

Pc 4 Photodynamic Therapy of U87-Derived Human Glioma in the Nude Rat

John E. George III, MD,¹ Yusra Ahmad, MPH,² Davood Varghai, MD,³ Xiaolin Li,⁴ Jeffrey Berlin,⁵ David Jackowe,³ Marc Jungermann,³ Michael S. Wolfe, MD,¹ Lothar Lilge, PhD,⁶ Ali Totonchi, MD,¹ Rachel L. Morris,⁷ Allyn Peterson,³ W. David Lust, PhD,³ Malcolm E. Kenney, PhD,⁵ Charles L. Hoppel, MD,⁴ Jiayang Sun, PhD,⁸ Nancy L. Oleinick, PhD,⁷ and David Dean, PhD^{3*}

¹Department of Surgery, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106

²Department of Anatomy, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106

³Department of Neurological Surgery, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106

⁴Department of Pharmacology, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106

⁵Department of Chemistry, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106

⁶Department of Medical Biophysics, University of Toronto, 610 University Avenue Rm 7-416, Toronto, Ontario M5G 2M9, Canada

⁷Department of Radiation Oncology, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106

⁸Department of Statistics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106

Background and Objectives: As a potential therapy for malignant glioma, we tested the phthalocyanine photosensitizer Pc 4 for: (1) rapid clearance from the vasculature, (2) specificity for glioma, and (3) tumoricidal photosensitizing capability.

Study Design/Materials and Methods: Parenchymal injection of U87 cells into athymic rat brains ($N = 100$) was followed after 12 days by tail vein injection of 0.5 mg/kg Pc 4. After 1 day, the tumor was illuminated with either 5 ($N = 11$) or 30 ($N = 16$) J/cm² red light at 672 nm. Sacrifice was 1 day later. The brains from these 27 animals underwent H&E (necrosis) and TUNEL assay (apoptosis) histology. Pc 4 concentration of explanted brains and tumors ($N = 16$), and all blood samples ($N = 52$) were determined by HPLC-MS 1 day post Pc 4 administration.

Results: Tumor-specific apoptosis was almost uniformly seen; however, necrosis was found mostly in the high-light-dose group. Pc 4 concentration in bulk tumor averaged 3.8 times greater than in normal brain.

Conclusions: These results warrant expanding this pre-clinical study to seek effective baseline Pc 4 drug- and light-doses and infusion-to-photoradiation timing that would be necessary for a Pc 4-mediated PDT clinical trial for glioma patients. *Lasers Surg. Med.* 36:383–389, 2005.

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INTRODUCTION

CBTRUS [1] estimates that 17,000 new cases of primary malignant brain tumors present annually, which is approximately 1.32% of all cancer diagnoses. The prognosis for patients with malignant gliomas remains poor despite many years of investigation [2]. The use of photodynamic

therapy (PDT) for glioma patients has been investigated clinically with several photosensitizers, including boronated porphyrin, or BOPP [3], PhotofrinTM (Axcan Pharma, Birmingham, AL) [4], and 5-aminolevulinic acid-induced porphyrin, or ALA [5]. The phthalocyanine Pc 4 has shown potential for tumor-specific ablation using far-red photoactivating light (i.e., 672 nm) [6]. During PDT the excited photosensitizer transfers energy to ground state oxygen (³O₂) thereby forming highly reactive singlet oxygen (¹O₂) and other reactive oxygen species (ROS) [7–9]. The formation of ROS results in oxidative damage to cellular proteins, membranes, and membranous organelles, which are sites of photosensitizer accumulation [10]. Lam et al. [11] have verified the effect of singlet oxygen on mitochondria following photosensitization by Pc 4. Although PDT also causes DNA damage [12–14], this is not thought to be the mechanism responsible for cell death [15,16].

It has been shown that Pc 4 binds to cytoplasmic organelle membranes, especially the mitochondrial membrane and the endoplasmic reticulum [11,17–19]. Although the peripheral benzodiazepine receptor (PBR), a protein of the outer mitochondrial membrane, has been proposed as a site of localization of some porphyrin photosensitizers, Morris et al. [18] found only low-affinity binding of Pc 4 to

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*Correspondence to: David Dean, PhD, Department of Neurological Surgery, HH5, MS 5042, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106.

