Enhanced *in vitro* uptake and retention of $^3$H-tetraphenylphosphonium by nervous system tumor cells

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Photodynamic therapy is a promising treatment for human brain tumors because of the selective retention of certain compounds by tumor cells. Certain lipophilic cationic compounds, such as tetraphenylphosphonium (TPP), are selectively taken up by a variety of carcinomas. Although preferential retention of TPP has been demonstrated for the breast carcinoma cell line MCF-7, this compound had not been tested previously on cells derived from nervous system tumors. In the present study, tritiated-TPP ($^3$H-TPP) uptake and retention for eight different cell cultures of three histologically different types of nervous system tumors was measured and the data were compared to a positive control (MCF-7) and negative controls (normal African Green monkey kidney epithelium (CV-1) and the normal human fibroblast (WI-38) cell lines). Uptake and retention characteristics could be grouped by specific pathological tumor types, but individual tumor variability was notable. Malignant astrocytoma (grade III/IV glioblastoma) and malignant neurofibrosarcoma cells showed preferential uptake and retention of $^3$H-TPP relative to meningioma cells and normal controls. A clonogenic assay utilizing the cytotoxic lipophilic cationic compound dequinium showed strong retainers of $^3$H-TPP to be more susceptible to the effects of dequinium than weak retainers. These data demonstrate that certain human and experimental animal nervous system tumor cell lines retain lipophilic compounds possessing a delocalized positive charge. Lipophilic cationic compounds may be useful in the intraoperative delineation of tumor margins and in the photodynamic therapy of certain nervous system tumors.

**Key Words** • photodynamic therapy • central nervous system neoplasm • tumor marker • lipophilic cationic compound

Photodynamic therapy takes advantage of photoactive compounds that concentrate in tumor tissue but which are relatively nontoxic in the absence of light. Activation of such compounds by laser or filtered high-intensity light of an appropriate wavelength can produce two effects: 1) the compound may visibly fluoresce, allowing for differentiation of tumor from normal tissue; and/or 2) the compound may be activated to induce toxicity to the tumor cells and/or the supporting tissues.

Malignant central nervous system (CNS) tumors are potentially ideal targets for photodynamic therapy. At surgery the boundary between tumor and normal tissue is often difficult to differentiate. Even with aggressive surgery, it is impossible to resect many tumors completely because tumor cells can infiltrate into regions of otherwise normal parenchyma. Compounds that are selectively accumulated and retained by tumor cells relative to normal neural tissue and supportive cell types could provide a valuable method for selective intraoperative delineation of tumor from normal tissue. In addition, selective killing of nests of abnormal tumor tissue interdigitating within regions of normal parenchyma could be achieved by photoactivating these dyes.

Interest in photodynamic therapy for the treatment of solid tumors has stimulated a search for photoactive compounds that specifically target tumor cells. Hematoporphyrin derivative (HpD) has been the most widely studied photodynamic agent. The efficacy of HpD has been limited because it is activated at a wavelength with little tissue penetration and it has poor selectivity for tumors relative to normal tissues, such as skin. Several porphyrin analogs, including chlorins, phthalocyanins, purpurins, phophorhobides, and benzoporphyrins, which have improved photochemical properties, are being investigated. However, the selectivity of these compounds, which is of crucial importance in brain-tumor therapy, may limit their efficacy.

Another class of photoactive tumor-selective agents, lipophilic cationic compounds, can potentially provide
**In vitro** uptake of $^3$H-TPP by CNS tumor cells

Fig. 1. **Upper:** Structure of tetrphenylphosphonium chloride, [phenyl-(3)]. **Lower:** Structure of dequaininium chloride.

very high degrees of selectivity for tumors. We report the selective uptake and retention of a lipophilic cationic compound, tritiated tetrphenylphosphonium ($^3$H-TPP) (Fig. 1 upper), by seven human tumor specimens representing three histologically different types of CNS tumors. The characteristics of these cells are compared to those of the established rat C6 glioma cell line and to normal neonatal rat cortical cell and Schwann cell preparations as well as to control of human breast carcinoma (MCF-7), normal African Green monkey kidney epithelium (CV-1), and normal human fibroblast (WI-38) cell lines. Furthermore, we demonstrate an enhanced cytotoxic effect of the lipophilic cationic compound dequaininium (Fig. 1 lower) on cell populations that retain $^3$H-TPP strongly. These data indicate that lipophilic cationic compounds may prove useful as photosensitizers for some types of human CNS tumors.

**Materials and Methods**

**Cell Lines and Tissue Culture**

The human breast carcinoma (MCF-7), normal African Green monkey epithelium (CV-1), normal human fibroblast (WI-38), and rat C6 glioma cell lines were obtained from established lines at our institution. Human tumor cell cultures were obtained from surgical specimens of one malignant neurofibrosarcoma, three malignant astrocytomas (grade III/III glioblastoma), and three meningiomas (meningiomas A and B were benign and C was malignant). After initial tissue diagnosis by frozen section, a portion of each specimen was used for routine pathological studies and for cell culture.

