Photodynamic Therapy Using Photofrin in Combination With Buthionine Sulfoximine (BSO) to Treat 9L Gliosarcoma in Rat Brain

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Background and Objective: The reactive oxygen mechanisms associated with cell damage after photodynamic therapy (PDT) may be exploited to enhance tumor destruction. Pharmacological reduction of glutathione (GSH), an inhibitor of reactive oxygen species, can be induced by administration of buthionine sulfoximine (BSO).

Study Design/Materials and Methods: BSO was administered in combination with Photofrin as the photosensitizer in order to promote PDT induced cell damage. Photofrin (12.5 mg/kg) or Photofrin with BSO (440 mg/kg) were administered to male Fischer rats (n = 27) containing an intracerebral 9L gliosarcoma or to non tumored rats. Brain tumor or non tumored brain was treated with an optical (632 nm) irradiance of 140 J/cm². Animals were sacrificed 24 h after PDT and the volume of tissue necrosis was measured. Brain Photofrin concentration was measured in tumor and in non tumor bearing animals administered either Photofrin or Photofrin with BSO. GSH was measured by high pressure liquid chromatography in tumor and homologous non tumor tissue in animals administered BSO or control solution.

Results: The volume of tumor necrosis was significantly greater in animals administered Photofrin and BSO than in animals administered only Photofrin. No differences were detected in non tumored tissue damage between groups. No differences in Photofrin concentration were detected in tumored or non tumored animals between animals administered Photofrin and animals administered Photofrin and BSO. BSO administration preferentially and significantly reduced GSH in tumor compared to non tumor tissue.
INTRODUCTION

Primary malignancies of the CNS comprise a group of highly heterogeneous and lethal neoplasms [1]. Even external beam radiation therapy and adjuvant chemotherapy with nitrosourea-based regimens following surgical resection have provided only modest improvements in the survival of patients with high-grade gliomas [2]. Unfortunately, malignant gliomas of the brain still have a poor prognosis, and no satisfactory treatment of these tumors is available [3].

Photodynamic therapy (PDT) of gliomas has been performed in experimental animal tumors and in human patients [4–6]. Although PDT can selectively destroy malignant tissue, based on the ability to localize the activating light and to concentrate the photosensitizers within the tumor, PDT has been infrequently employed in the clinical environment one of the reasons being severe phototoxicity to normal brain tissue. Thus it is warranted to identify ways to improve the tumoricidal capability of PDT without increasing toxicity to normal brain tissue.

Glutathione (r-glutamylcysteinylglycine) is an intracellular nonprotein sulfhydryl donor [7], which regulates intracellular free radical concentration [8]. GSH is present in high concentrations in human tumor cells [9,10], and decreases the cytotoxic effects of electrophilic alkylating agents and radiation [11,12]. Decreasing intracellular GSH levels enhances tumor cell chemosensitivity [13]. Glutathione depletion is an attractive strategy for increasing sensitization of tumor cells to PDT. Buthionine sulfoximine (BSO) has been used to deplete glutathione in vivo [11,14] and in vitro [11,15]. The active diastereoisomer, L-buthionine-S-sulfoximine inhibits r-glutamylcysteine synthetase, the rate-limiting enzyme in the pathway of glutathione biosynthesis [16,17]. In vitro studies by Miller and Henderson have demonstrated that BSO promotes PDT induced cell death [18]. However, to out knowledge, in vivo studies of PDT combined with BSO to reduce glutathione levels have not been performed. The aim of the present study is to improve the tumoricidal activity of PDT on the 9L-gliosarcoma in the rat brain using BSO as an adjuvant to the photosensitizer Photofrin.

MATERIALS AND METHODS

9L-Gliosarcoma Implantation in Fischer Rats.

Male Fischer rats (n = 26) (Charles River Breeding, Wilmington, MA) with body weights of 180–250 g were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg) administered intramuscularly (im). Atropine was also injected (im) at the time of anesthesia induction at a concentration of 0.04 mg/kg. Once fixed in a stereotaxic device, the scalp was reflected, the cranium exposed, and a 2–3 mm incision made directly down the midline. By using a high-speed drill, a 5 mm craniectomy was made on the right hemisphere anterior to the coronal suture. The tip of a 10 μl Hamilton syringe was inserted 3 mm beneath the dura and 104 9L cells in 5 μl minimum essential medium (MEM) were injected intracerebrally. The craniectomy was covered with a film of polyvinyl chloride glued to the surrounding intact bone and the incision was closed with 4-0 silk suture (Ethicon, Somerville, NJ). The tumors were allowed to grow to a diameter of 3.5 mm in 14 days.