E-mail: dxd35@cwru.edu

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the PBR. However, in addition to these results from confocal microscopy, there is now considerable evidence that Pc 4 binds to the mitochondria of cancer cells. Pc 4-mediated PDT causes immediate photodamage to Bcl-2 and Bcl-xL [19–21], anti-apoptotic proteins that are located on the outer mitochondrial membrane and endoplasmic reticulum. Bcl-2 and/or Bcl-xL are often overexpressed in a variety of cancers, including gliomas, where they are thought to contribute to the increased resistance of tumor to treatment [22,23]. Photodamage requires that the targeted proteins reside within range ($<0.1 \mu\text{m}$) of a site of singlet oxygen production, that is, very close to sites of sufficient photosensitizer concentration. Pc 4-mediated PDT regimens that photodamage Bcl-2 have been shown to lead to activation of Bax, induction of apoptosis, and elimination of otherwise resistant tumor cells [21,23,24]. Additionally, Pc 4 can undergo fluorescence resonance energy transfer (FRET) from nonyl-acridine orange (NAO), a highly specific probe of the phospholipid cardiolipin [25]. Because cardiolipin is found only in the mitochondrial inner membrane, and at the contact sites between the inner and outer membranes, and because FRET occurs only when the donor and acceptor are within 10 nm of each other, Pc 4 detected in that study must have found residence in close association with mitochondrial cardiolipin.

High specificity of Pc 4 for tumor is necessary if it is going to find success as a photosensitizer for PDT of malignant glioma. Normal brain tissue is known to be highly sensitive to relatively low concentrations of other photosensitizing drugs [26,27]. Photosensitizers that are larger molecules tend not to cross the blood-brain barrier (BBB), whereas tumors actively break down the BBB to create their own blood supply. In these cases, tumor tissue is much more likely to receive a high dose of intravenously administered photosensitizer, adding to the selectivity of PDT. The roughly 700 Da Pc 4 molecule is similar in size to other photosensitizers that exhibit higher uptake in tumor than normal brain tissue.

Of nearly equal importance to tumor selectivity for drug, is determining whether drug-infused tumor is sensitive to illumination. Studies of Pc 4 in ectopic, non-glial xenograft tumors have shown a rapid increase in apoptosis within the treated tumors [17]. Thus, effective PDT of glial tumors will depend on both (a) the differential in normal tissue/tumor drug uptake, and (b) the intrinsic PDT sensitivity of both tissues to photoirradiation. Lilge et al. [28] found significantly more apoptosis in normal brain that received PhotofrinTM followed by PDT at a low radiant exposure (1 J/cm^2), a dose that did not cause necrosis, than with a high radiant exposure (17 J/cm^2), which universally induced necrosis. However, they found that the shift from apoptosis to necrosis might not be linear as light and/or drug dose is increased. Indeed, apoptosis and necrosis may be competing or possibly even antagonistic responses to photosensitization depending on drug concentration and uptake inhomogeneity, as well as radiant exposure, and tumor sensitivity to both. The selectivity and sensitivity of Pc 4 in glioma and other brain tissue is currently unknown. However, in the mouse, circulating Pc 4 was seen to clear

from the plasma compartment within 24 hours and inadvertent skin photosensitivity resolved within 4 days [29]. However, the pharmacokinetics and tissue response reported using this transplantable murine tumor model [29] may not be applicable to glioma.

Several glioma models have been studied for the assessment of PDT photosensitizer selectivity and sensitivity, including the VX2 rabbit [30] and 9L rat [26,31] gliosarcoma brain tumor models. Gliosarcoma may be a minor constituent in grade III and IV glioma, although, it is usually not present. However, because they are not of CNS origin, unlike glioma, these models have: (a) a lower “take” rate of injected cells converting to tumors compared to the U87 model, (b) generally poor intrusion of the tumor into the brain, and (c) no associated edema, something commonly found with glioma. The J3T canine glioma [32] model overcomes some of these problems, but it is expensive and difficult to husband. The use of athymic rats and the C6 cell line have also shown promise as a better model for the glioma patient [33]; however, the selectivity of drugs that have been studied in humans appears to be relatively low [34–36].

A collaboration between Henry Ford Hospital and the University of Toronto [31,37] has demonstrated the utility of the human-derived U87 glioma cell line [38–40] for the creation of parenchymal brain tumors in the nude rat for the study of PDT photosensitizer selectivity and specificity. These workers found that U87 tumors result in $>90\%$ of animals following a single infusion. In the study reported here, we implanted U87 cells into the brain parenchyma of nude rats and subsequently administered Pc 4 and photoirradiating light to determine: (a) the kinetics of clearance of drug from the plasma compartment, (b) the specificity of Pc 4 for glioma versus normal brain tissue, and (c) the possibility of inducing tumor necrosis and/or apoptosis by Pc 4-mediated PDT. These pilot experiments justify further pre-clinical study of Pc 4 dose, infusion-to-photoirradiation duration, and radiant exposure for a glioma patient Phase I (i.e., dose escalation) clinical trial of Pc 4-mediated PDT.

MATERIALS AND METHODS

U87 Human Glioma Model

U87 cells (ATCC, Manassas, VA) were maintained in MEM medium (Gibco #41500–018, Carlsbad, CA) containing 1 mM sodium pyruvate, 10% fetal bovine serum, and 1% penicillin/streptomycin. Approximately 250,000 cells were implanted in 100 athymic (nude) rats (Harlan Industries, Inc., Indianapolis, IN). Cell injection was via Hamilton syringe. The site of injection was 2 mm lateral and 2 mm posterior to the bregma at a depth of 2 mm.

