytical grade or better. Dulbecco's PBS, RPMI 1640 liquid medium, fetal calf serum (FCS), and trypsin/versene were purchased from Commonwealth Serum Laboratories (Parkville, Australia); HpD was purchased and prepared as described (11).

The C6 rat glioma cell line was obtained from the American Type Culture Collection.

Methods. *In vitro cytotoxicity of C6 cells exposed to BOPP or HpD.* C6 cells were cultured and harvested as described (22) and a single cell suspension (200 cells per 3 ml) in RPMI 1640 medium supplemented with 10% FCS was prepared. Three-milliliter aliquots were then seeded into 25-cm² flasks, which were wrapped in foil, and incubated at 37°C for 6 hr, whereupon the medium was removed and replaced with 3 ml of RPMI 1640 medium containing either BOPP or HpD at concentrations of 0–200 μg/ml. The flasks were incubated for a further 18 hr to allow for sensitizer uptake, at which time the medium and exogenous sensitizer were removed and the adherent cells were washed twice with 5 ml of fresh sensitizer-free medium, prior to addition of 3 ml of fresh medium. The flasks were incubated for a further 7 days and the resultant colonies were fixed, stained, and counted as described (22). At all times before fixation, the flasks were wrapped in aluminum foil and all procedures were carried out in dim light.

In vitro phototoxicity of C6 cells exposed to BOPP or HpD. Three-milliliter aliquots of a suspension of C6 cells (200 cells) were added to foil-wrapped 25-cm² flasks as described above. After allowing 6 hr for adherence, the cells were washed as described above and incubated for 18 hr in RPMI 1640 medium containing either BOPP at 0–80 μg/ml or HpD at 6 μg/ml. The foil was then removed from the flasks in a dark room, and the flasks were placed on a light-box and exposed to red-filtered light from a fluorescent source (NEC 15-W standard cool white; Nippon Electrical, Tokyo) for between 0 and 120 min. The red light output was achieved by placing filters between the flasks and the opaque Perspex top of the light box. This filter combination (Roscoe Supergel nos. 15 Deep straw and 25 Orange red; Masson Photographics, Bulleen, Australia) allowed transmission only of light > 600 nm at an energy dose of 0.2 J·min⁻¹·cm⁻². After light exposure, the flasks were rewrapped in foil and incubated for a further 7 days, when the adherent colonies were fixed, stained, and counted as described above.

Cell culture and intracranial tumor implantation. The C6 glioma cells were cultured in RPMI 1640 medium supplemented with 10% FCS as described (11, 22). The cells were harvested with trypsin/versene while in the logarithmic phase of growth; before implantation a single cell suspension (4 × 10⁶ cells per ml) was prepared, and tumor implantation was performed as described (11, 22). Rats bearing the intracerebral C6 tumor used in this study were typically treated 10–12 days postimplantation of the tumor cells. The rats designated as the non-tumor-bearing group were given the same operative procedure as detailed above, except that no tumor cells were injected.

Preparation and administration of BOPP. The photosensitizer BOPP was prepared as described as a dried powder of the potassium salt (19, 20) and was stored under desiccation *in vacuo* at −20°C. Typically, solutions of 10 mg/ml in 0.9%

isotonic NaCl were prepared and were stored in darkness at 4°C for no more than 24 hr before administration via i.v. tail vein injection into rats.