Photodynamic Therapy

Male Fischer rats were treated 14 days after tumor implantation. Photofrin (12.5 mg/kg) in a 5% dextrose solution plus BSO (440 mg/kg) (Photofrin + BSO) or Photofrin in dextrose solution alone was injected intraperitoneally 24 h prior to light treatment. Each group contained five rats. Normal brain was subjected to PDT in separate non-tumor bearing rats, treated with either Photofrin + BSO (n = 4) or Photofrin alone (n = 5). All treatment parameters and surgical procedures, including craniectomy (made at the time of light irradiation), were identical to the tumor-bearing animals.

Surgery and PDT laser treatment were performed, as described elsewhere [5,6]. Briefly, animals were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg). Body temperature was
monitored with a rectal probe and maintained by use of a water recirculating K-module. Light (632 ± 2 nm) from an argon-pumped dye laser was delivered via a fiber optic equipped with a microlens at the distal end. The brain was illuminated through the 5 mm craniectomy. The irradiance was maintained at 100 mW/cm² during treatment, and an optical radiant exposure of 140 J/cm² delivered.

**Histopathology**

Animals were sacrificed 24 h post PDT and brain tissues were processed to determine the volume of pan necrosis and incomplete necrosis, as described elsewhere [5,6,19]. Briefly, under general anesthesia induced with ketamine (80 mg/kg) and xylazine (13 mg/kg) administered intramuscularly (im), the animals were perfused via the left ventricle with neutral buffered formalin following vascular wash out with heparinized saline. The brain was removed and the brain tissue, encompassing the entire tumor, was cut into four coronal blocks (3 mm thick). Tumor-bearing brain sections were routinely processed, placed in paraffin, and subsequently five equally spaced sections (6 μm thick) were obtained from the blocks encompassing the tumor. These sections were stained with Hematoxylin and Eosin (H&E) for light microscopic examination (×2.5 magnification) and image analysis. The volume of tumor tissue damaged by PDT was measured using light-microscopic analysis and a Global Lab Image analysis program (Data Translation, Marlboro, MA) with the observer blinded to the treatment conditions. In each coronal section, the area (mm²) of the PDT damaged tumor was measured by tracing the damaged tissue boundary on the computer screen, and the volume of necrosis (mm³) was determined by multiplying each area with the respective section interval thickness. The volume of PDT damaged tumor, including massive complete tumor necrosis and multifocal incomplete tumor necrosis, were calculated. Identical analyses were performed on normal brain tissue subjected to PDT.

**Photofrin Concentration Analysis**

Fourteen days post tumor implantation (9L-gliosarcoma), separate groups of animals (two groups of four animals each) were injected (ip) with Photofrin + BSO or Photofrin alone, respectively, at a dose of 12.5 mg/kg (Photofrin) and 440 mg/kg (BSO). Twenty-four hours after Photofrin or Photofrin × BSO administration, the tumor bearing animals were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg) and decapitated. The brains from all animals were quickly removed. The tumor, brain adjacent to tumor (BAT), and contralateral non-tumor bearing hemisphere were dissected and frozen at −70°C. Two additional groups (n = 4) of normal non-tumor bears were injected (ip) with Photofrin + BSO or Photofrin alone, respectively, and were subjected to the identical experimental procedures used for measurement of Photofrin concentration as the tumor bearing animals.

The Photofrin concentration analysis procedure is described elsewhere [20]. Briefly, each tissue sample of approximately 0.1 g wet weight (w_t) was immersed in 2 ml (V_s) of Solvable (Dupont-Biotechnology Systems, Boston, MA) and placed into a shaking water bath at 50°C for 1 h. The sample was then mechanically homogenized and up to five 200 μl aliquots of the resulting homogenate and an equal number of 200 μl aliquots of each sample spiked with a known amount of Photofrin (V_sp = 50 μl at C_sp = 37.5 μg/ml), were diluted with 1 ml Solvable and 3 ml distilled water and returned to the water bath for another hour. The Photofrin used to spike some of the aliquots was first monomerized by diluting the stock solution with methanol to 0.075 mg/ml, with further dilution to C_sp using distilled water.

Samples were diluted with double-distilled water to achieve an OD < 0.1 at 405 μm the excitation wavelength. For analysis, the porphyrin emission intensity was integrated from 590 to 730 nm.