Photodynamic Therapy

After 12 days all but the 5 control rats received Pc 4 (0.5 mg/kg) MEK lab, CWRU by tail vein injection. Twenty four hours after Pc 4 administration, all but 16 animals underwent re-opening of their craniotomies and photoirradiation of their brain using a diode laser emitting at 672 nm (250-HHL-670 model of a 250 mW AOC, Applied

Optics Corp., South Plainfield, NJ) that delivered one of two red light radiant exposure levels, both at an irradiance of 50 mW/cm². The low radiant exposure of 5 J/cm² was applied over 100 seconds and the high radiant exposure of 30 J/cm² was applied over 600 seconds. Brains from the 16 animals not receiving photoirradiation were used for high performance liquid chromatography/tandem mass spectrometry (HPLC/MS) determination of Pc 4 concentration in normal brain and tumor tissue. The laser spot size for both groups was 0.8 cm in diameter. One day later, each animal was anesthetized for sacrifice, and 5 cc of blood was collected from the left cardiac ventricle before its vascular system was flushed with 100 ml of phosphate buffered saline (PBS). Then the stopcock was switched to allow 250 ml of paraformaldehyde (PFA) into the cannula to fix the animal's brain. The blood sample was centrifuged for 10 minutes at 3,000g and the resulting plasma aspirate was frozen at -80°C for subsequent HPLC/MS to determine the Pc 4 concentration.

Next, the animal was decapitated and the brain was removed. The brains of the 16 animals analyzed by HPLC/MS for Pc 4 concentration were manually dissected. After dissection, the entire tumor and a sample of contralateral normal cerebrum were placed directly in liquid nitrogen for storage. The rest of the brains were analyzed histologically, beginning with complete submersion in PFA for 24 hours, then refrigeration in sucrose. Histological sectioning began three or more days later.

Determination of Pc 4 Concentration by HPLC/MS

Pc 4 concentration in rat plasma, normal brain, and tumor specimens was determined by HPLC/MS using an Agilent (Wilmington, DE) 1,100 series quaternary gradient pump, solvent degassing unit, automatic liquid sampler, and thermostatic column compartment. The atmospheric pressure ionization triple quadrupole mass spectrometer used was a ThermoFinnigan (San Jose, CA) TSQ 7000 model equipped with an electrospray ion source. Nitrogen was used as the sheath and auxiliary gas in the electrospray source. Argon was used as the collision gas. The ion source optics and analyzer voltages were optimized for Pc 4 detection.

Frozen rat plasma samples were allowed to thaw in an ice bath. Aliquots of 200 µl of plasma were transferred to glass test tubes. An internal standard solution (silicon phthalocyanine Pc 34; 100 pmol/ml, 10 µl) was added to these tubes, and, then, the contents of each tube were vortex-mixed. Volumes of 3.0 ml of hexane:isopropanol (9:1, v:v) were added to each tube, and the tubes were again vortex-mixed. Next, the tubes were centrifuged at 1,760g to facilitate phase separation. The organic supernatant layers were transferred to clean glass test tubes and evaporated under dry nitrogen in a water bath at 37°C. The resultant residues were reconstituted in 40 µl of chromatographic mobile phase, centrifuged at 13,000g, and the supernate was transferred to autosampler vials. Aliquots of 5 µl were injected into the liquid chromatograph.

The chromatographic separation was accomplished on a Varian (Palo Alto, CA) Omnisphere 3 C18 column (4.6 mm

i.d.×100 mm; 30 µm particle diameter). The chromatographic column was protected against particulate and highly apolar sample constituents by an integral 2 mm i.d.×10 mm cartridge pre-column, which contained the same stationary phase. The chromatographic eluent consisted of methanol: 0.05M ammonium formate buffer (80:20::v:v); the flow rate was 0.5 ml/min. Pc 4 and Pc 34 were detected by monitoring collision-induced transitions of m/z 718 to m/z 557 and m/z 746 to m/z 557, respectively. Plasma samples and concurrent quality control specimens were prepared and analyzed in duplicate, and Pc 4 concentrations were calculated in relation to calibration curves established over the range of 0.078–100 pmol/ml of Pc 4 in plasma during each episode of sample analysis.

Normal tissue and tumor tissue specimens were dissected from rat brains prior to freezing. Precisely weighed specimens of 0.1–0.3 g were combined with a threefold volume multiple of PBS solution (1.2×10⁻³M KH₂PO₄, 2.9×10⁻³M Na₂HPO₄, 1.54×10⁻¹M NaCl, pH 7.4), and homogenized in a glass mortar and pestle apparatus. Aliquots of 200 µl of homogenate were extracted and prepared in duplicate for chromatographic analysis as described above for plasma specimens, and Pc 4 concentrations were calculated in relation to calibration curves established over the range of 0.078–100 pmol of Pc 4 per g wet weight of tissue during each episode of sample analysis.