PDT of tumor-bearing and non-tumor-bearing rats. PDT was administered to tumor-bearing and non-tumor-bearing rats 24 hr postsensitization at doses of 5, 10, 25, or 50 mg of BOPP per kg of body weight. The protocol for administration of PDT was as described (11). Briefly, light of 628 nm from a gold metal vapor laser (Quentron, Adelaide, Australia) was administered via a 600-μm (inner core diameter) quartz optical fiber, which was fixed in a vertical position 6 mm over the exposed brain so that the entire red light spot completely covered the exposed brain or tumor and was within the limits of the craniotomy site. The power output at the fiber tip was determined with a power meter (model 361; Scientech, Boulder, CO) and was typically between 800 and 1000 mW. The dose of light administered was between 0 and 500 J/cm² of the irradiation area. The surface of the brain was continuously irrigated with 0.9% isotonic saline at room temperature during the procedure, as this has been previously shown to prevent any temperature increase in the tissue caused by the administration of laser light at the power densities used in these studies (11). After the administration of PDT the craniotomy site was covered with a single layer of Surgicel (Johnson and Johnson, North Ryde, Australia), and the scalp incision was closed with wound clips. The animals were then returned to cages and kept in normal room light, with no evidence of cutaneous photosensitivity until sacrifice, and fed water and food ad libitum.

Quantitation of PDT necrosis mediated by BOPP. The rats were sacrificed 5 days postadministration of PDT, as previous studies have shown this to be the optimal time to determine phototoxic necrosis in normal brain or tumor (11). The brains were then removed, fixed, and sectioned, and the extent of cerebral edema and the depth and extent of brain or tumor necrosis were determined as described (11).

Regrowth of intracranial C6 tumor after PDT mediated by BOPP or HpD. Tumor-bearing rats were sensitized via i.v. administration of either 25 mg of BOPP per kg of body weight 24 hr pre-PDT or 20 mg of HpD per kg of body weight 6 hr pre-PDT, drug dose regimes that have been shown to result in maximal selective tumor destruction (ref. 11; see Fig. 4). PDT was administered as described above, except that the laser used was a potassium titanium phosphate/neodymium/yttrium/aluminum-garnet pumped dye laser (Laserscope Surgical Systems, San Jose, CA) producing 1000 mW of 628-nm light at the fiber tip. Light was then administered at dosages of 25 J/cm² and 250 J/cm² for the BOPP-sensitized and HpD-sensitized animals, respectively. These light and sensitizer dosages had previously been shown to mediate the maximum depth of selective tumor necrosis using BOPP (see Fig. 4) or HpD (11). After PDT, the animals were returned to cages, fed water and food ad libitum, and continually monitored for any signs of neurological deficit. If any deficit or distress was apparent, the animal was sacrificed. Death of the animals was not used as an endpoint of the study, and at 21 days post-PDT all surviving animals were sacrificed. The brains of all animals were removed, fixed, sectioned, and stained, and the PDT effects were quantitated as described above.

RESULTS

The cytotoxicity of BOPP or HpD on C6 glioma cells *in vitro* is shown in Fig. 1. BOPP is less toxic to glioma cells than HpD in the absence of light, with 50% colony survival (CS_{50}) ≈120 μg/ml and 90% colony survival (CS_{90}) ≈20 μg/ml, compared to the corresponding values for HpD of 10 and 6 μg/ml, respectively. The *in vitro* phototoxicity studies shown in Fig. 2 demonstrate that BOPP can mediate significant phototoxicity in glioma cells. The data from the survival curves for the phototoxicity of cells exposed to 20 μg of BOPP per ml or to
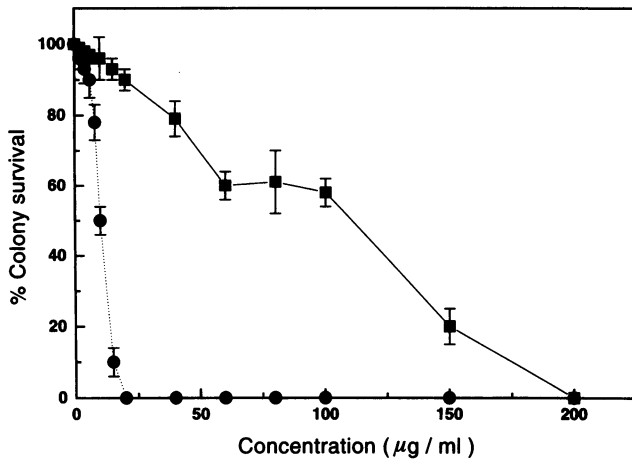
FIG. 1.   *In vitro* cytotoxicity of BOPP and HpD. Rat C6 glioma cells were incubated in the dark in RPMI 1640 medium/10% FCS containing either BOPP (■) or HpD (●) for 16 hr at the concentrations indicated, washed twice in sensitizer-free medium, resuspended in fresh medium, and incubated in the dark for a further 7 days to allow colony formation. Colony survival was determined as percentage survival at 0 μg of sensitizer per ml. Data represent mean colony survival in three flasks at each concentration, and error bars represent 1 SEM.