The Photofrin concentration in the sample was calculated as

\[ C_t = \left( \frac{I_{sp}}{I_{nsp}} \right) \left( \frac{D_{sp}}{D_{nsp}} \right) \left( 4.2 \right)^{-1} \left( \frac{V_t + V_s}{V_t} \right) C_{sp} \left( 4.25 \right) \left( 0.05 \right) \left( 4.2 \right) \left( 4.25 \right) \left( 0.2 \right) \]

where \( I_{sp}, I_{nsp}, D_{sp}, \) and \( D_{nsp} \) are the intensities and dilution factors to reduce the OD below 0.1 for the spiked and unspiked aliquots, respectively, and \( V_t \) the volume of the wet tissue sample assuming a density of 1. \( V_s \) and \( V_{sp} \) is the sample volume after adding Solvable for the unspiked and the spiked samples, respectively. The ratio 4.2/4.25 corrects for the volume difference of unspiked and spiked aliquots and 4.2/0.2 was derived from the dilution step when splitting the various aliquots.
Determination of Intracellular Glutathione by HPLC

Two groups of four animals each were tested 14 days post tumor implantation (9L-gliosarcoma). One group of animals was injected (ip) with BSO at a dose of 440 mg/kg. A control group of animals was injected (ip) with an equivalent volume of 5% dextrose solution. Twenty-four hours after BSO or dextrose solution administration, the animals of both treatment and control groups, were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg) and decapitated. The brain from all animals were quickly removed. The tumor and contralateral non-tumor-bearing hemisphere were dissected, frozen, and stored at −70°C. For analysis, the frozen tissues were thawed in water, blotted dry, and weighed. After weighing, the tissues were homogenized in ice-cold 5% 5-sulfosalicylic acid (SSA) containing 0.25 mM EDTA (Calbiochem, San Diego, CA) by using a manual glass homogenizer. The homogenates were centrifuged at 10,000g for 5 min in an Eppendorf Centrifuge (model 5415C) at 5°C. The GSH in the supernatant was derivatized with monobromobimane (mBBr; 3,7-dimethyl-4-bromomethyl-6-metlyl-1,5-diazobicyclo octa-3,6-diene-2,8-dione) (Calbiochem, San Diego, CA), according to a procedure described by Anderson [21], with some minor modifications. Analysis of GSH was performed by using a Waters 600E HPLC system equipped with a Waters 474 Scanning Fluorescence Detector (Waters, Ltd., Mississauga, Ontario, Canada). The GSH-mBBr derivative was analyzed using a Nova-PakC18 reverse-phase cartridge column (60A, 4 μm, 3.9 mm x 150 mm). The fluorescence detector was operated at an excitation wavelength of 375 nm and an emission wavelength of 465 nm.

RESULTS

Photofrin Tissue Levels

The tissue concentrations of Photofrin for the Photofrin + BSO and Photofrin alone treatment groups in tumor tissue, BAT, and contralateral hemisphere normal brain tissues, and in brain from non tumored animals are shown in Table 1. In all animals from both treatment groups, Photofrin concentration was significantly higher ($P < 0.05$) in tumor than in the contralateral hemisphere or BAT. No significant differences in Photofrin concentration were detected between tumored or non tumored animals administered Photofrin and animals administered Photofrin + BSO.

Effect of Photodynamic Therapy on the Intracerebral Tumor

On microscopic histological analysis performed at 16 days after tumor implantation, uniform tumor masses were clearly identified by H&E staining. BAT was identified as the area around the main tumor mass of approximately 0.5 to 1 mm in depth that was slightly darker than the surrounding normal tissue. Cellular damage was not detected in tumors of the control group without PDT treatment. Photodynamic therapy induced lesions in tumor were irregularly shaped and marked by necrosis and focal hemorrhage. PDT induced lesion volumes within the tumor and the normal brain for the Photofrin + BSO and Photofrin alone treatment groups are shown in Table 2. The lesion volume within the tumors in the Photofrin + BSO group was significantly greater ($P < 0.05$) than in the Photofrin alone animal group. Normal brain control animals showed no surgery-associated mechanical damage, and their PDT induced lesions were necrotic and hemorrhagic. No significant differences in lesion vol-

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### TABLE 1. BSO-PDT Tissue Concentration of Photofrin μg/g

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Photofrin + BSO (n = 4) tissue concentration ± SE</th>
<th>Photofrin (n = 4) tissue concentration ± SE</th>
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</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>$5.2 \pm 1.5^*$</td>
<td>$4.9 \pm 0.7^{**}$</td>
</tr>
<tr>
<td>Brain adjacent to tumor</td>
<td>$0.6 \pm 0.2$</td>
<td>$0.7 \pm 0.2$</td>
</tr>
<tr>
<td>Normal brain (tumored rat)</td>
<td>$0.3 \pm 0.0$</td>
<td>$0.8 \pm 0.2$</td>
</tr>
<tr>
<td>Normal brain (non tumored rat)</td>
<td>$0.7 \pm 0.1$</td>
<td>$0.7 \pm 0.2$</td>
</tr>
</tbody>
</table>

*$P < 0.05$ compared to Brain Adjacent to Tumor and Normal Brain in Photofrin + BSO animals.