Brain Histology

Each brain was embedded in OCT (Optimal Cutting Temperature) from Sakura Tissue Tek (Torrance, CA) and frozen for 2 or more hours at -80°C before Cryostat (Model JUNG CM3000, Leica, Nussloch, Germany) sectioning. Tumor detection was facilitated with thionin (Fisher Scientific, Fairlawn, NJ) staining. Before and after tumor detection, the tissue was advanced at 200 µ intervals. Slides were stored in a Fisher #3–447 (Fairlawn, NJ) slide box at -80°C prior to staining. In the non-tumor region, slide triplets were prepared with: thionin, Hematoxylin and Eosin (H&E), and TUNEL Assay. In the tumor region, thionin staining ceased and was replaced with an alternating series of H&E and DeadEnd™ Fluorometric TUNEL Assay (Promega, Madison, WI). To enhance H&E (Fisher Scientific), soak time was reduced in: xylene from 7 to 2 minutes, hematoxylin from 15 to 8 minutes, and eosin from 3 to 1 minutes. We collected 20–100 slides per brain, depending on tumor size.

RESULTS

Survival and Tumorigenesis

Of the 100 nude rats implanted with U87 cells, 35 died prior to receiving drug. Behavioral observations and necropsies revealed that most of the pre-mature deaths were due to pre-sacrifice anesthesia or, apparently, tumor mass effects. Twelve days appeared necessary for the tumor to reach a size deemed reasonable for study, but tumor sizes varied greatly among these animals, ranging from 3 to 15 mm in maximum length. Of the 65 animals that received drug, 16 specimens were analyzed by HPLC/MS for Pc 4

concentration in normal brain, tumor, and plasma. Plasma from 49 animals, including the 5 controls and 44 entering histology, was also analyzed for Pc 4 concentration. The five controls received no drug or light. The remaining 44 animals entering histology received Pc 4 and either low- or high-radiant exposure. However, of these 44 animals, only 27 provided histological data to the study (i.e., 11 low radiant exposure, 16 high radiant exposure). Of the 17 remaining rat brains intended for histological study, 3 animals expired after photoirradiation but prior to sacrifice, 4 were lost when a freezer failed unexpectedly, and 10 were found to have developed no tumor following the injection of U87 cells.

HPLC/MS Tissue and Blood Pc 4 Concentration Assay

The results of the HPLC/MS tissue assays for Pc 4 are reported in Table 1. Please recall that blood was collected at sacrifice. Three of the 65 animals receiving drug did not survive, and 10 additional animals did not contribute plasma samples for an N of 52. These data were correlated with the 16 animals contributing tumor and normal brain tissue. Tumor tissue drug uptake was never lower than in normal brain tissue. However, there were marked differences in total concentration from animal to animal, resulting in large standard deviations. The range of Pc 4 concentration values in tumor tissue was 2.4–37.0 pmol/g, and in normal brain tissue was 0.85–7.6 pmol/g, and in plasma was 7.0–29.9 pmol/ml. The statistical significance of the difference between tumor, normal brain, and plasma Pc 4 concentrations in these data was determined via Wilcoxon Signed Rank test (see Table 1). We found that drug concentration was significantly ($P = 0.00024$) greater in tumor than normal brain, significantly ($P = 0.00036$) greater in plasma than in normal brain, and was less ($P = 0.0647$), but not statistically significantly so, if a $\alpha < 0.05$ significance level is used, in tumor than in plasma. These data are for the matched samples of 16 tumor, normal brain, and plasma tissues. The plasma average (14.3 pmol/ml or 10.2 ng/ml) and standard deviation (5.85 pmol/ml or 4.2 ng/ml) that includes the full sample ($N = 52$) is very similar to that matched data ($N = 16$).

Brain Histology

The histological sample included 11 animals receiving the low radiant exposure and 16 animals receiving the high radiant exposure (Table 2). All but two of the high radiant exposure specimens presented evidence of necrosis, whereas only one of the low radiant exposure specimens presented evidence of necrosis (Table 2). The category of “mild” necrosis was used when only a limited area of the tumor showed necrosis. We found no indication of non-specific necrosis in normal brain areas (Fig. 1).

Mild necrosis was seen in one animal that had received a low radiant exposure and three that received a high radiant exposure. We hand traced the boundary of the area of the tumor and, separately, traced the area of necrosis on the central slide, three times. Variation between tracings was acceptably low (difference range: 5.2%–14.9%). Using the average values, we calculated the percent of live (i.e., non-necrotic) tissue remaining in these four tumors (Table 3).

All specimens exhibited apoptosis, especially at the periphery. However, the incidence of apoptosis tended to be more dispersed in the low radiant exposure group than in the high radiant exposure group (Fig. 2A). Apoptosis was also observed in blood vessels in tumor or normal brain areas (Fig. 2B). No apoptosis was observed in normal brain areas, other than in blood vessels.