6 μg of HpD per ml are the most directly comparable, since these concentrations correspond to the respective CS_{90} values for each sensitizer determined in the absence of light. The kinetics of cell kill are similar for both curves, and both show an initial lag phase, characteristic of the cells' ability to repair sublethal phototoxic damage, followed by a rapid increase in the rate of cellular death.

Ten days post intracranial implantation into Wistar rats, the tumors were of an appropriate size to determine the depths of tumor kill able to be achieved after BOPP-mediated PDT. The doses of BOPP administered to the rats (5, 10, 25, or 50 mg per kg of body weight) have been shown in a mouse model to result in extremely selective uptake of the drug into tumor, with very low levels in surrounding normal brain and blood (20). A dose
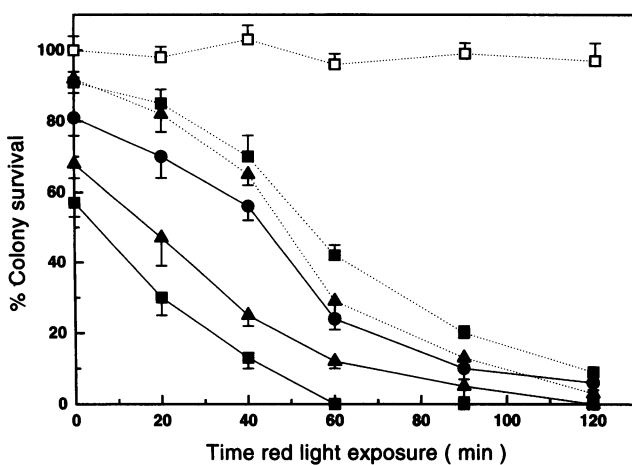

FIG. 2.   *In vitro* phototoxicity of BOPP and HpD. Rat C6 glioma cells were incubated in the dark in RPMI 1640 medium/10% FCS containing BOPP at 0 (□··□), 20 (■··■), 40 (●—●), 60 (▲—▲), or 80 (■—■) μg/ml, or HpD at 6 μg/ml (▲—▲) for 16 hr. The cells were then washed twice with fresh medium, resuspended in fresh medium, and then exposed to red light for the times indicated and incubated in the dark for a further 7 days to allow colony formation. The colony survival was determined as percentage mean survival in the flasks with 0 μg/ml and 0 min exposure. Data represent mean colony survival in three flasks at each point, and error bars represent 1 SEM.
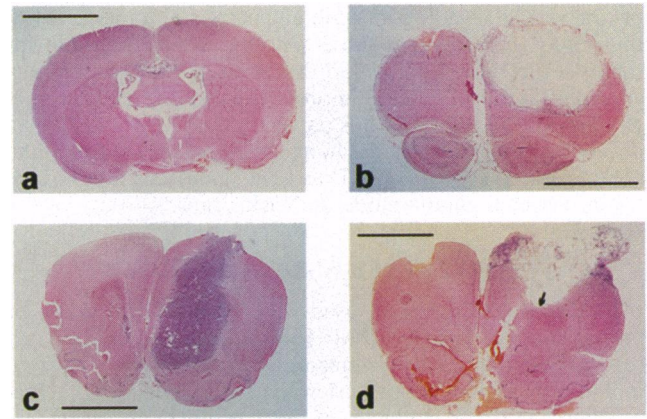