The astrocytoma specimens were minced, centrifuged, and digested with collagenase type III* and 0.25% trypsin at 37°C, resuspended in F-10 medium with 10% fetal bovine serum (FBS) in plastic flasks, and grown at 37°C with 5% CO$_2$. The meningioma and neurofibrosarcoma specimens were prepared similarly, but with collagenase type II used for digestion.

Neonatal rat cortical cell cultures were prepared as described by Lam and as modified by Huettner and Baughman. The cortex was dissected from the brains of a litter of neonatal rats and then minced and digested in a papain-ethylenediaminetetra-acetic acid solution for 90 minutes at room temperature. The product was triturated with 1 mg/ml ovomucoid inhibitor solution† and passed through a fine nylon mesh before resuspension in Dulbecco’s minimum essential medium and transfer to tissue culture flasks.‡ Cortical cell cultures from the initial preparation and from passage No. 1 were used in two consecutive retention experiments. The gross cortical preparation contained a mixture of neurons and glial cells. Neonatal rat Schwann cell cultures were prepared in our laboratory, as previously described by Brockes, et al.¶

**Uptake and Retention Experiments**

Uptake and retention experiments were performed as described previously. Cells were grown on 12-mm square glass coverslips with a uniform seeding density of $5 \times 10^5$ cells per coverslip (100 μl of a $5 \times 10^5$ cells/ml suspension) in F-10 medium with 10% FBS. After 24 hours, the cells were incubated with $^3$H-TPP in F-10 medium without FBS at a final concentration of 0.5 μCi/μl. For uptake experiments, samples were incubated continuously in $^3$H-TPP medium and data points were taken in triplicate at the indicated times. For retention experiments, samples were incubated for 60 minutes in $^3$H-TPP-containing medium, rinsed twice with phosphate-buffered saline (PBS, pH 7.4), and subsequently incubated in F-10 medium without FBS. At the indicated times, coverslips were removed in triplicate from the dish, rinsed five times with PBS for 15 seconds, placed in scintillation vials, and counted in a liquid scintillation spectrophotometer using Aquasol.*

A series of blank coverslips were treated in parallel to correct for nonspecific absorption.

Uptake curves were generated by plotting an averaged absolute number of counts per minute versus time while retention curves represented a percentage of the initial uptake value at 60 minutes after exposure to $^3$H-TPP. The ratio of the absolute number of counts per minute for each specimen relative to the positive control, MCF-

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* Collagenase types II and III supplied by Worthington Biochemical Co., Freehold, New Jersey.
† Ovomucoid inhibitor solution supplied by Boehringer-Mannheim, Indianapolis, Indiana.
‡ Tissue culture flasks manufactured by Becton-Dickinson Labware, Oxnard, California.
TABLE 1

<table>
<thead>
<tr>
<th>Cell Line or Preparation</th>
<th>Passage No.</th>
<th>Percent Uptake*</th>
</tr>
</thead>
<tbody>
<tr>
<td>human cortical cells</td>
<td>1</td>
<td>150.4 ± 23.4</td>
</tr>
<tr>
<td>rat C6 glioma</td>
<td></td>
<td>136.6 ± 11.1</td>
</tr>
<tr>
<td>human breast carcinoma MCF-7</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>human neurofibrosarcoma</td>
<td>3</td>
<td>64.3 ± 23.4</td>
</tr>
<tr>
<td>rat schwann cells</td>
<td>3</td>
<td>59.8</td>
</tr>
<tr>
<td>human malignant gliomas</td>
<td>1</td>
<td>69.2</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>39.1 ± 1.6</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>52.6 ± 8.4</td>
</tr>
<tr>
<td>human fibroblast WI-38</td>
<td>16</td>
<td>30.0 ± 5.4</td>
</tr>
<tr>
<td>human meningiomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>30.1 ± 9.8</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>25.4</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>15.8</td>
</tr>
<tr>
<td>monkey epitheliun CV-1</td>
<td></td>
<td>0.4 ± 0.2</td>
</tr>
</tbody>
</table>

* Uptake of $^3$H-TPP expressed as a percentage of the positive control MCF-7, mean ± standard error of the mean. — = long-term cell line.

7, at the initial time point for each experiment was determined (Table 1). The mean and standard error values were calculated for each set of data points.

Clonogenic Assay

Clonogenic assays were performed as previously described. Briefly, about 40 cells were seeded in each well of a 24-well tissue culture plate† and incubated overnight. The next day, the growth medium was replaced with medium containing dequillinum chloride‡ at various concentrations. For limited-exposure experiments, the medium containing dequillinum was removed after 3 hours by rinsing each well with Hanks' balanced salt solution and adding drug-free medium. Other cells were exposed to dequillinum continuously for 1 week before staining. Colony-forming units were counted and the concentration of dequillinum required to inhibit growth was determined 1 week later by staining with 10% Giemsa stain.