DISCUSSION

The goals of this study were to determine whether there was selective uptake for Pc 4 in glioma tissue and whether that tissue, if so infused, was sensitive to PDT initiated by illumination with 672 nm red light. We found both to be the case.

It is thought that normal brain tissue is protected by the BBB, and that tumors such as the U87 human glioma utilized in this study substantially break down the BBB. Selective uptake in tumor is expected to protect against inadvertent necrosis of normal brain tissue. On average the ratio of concentrations of Pc 4 in tumor:normal-brain was 3.89:1.0. It is possible that the concentration would have been reduced in plasma, and thus the ratio higher, had we allowed longer than one day for the drug to circulate and

TABLE 1. Pc 4 Concentrations Determined by HPLC/MS

Pc 4 Concentration	Normal brain ($N = 16$)		Tumor ($N = 16$)		Plasma ($N = 16$)	
	Average	Standard deviation	Average	Standard deviation	Average	Standard deviation
pmol/g or ml	3.7	1.7	14.4	10.6	18.3	4.5
ng/ml	2.7	1.2	10.3	7.6	13.1	3.2

Pc 4 concentrations are expressed in both pmol/g and ng/g, for normal brain and tumor and in pmol/ml and ng/ml for plasma. Note that 16 brains with tumor were analyzed. The normal brain tissue was taken from the cerebral hemisphere opposite the tumor. Note that plasma was taken from more than the 16 specimens (i.e., 52) contributing brains with tumor for HPLC/MS analysis, however the data presented here are for the matched plasma samples. The brains from these of 27 these additional 36 animals contributing plasma were analyzed histologically.

TABLE 2. Pc 4-Mediated PDT Histology

No. specimens	Radiant exposure	Necrosis (hematoxylin & eosin)				Apoptosis (TUNEL assay)
		None	Mild	Frank	Total	Total
11	Low	10	1	0	1	11
16	High	2	3	11	14	16

Necrosis was analyzed by Hematoxylin and Eosin preparation. Three categories of necrosis were observed: none, mild, and frank. Specimens were classified with mild necrosis if a recognizable area of viable tumor tissue could be identified and bounded with a manual drawing tool. These areas were then quantified and are reported in Table 3. Specimens were classified with frank necrosis if no recognizable area of viable tumor tissue could be identified. Apoptosis was analyzed by TUNEL Assay preparation. Note that all 27 specimens prepared histologically demonstrated apoptosis.

clear the vasculature. Given our exploratory study protocol, we cannot ascertain whether the drug concentration differential between tumor and plasma would have increased had we allowed a longer circulation time. Pc 4 must also “safely clear” the vasculature to eliminate the possibility of inadvertent photosensitization of the skin and eyes. The observation of apoptosis in the cells lining blood vessels (Fig. 2B) suggests that a longer delay for PDT illumination might be beneficial.

In no specimen was the Pc 4 concentration higher in normal brain than in tumor. In one of the specimens, the tumor/normal brain tissue Pc 4 concentration ratio was 1.0, and two others presented only 10% more drug in tumor than normal brain. All three of these outliers had very low concentrations of drug (range 3.4–5.1 pmol/g), suggesting the possibility that these three specimens had very small live tumor contributions to the assayed sample or that these small tumors had not caused sufficient disruption to the

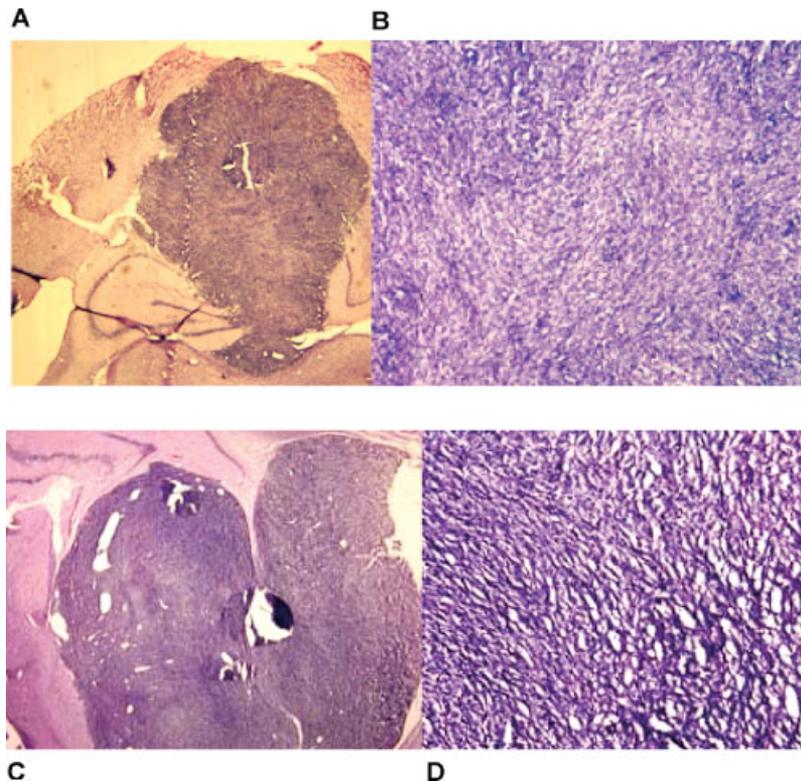


Fig. 1. Hematoxylin & Eosin-stained, Pc 4-photosensitized U87 tumors: **A–B**: shows the tumor from specimen 27. It received a low radiant exposure (i.e., 5 J/cm²). This tumor shows no areas of necrosis (note: there is minor histological preparation artefact). **C–D**: Shows the tumor from Specimen