FIG. 3.   Effect on tumor or normal brain of BOPP-sensitized PDT. (*a*) Non-tumor-bearing normal brain sensitized with 25 mg of BOPP per kg of body weight i.v. and exposed to 628-nm light (25 J/cm²) 24 hr postsensitization. (*b*) Non-tumor-bearing normal brain sensitized as in *a* and exposed to light at 200 J/cm². (*c*) Unsensitized tumor in normal brain exposed to light at 200 J/cm². (*d*) Tumor in normal brain sensitized with 25 mg of BOPP per kg of body weight i.v. and exposed to light at 25 J/cm². Arrow, point of maximum depth of tumor kill. (Bars = 5 mm.)

of 25 mg of BOPP per kg of body weight, in combination with light at 25 J/cm², mediated significant phototoxic tumor necrosis (Fig. 3*d*). Administration of the same doses of BOPP and light to non-tumor-bearing animals resulted in no apparent edema or toxicity in normal brain (Fig. 3*a*), although higher light doses were able to induce phototoxic necrosis in the brain of sensitized non-tumor-bearing rats, even though the levels of BOPP in brain were extremely low (Fig. 3*b*). Significant macrophage infiltration and an inflammatory response were evident at the margins of the lesion (Fig. 3*b*). The administration of BOPP alone in doses of 50 mg per kg of body weight or laser light alone, in combination with saline irrigation, in doses up to 200 J/cm² (Fig. 3*c*) caused no detectable tumor or normal brain necrosis or edema.

The BOPP-mediated PDT of the C6 glioma 5 days post-therapy is shown in Fig. 4. At all doses of BOPP, phototoxic necrosis of tumor was apparent, although normal brain damage could be induced at high doses of light. The light-dose threshold for brain necrosis was dependent on the dose of BOPP. Normal brain damage was apparent at 25 J/cm² after a BOPP dose of 50 mg per kg of body weight but was absent at lower BOPP doses. The maximum depth of tumor necrosis of 4.5 mm was induced by the combination of 25 mg of BOPP per kg of body weight and 25 J/cm² of laser light. Arrows indicate light doses that mediated maximum tumor kill with sparing of normal brain.

The results of the study of tumor regrowth in rats post-PDT is shown in Table 1. While the number of animals in each group is small, the data suggest that there is a difference in the time to occurrence of neurological symptoms in the BOPP-sensitized animals compared to those sensitized with HpD. All animals that were sacrificed with symptoms of neurological deficit before day 21 post-PDT exhibited obvious histological tumors. However, the brains from the BOPP-treated animals that survived for 3 weeks before sacrifice at day 21 were substantially tumor-free, whereas all of the HpD-treated animals had obvious tumors (data not shown). All animals in both groups showed inflammatory responses in the brain similar to those previously noted in PDT-treated rats, which may be indicative of a small degree of treatment-induced edema or phagocytic and macrophage infiltration and removal of tumor debris from the treatment site (11).

DISCUSSION

The treatment of cerebral glioma remains a major challenge because of the infiltrative nature of these tumors. They gen-

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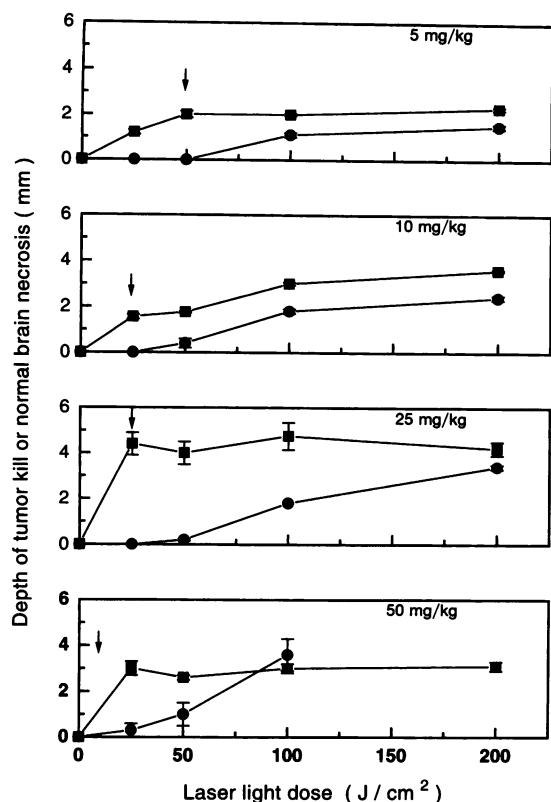*Proc. Natl. Acad. Sci. USA 92 (1995)*   12129