**$P < 0.05$ compared to Brain Adjacent to Tumor and Normal Brain in Photofrin animals.
volume of normal brain tissue were detected between the two animal groups of Photofrin + BSO and Photofrin alone treatments.

The tumor and normal brain concentrations of GSH in animals differed between animals given or not given BSO. In normal brain samples, the concentration of GSH decreased from a control value of $1.03 \pm 0.16 \mu$mol/g tissue (mean ± SD) to $0.67 \pm 0.02 \mu$mol/g tissue in BSO-treated rats ($n = 4$, $P = 0.02$). In the tumor samples, the GSH concentration decreased from a control value of $1.46 \pm 0.11 \mu$mol/g tissue to $0.48 \pm 0.12 \mu$mol/g tissue in BSO-treated rats ($n = 4$, $P = 0.003$).

**DISCUSSION**

The specific uptake ratio given by Photofrin concentration in tumor vs. normal tissue is comparable to previously published results [5,19]. We did not detect significant differences in the specific uptake of Photofrin by tumor tissues between the Photofrin + BSO and Photofrin alone groups (Table 1). These data are consistent with in vitro data that BSO has no effect on porphyrin uptake [18]. Although Photofrin levels are not increased by BSO administration, our data indicate that PDT using Photofrin combined with BSO significantly increases brain tumor destruction compared to PDT using Photofrin alone. The enhanced treatment of the 9L-gliosarcoma with Photofrin + BSO may therefore be attributed to the decreased tumor intracellular concentration of GSH induced by BSO administration. The tumor intracellular GSH concentrations in BSO injected animals are approximately three times lower than those in animals not injected with BSO. GSH deficiency sensitizes cells to effects of radiation, oxidative reactions to various toxic compounds [22] and to PDT with Photofrin as the photosensitizer [18].

There are multiple pathways by which depletion of GSH by BSO enhances PDT-induced tissue damage. Our data exclude an effect of BSO administration on Photofrin concentration. In vitro data from Miller and Henderson suggest that $^{1}\text{O}_2$ production is not affected by BSO or GSH [18]. GSH can scavenge free radicals and singlet oxygen. A primary target of PDT is the cellular membrane [23,24]. By reducing reactive oxygen species, GSH reduces membrane lipid peroxidation [25,26]. Thus, BSO by lowering GSH likely augments membrane damage and thereby promotes cellular destruction. GSH can also repair sublethal DNA single strand breaks [27]. However, in vitro data from Miller and Henderson [18] on PDT induced damage does not support a DNA repair function of GSH.

BSO is a synthetic amino acid analog. It is one of a family of sulfoximines synthesized as inhibitors of r-glutamylcysteine synthetase [16,17], the inhibition of which causes decreased glutathione biosynthesis. When exposed to BSO, cellular glutathione is effectively depleted in tissue exhibiting moderate to high rates of glutathione utilization [7,8].

Our data indicate significant glutathione reduction in normal brain tissues following BSO injection. However, GSH was preferentially reduced in tumor compared to non tumor tissue. This is consistent with previous reports by Skapek et al. [28]. The differential effect of BSO on GSH levels in brain tumors and normal brain may be due to differential rates of entry of BSO into the two tissues [28]. The comparable PDT induced non-tumored brain tissue damage in BSO and non BSO treated animals may therefore be attributed to a reduced differential depletion of GSH in these tissues.

In summary, our data suggest that BSO selectively enhances the brain tumor response to PDT with Photofrin. Further investigations on the dose response on BSO on GSH and tumored and non tumored tissue damage are therefore warranted.

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**TABLE 2. PDT-Induced Lesion Volumes**

<table>
<thead>
<tr>
<th>Energy fluence (J/cm²)</th>
<th>Photosensitizer</th>
<th>Lesion volume ± SD (mm³) normal brain</th>
<th>Lesion volume ± SD (mm³) tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>140</td>
<td>Photofrin</td>
<td>5.6 ± 2.3 ($n = 5$)</td>
<td>4.5 ± 2.2 ($n = 5$)*</td>
</tr>
<tr>
<td>140</td>
<td>Photofrin + BSO</td>
<td>5.4 ± 2.1 ($n = 4$)</td>
<td>9.5 ± 4.7 ($n = 5$)</td>
</tr>
</tbody>
</table>

*Significantly different from Photofrin + BSO animals, $P < 0.05$. 
REFERENCES