28 (C-D). It received a high radiant exposure (i.e., 30 J/cm²). This tumor shows frank necrosis with debris from cellular structures. Magnifications of specimens: 1A: 20×, 1B: 100×, 1C: 40×, 1D: 100×.

TABLE 3. Analysis of Mild Necrosis (i.e., Residual Live Tissue) in Four Tumors (H&E Preparation)

Specimen	Radiant exposure	Live tissue percentage of tumor
PR 94	Low	26.8%
PR 43	High	3.2%
PR 46	High	23.8%
PR 83	High	3.7%

Four of the tumors prepared histologically presented mild necrosis. The boundaries of the entire tumor and the residual area of live tissue were separately hand circumscribed. The amount of the latter was divided by the former and is presented in the third column. These measurements were repeated (see text).

BBB. It is also possible that the low differential in these outlier specimens was due to averaging of the tumor's necrotic core and/or normal tissue assayed along with the live tumor tissue. No attempt was made to differentiate necrotic core from live tumor when the tumors were harvested from the explanted brains for HPLC/MS analysis. As to a possible averaging of normal brain tissue along with the tumor sample, it should be noted that the margin of the tumor was generally well delineated, but due to the microscopic and invasive nature of this disease, the tumor/normal brain margin can be difficult to detect on visual inspection in some regions. Moreover, it was deemed most important to be sure to include the live margin of the tumor in the tumor sample. Thus, we may have erred on the side of including surrounding normal tissue if the boundary did not appear distinct, with the overall result that the actual tumor to normal ratio for Pc 4 uptake may have been higher than what is reported here.

The prevalence of necrosis in the high radiant exposure group was expected. The detection of apoptosis in both groups was equally encouraging. Together these findings do nothing to dampen the possibility that Pc 4 might eventually be used in a predictable fashion to sensitize PDT of glioma in human patients. The preliminary results from this study are not sufficient to suggest an effective time for

drug circulation nor an effective drug dose or radiant exposure for Pc 4-mediated PDT. However, the preferential tumor concentrations of Pc 4, and what appear to be Pc 4-mediated PDT-induced necrosis and apoptosis, are sufficient justification for further pre-clinical research to determine whether there are safe (i.e., drug uptake and PDT photosensitization specific to tumor, and rapid rate of clearance from the vascular compartment) and effective (i.e., high sensitivity of tumor) radiant exposure and Pc 4 dose levels for PDT of U87-derived glioma in the nude rat model. It would also be useful to determine whether Pc 4 can be profitably used for intra-operative tumor localization via fluorescence. Determination of safe and efficacious Pc 4-mediated-PDT and fluorescence localization of U87-derived glioma would set a strong baseline for translating Pc 4 to a clinical trial for human glioma patients.

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REFERENCES

1. CBTRUS (Central Brain Tumor Registry of the US). 2002–2003. Primary Brain Tumors in the United States, Statistical Report (<http://www.cbtrus.org/factsheet.htm>).
2. McLendon RE, Halperin EC. Is the long-term survival of patients with intracranial glioblastoma multiforme overstated? *Cancer* 2003;98:1745–1748.
3. Rosenthal MA, Kavar B, Uren S, Kaye AH. Promising survival in patients with high-grade gliomas following therapy with a novel boronated porphyrin. *J Clin Neurosci* 2003;10:425–427.
4. Schmidt MH, Meyer GA, Reichert KW, Cheng J, Krouwer HG, Ozker K, Whelan HT. Evaluation of photodynamic therapy near functional brain tissue in patients with recurrent brain tumors. *J Neurooncol* 2004;67:201–207.
5. Stummer W, Novotny A, Stepp H, Goetz C, Bise K, Reulen HJ. Fluorescence-guided resection of glioblastoma multiforme by using 5-aminolevulinic acid-induced porphyrins: A prospective study in 52 consecutive patients. *J Neurosurg* 2000;93:1003–1013.
6. Oleinick NL, Antunez AR, Clay ME, Rihter BD, Kenney ME. New phthalocyanine photosensitizers for photodynamic therapy. *Photochem Photobiol* 1993;57:242–247.
7. Weishaupt K, Gomer CJ, Dougherty T. Identification of singlet oxygen as the cytotoxic agent in photoinactivation of a murine tumor. *Cancer Res* 1976;36:2326–2329.
8. Moan J, Berg K. Phototherapy of cancer: Experimental research. *Photochem Photobiol* 1992;55:931–948.