FIG. 4. Depth of tumor kill and normal brain necrosis after PDT of BOPP-sensitized rats. Depth of intracranial C6 tumor kill (■) or normal brain necrosis (●) in Wistar rats sensitized i.v. with BOPP at the doses indicated and exposed to various doses of 628-nm light from a gold metal vapor laser. Values shown represent mean depth of kill in a minimum of three animals at each data point and error bars represent 1 SEM. Arrows, dose of light that results in selective tumor necrosis with sparing of normal brain.

Table 1. Tumor regrowth in rats post-PDT

Sensitizer	Rat	Comments
BOPP*†	1	Sacrificed 8 days post-PDT; neurological symptoms, histological tumor present.
	2	Sacrificed 17 days post-PDT, neurological symptoms, histological tumor present.
	3	Sacrificed 21 days post-PDT, neurological symptoms, small histological tumor present.
	4	Sacrificed 21 days post-PDT, no neurological symptoms, no histological tumor apparent.
	5	Sacrificed 21 days post-PDT, no neurological symptoms, no histological tumor apparent.
HpD‡§	6	Sacrificed 8 days post-PDT, neurological symptoms, histological tumor present.
	7	Sacrificed 8 days post-PDT, neurological symptoms, histological tumor present.
	8	Sacrificed 8 days post-PDT, neurological symptoms, histological tumor present.
	9	Sacrificed 21 days post-PDT, neurological symptoms, histological tumor present.

Significance in difference in time to sacrifice at $P < 0.05$ by Student's paired t test.
*Dosimetry, 25 mg of BOPP per kg of body weight; 628-nm light at 25 J/cm^2.
†Dosimetry, 20 mg of HpD per kg of body weight; 628-nm light at 200 J/cm^2.
‡Mean time to sacrifice, 17.6 days; median time to sacrifice, 21 days.
§Mean time to sacrifice, 11.3 days; median time to sacrifice, 8 days.

erally do not metastasize throughout the body but rather kill by local regrowth, making use of locally targeted binary therapies, such as PDT and BNCT, very attractive (9). In this report, we have studied the action of a sensitizer, BOPP, which, because of its porphyrin component, is a photosensitizer for PDT and, because it consists of ≈30% (wt/wt) boron (19), has the property of also being a sensitizer for BNCT. In comparison to HpD, BOPP is significantly less toxic to glioma cells *in vitro* in the absence of light (Fig. 1), although our studies have shown that both have approximately equal toxicity when administered to animals *in vivo* (J.S.H., S.B.K., S.S.S., and A.H.K., unpublished observations).

The data presented in Figs. 2–4 and Table 1 show that BOPP can act as a potent photosensitizer of cerebral glioma *in vitro* and *in vivo*, and with the appropriate dosimetry selective destruction of tumor with sparing of normal brain can be achieved. We have previously shown that administration of BOPP to mice bearing the same C6 tumor used in this study results in extremely selective sensitizer uptake into the tumor (20). The selectivity of uptake into the tumor was nearly 10 times that observed for HpD, the sensitizer used in most human clinical trials of PDT of glioma (9, 13, 16, 17). In addition, fluorescence microscopy with a confocal microscope has demonstrated that BOPP is present in the tumor cells invading normal brain (20). These findings are critical since it is these cells that are the primary target of adjuvant therapies.

The data in Fig. 4 show that BOPP is a potent photosensitizer, which on a weight basis is ≈10 times more phototoxic *in vitro* than HpD. We have previously reported that an optimum tumor necrosis depth of ≈5 mm, with sparing of normal brain, could be achieved with a dose of 20 mg of HpD per kg of body

weight and 200 J/cm^2 of 628-nm light (11). In this study, the optimum dosimetry to achieve the same depth of tumor kill was 25 mg of BOPP per kg of body weight and only 25 J/cm^2 of light administered 24 hr postsensitization. It is difficult to directly compare the two sensitizer doses since the molecular weights of the two drugs are so different. BOPP, which is a singular chemical entity has a molecular weight of ≈1300 (19), whereas HpD is a complex mixture of porphyrins, which exist in varying aggregation states (for review, see ref. 9). The molecular weight of the monomeric hematoporphyrin is ≈600, so it may be considered that on a molar basis the dose of BOPP required to achieve maximal tumor kill is half that required for HpD. In this study, PDT was administered 24 hr postsensitization, the time point we have previously shown corresponds to the maximal levels of BOPP in tumor (20). While the maximum tumor/normal brain uptake ratio was evident 48 hr post administration of BOPP (20), PDT of animals at this time point (data not shown) did not result in the same depth of tumor kill as at 24 hr (Fig. 4). This suggests that the limiting factor in tumor phototoxicity was the sensitizer level in the tumor, rather than the differential concentration between tumor and normal brain. However, the data in Fig. 4 also show that the level of BOPP in tumor must be below an upper threshold, since the depth of tumor kill decreased after a dose of 50 mg/kg. This may be due to higher tumor sensitizer levels 24 hr after a dose of 50 compared to 25 mg/kg. Our previous studies in an identical mouse model have shown a BOPP level of 140 μg per g of tumor after the former dose, compared to 60 μg/g after the latter dose (20). High levels of BOPP may result in increased absorbance of the activating light near the superficial margin, resulting in an attenuation of the light dose to the deeper tumor regions, with consequent reduced necrosis. This attenuation of light may be a critical factor in some clinical situations where high sensitizer levels may be present in some tumors.

The reason for the more potent tumor kill with BOPP compared to HpD is probably related to their respective

subcellular localization. Using confocal laser scanning microscopy, in conjunction with centrifugal organelle fractionation, we have shown that BOPP is almost exclusively localized in the mitochondria of glioma cells (20, 21). HpD localized in a variety of subcellular sites, including the cytoplasm, lysosomes, and mitochondria (9, 21, 23), probably reflecting the heterogeneity of the HpD mixture (9). It has previously been shown that the mitochondria of tumor cells may be critical subcellular targets for PDT (24). The data in Fig. 4 and Table 1 suggest that specific localization of high concentrations of BOPP to mitochondria may result in an increased efficacy of BOPP-sensitized PDT compared to HpD PDT. It may also explain the greater degree of tumor eradication and an increased time interval between therapy and tumor regrowth in BOPP-sensitized animals. This preliminary finding indicates the requirement for a more complete study of long-term survival after BOPP-mediated PDT. It may have great significance since it provides a rational basis for future drug design based on the site of localization of the sensitizer.

These findings demonstrate that with appropriate dosimetry, BOPP is a more potent photosensitizer of glioma than HpD. This may have direct clinical implications where the time constraints of surgery require the adjuvant PDT procedure to be completed in the minimum possible time. In many HpD-sensitized treatments of cerebral glioma, or larger systemic tumors such as mesothelioma, the residual tumor bed is so large that the delivered light dose is lower than that required to achieve maximum tumor eradication. The use of a more potent photosensitizing drug such as BOPP may overcome this problem. In addition, the lower activating light threshold of BOPP may allow destruction of deeper nests of tumor compared to HpD.

In addition, the attraction of using BOPP as a BNCT sensitizer allows the possibility of combination PDT/BNCT treatments, which may substantially improve the degree of local control of tumors such as cerebral glioma.

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