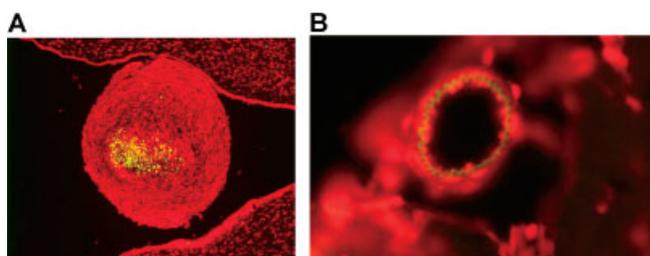


Fig. 2. TUNEL-stained, Pc 4-photosensitized U87 tumors: (A) is Specimen 87. It was exposed to the high radiant exposure (i.e., 30 J/cm²). The area surrounding the central concentration of apoptosis was largely necrotic. B: Shows areas of apoptosis in a blood vessel in specimen 79. Magnifications of specimens: A: 100× and 2B: 400×.

9. Macdonald IJ, Dougherty TJ. Basic principles of photodynamic therapy. *J Porphyrins and Phthalocyanines* 2001;5: 105–129.
10. Oleinick NL, Morris RL, Belichenko I. The role of apoptosis in response to photodynamic therapy: What, where, why, and how. *Photochem Photobiol Sci* 2002;1:1–21.
11. Lam M, Oleinick NL, Nieminen AL. Photodynamic therapy-induced apoptosis in epidermoid carcinoma cells. Reactive oxygen species and mitochondrial inner membrane permeabilization. *J Biol Chem* 2001;276:47379–47386.
12. Gomer CJ. DNA damage and repair in CHO cells following hematoporphyrin photoradiation. *Cancer Lett* 1980;11:161–167.
13. Moan J, Waksvik H, Christensen T. DNA single-strand breaks and sister chromatid exchanges induced by treatment with hematoporphyrin and light or by X-rays in human NHIK 3025 cells. *Cancer Res* 1980;40:2915–2918.
14. Ramakrishnan N, Oleinick NL, Clay ME, Horng MF, Antunez AR, Evans HH. DNA lesions and DNA degradation in mouse lymphoma L5178Y cells after photodynamic treatment sensitized by chloroaluminum phthalocyanine. *Photochem Photobiol* 1989;50:373–378.
15. Gomer CJ, Rucker N, Murphree AL. Differential cell photosensitivity following porphyrin photodynamic therapy. *Cancer Res* 1988;48:4539–4542.
16. Wilson BC, Olivo M, Singh G. Subcellular localization of Photofrin and aminolevulinic acid and photodynamic cross-resistance in vitro in radiation-induced fibrosarcoma cells sensitive or resistant to photofrin-mediated photodynamic therapy. *Photochem Photobiol* 1997;65:166–176.
17. Trivedi NS, Wang HW, Nieminen AL, Oleinick NL, Izatt JA. Quantitative analysis of Pc 4 localization in mouse lymphoma (LY-R) cells via double-label confocal fluorescence microscopy. *Photochem Photobiol* 2000;71:634–639.
18. Morris RL, Varnes ME, Kenney ME, Li YS, Azizuddin K, McEnery MW, Oleinick NL. The peripheral benzodiazepine receptor in photodynamic therapy with the phthalocyanine photosensitizer Pc 4. *Photochem Photobiol* 2002;75:652–661.
19. Usuda J, Chiu SM, Murphy ES, Lam M, Nieminen AL, Oleinick NL. Domain-dependent photodamage to Bcl-2. A membrane anchorage region is needed to form the target of phthalocyanine photosensitization. *J Biol Chem* 2003;278: 2021–2029.
20. Xue LY, Chiu SM, Oleinick NL. Photochemical destruction of Bcl-2 oncoprotein during photodynamic therapy with the phthalocyanine photosensitizer Pc4. *Oncogene* 2001;20: 3420–3427.
21. Xue LY, Chiu SM, Fiebig A, Andrews DW, Oleinick NL. Photodamage to multiple Bcl-xL isoforms by photodynamic therapy with the phthalocyanine photosensitizer Pc 4. *Oncogene* 2003;22:9197–9204.
22. Fels C, Schafer C, Huppe B, Bahn H, Heidecke V, Kramm CM, Lautenschlager C, Rainov NG. Bcl-2 expression in higher-grade human glioma: A clinical and experimental study. *J Neurooncol* 2000;48:207–216.
23. Usuda J, Azizuddin K, Chiu SM, Oleinick NL. Association between the photodynamic loss of Bcl-2 and the sensitivity to apoptosis caused by phthalocyanine photodynamic therapy. *Photochem Photobiol* 2003;78:1–8.
24. Chiu SM, Xue LY, Usuda J, Azizuddin K, Oleinick NL. Bax is essential for mitochondrion-mediated apoptosis but not for cell death caused by photodynamic therapy. *Br J Cancer* 2003;89:1590–1597.
25. Morris RL, Azizuddin K, Lam M, Berlin J, Nieminen AL, Kenney ME, Samia AC, Burda C, Oleinick NL. Fluorescence resonance energy transfer reveals a binding site of a photosensitizer for photodynamic therapy. *Cancer Res* 2003;63: 5194–5197.
26. Lilge L, Wilson BC. Photodynamic therapy of intracranial tissues: A preclinical comparative study of four different photosensitizers. *J Clin Laser Med Surg* 1998;16: 81–91.
27. Lilge L, Portnoy M, Wilson BC. Apoptosis induced in vivo by photodynamic therapy in normal brain and intracranial tumour tissue. *Br J Cancer* 2000;83:1110–1117.
28. Lilge L, Ching E, Portnoy M, Molckovsky A, Wilson BC. Photofrin mediated PDT in normal rat brain: Assessment on apoptosis as a quantitative biological endpoint. In: Dougherty TJ, editor. *Optical methods for tumor treatment and detection: Mechanisms and techniques in photodynamic therapy IX*, Vol. 3909. Bellingham, WA: SPIE; 2000. 45–52.
29. Anderson C, Hrabovsky S, McKinley Y, Tubesing K, Tang HP, Dunbar R, Mukhtar H, Elmets CA. Phthalocyanine photodynamic therapy: Disparate effects of pharmacologic inhibitors on cutaneous photosensitivity and on tumor regression. *Photochem Photobiol* 1997;65:895–901.
30. Lilge L, Portnoy M, Wilson BC. PDT-induced apoptosis in brain tissue in vivo: Apoptosis following PDT in normal and VX2 tumor-bearing rabbit brain. In: Dougherty TJ, editor. *Optical methods for tumor treatment and detection: Mechanisms and techniques in photodynamic therapy VIII*, Vol. 3592. Bellingham, WA: SPIE; 1999. 28–36.
31. Jiang F, Lilge L, Belcuig M, Singh G, Grenier J, Li Y, Chopp M. Photodynamic therapy using Photofrin in combination with buthionine sulfoximine (BSO) to treat 9L gliosarcoma in rat brain. *Lasers Surg Med* 1998;23:161–166.
32. Rainov NG, Koch S, Sena-Esteves M, Berens ME. Characterization of a canine glioma cell line as related to established experimental brain tumor models. *J Neuropathol Exp Neurol* 2000;59:607–613.
33. Saini M, Bellinzona M, Meyer F, Cali G, Samii M. Morphometrical characterization of two glioma models in the brain of immunocompetent and immunodeficient rats. *J Neurooncol* 1999;42:59–67.
34. Stummer W, Stocker S, Novotny A, Heimann A, Sauer O, Kempfski O, Plesnila N, Wietzorrek J, Reulen HJ. In vitro and in vivo porphyrin accumulation by C6 glioma cells after exposure to 5-aminolevulinic acid. *J Photochem Photobiol B* 1998;45:160–169.
35. Obwegeser A, Jakober R, Kostrom H. Uptake and kinetics of 14C-labelled meta-tetrahydroxyphenylchlorin and 5-aminolevulinic acid in the C6 rat glioma model. *Br J Cancer* 1998;78:733–738.
36. Hebeda KM, Saarnak AE, Olivo M, Sterenberg HJ, Wolbers JG. 5-Aminolevulinic acid induced endogenous porphyrin fluorescence in 9L and C6 brain tumours and in the normal rat brain. *Acta Neurochir (Wien)* 1998;140(5):503–512; discussion 512–513. Erratum in: *Acta Neurochir (Wien)* 1998; 140(8):881.
37. Jiang F, Robin AM, Katakowski M, Tong L, Espiritu M, Singh G, Chopp M. Photodynamic therapy with photofrin in combination with buthionine sulfoximine (BSO) of human glioma in the nude rat. *Lasers Med Sci* 2003;18:128–133.
38. Engebraaten O, Hjortland GO, Hirschberg H, Fodstad O. Growth of precultured human glioma specimens in nude rat brain. *J Neurosurg* 1999;90:125–132.
39. Deisboeck TS, Berens ME, Kansal AR, Torquato S, Stemmer-Rachamimov AO, Chiocca EA. Pattern of self-organization in tumour systems: Complex growth dynamics in a novel brain tumour spheroid model. *Cell Prolif* 2001;34:115–134.
40. Lobel J, MacDonald IJ, Ciesielski MJ, Barone T, Potter WR, Pollina J, Plunkett RJ, Fenstermaker RA, Dougherty TJ. 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH) in a nude rat glioma model: Implications for photodynamic therapy. *Lasers Surg Med* 2001;29:397–405.