Results

Uptake Experiments

The uptake of $^3$H-TPP by various CNS tumor types was compared to that of MCF-7, a breast carcinoma cell line known to have increased uptake relative to normal cells (Table 1). Uptake was measured at 60 minutes after initial exposure to $^3$H-TPP. The data were derived from both the uptake and retention experiments studied. The mean uptake was 26.9% ± 6.6%

‡ Tissue culture plate manufactured by Becton-Dickinson Labware, Oxnard, California

‡ Dequillinum chloride supplied by Sigma Chemical Co., St. Louis, Missouri.
**In vitro** uptake of $^3$H-TPP by CNS tumor cells

**Fig. 4.** $^3$H-TPP retention curves for human malignant gliomas 1, 2, and 3, and breast carcinoma (MCF-7), human fibroblast (WI-38), and monkey epithelium (CV-1) cells. Data are expressed as a percentage of the initial uptake obtained after 60 minutes of exposure to $^3$H-TPP-containing media.

**Fig. 5.** $^3$H-TPP retention curves for human malignant neurofibrosarcoma (NFS), rat C6 glioma, breast carcinoma (MCF-7), and human fibroblast (WI-38) cells. Data are expressed as a percentage of the initial uptake obtained after 60 minutes of exposure to $^3$H-TPP-containing media.

**Fig. 6.** $^3$H-TPP retention curves for rat Schwann cell and cortical cell preparations, and breast carcinoma (MCF-7) and human fibroblast (WI-38) cells. Data are expressed as a percentage of the initial uptake obtained after 60 minutes of exposure to $^3$H-TPP-containing media.

Human glioma cells were studied in three of the retention experiments. The selective retention was variable for gliomas 1, 2, and 3. Glioma 1 and 2 cells retained $^3$H-TPP to a degree intermediate to that of the positive (MCF-7) and negative (WI-38, CV-1) controls, while Glioma 3 cells behaved similarly to WI-38 cells (Fig. 4).

The rat C6 glioma and the human malignant neurofibrosarcoma cells retained $^3$H-TPP strongly (Fig. 5), although to a slightly lesser extent than the MCF-7 cells. At the latest time point (190 minutes), the C6 glioma and the neurofibrosarcoma cells had retained 53.5% and 43.2% of their initial counts, respectively, while MCF-7 cells had retained 53.0%.

Retention characteristics for the neonatal rat Schwann cell and cortical cell preparations were assessed (Fig. 6); mean values at 180 minutes were 61.6% and 47.3%, respectively. Retention values for MCF-7 and WI-38 cells were consistent with the previously described results. The pooled background average for all retention experiments was not significant.

**Retention Experiments**

Representative retention curves for the cell types studied are summarized in Figs. 3 to 6. Retention data are expressed as a percentage of the initial uptake after 60 minutes of exposure to $^3$H-TPP containing media (retention time, $t = 0$). Meningiomas were studied in four different experiments. All three meningiomas retained much less $^3$H-TPP for a shorter period of time than did the positive control MCF-7. The retention characteristics of Meningiomas A, B, and C were all similar and paralleled those of the negative control WI-38 (Fig. 3). Retention of $^3$H-TPP by CV-1 fell off more rapidly than that of the meningiomas and WI-38. The MCF-7 cells behaved characteristically, retaining more than one-half of the accumulated $^3$H-TPP over the course of the assay.

**Clonogenic Assay**

Cell types that accumulate more lipophilic cationic compound receive a higher effective dose and will, therefore, be more sensitive to toxic lipophilic cationic compounds such as dequalinium. A clonogenic assay was used to compare the effect of dequalinium on C6 glioma, malignant neurofibrosarcoma, MCF-7, and WI-38 cells. For the 3-hour exposures, C6 glioma, neurofibrosarcoma, and MCF-7 cells demonstrated complete growth inhibition at a dequalinium concentration of 10 $\mu$M while WI-38 cells showed complete growth inhibition at a concentration of 30 $\mu$M (Figs. 7 and 9). With continuous exposure to dequalinium, complete growth inhibition occurred at a dequalinium concentration of 1 $\mu$M for C6 glioma, malignant neurofibrosar-
Fig. 7. Results of 3-hour exposure to dequalinium clonogenic assay. Rat C6 glioma, neurofibrosarcoma (NFS), and breast carcinoma (MCF-7) cells demonstrated increased sensitivity relative to human fibroblast (WI-38) cells.

Fig. 8. Results of continuous exposure to dequalinium clonogenic assay. Rat C6 glioma, neurofibrosarcoma (NFS), and breast carcinoma (MCF-7) cells demonstrated increased sensitivity relative to human fibroblast (WI-38) cells.

...coma, and MCF-7 cells, but required a concentration of 3 μM for WI-38 cells (Figs. 8 and 9). Thus, under these conditions dequalinium is three times more toxic to C6 glioma, neurofibrosarcoma, and MCF-7 cells than to the WI-38 cells.

Discussion

Photodynamic Agents

For a photodynamic agent to be effective in the treatment of CNS tumors, it must first be selectively accumulated and/or retained by neoplastic cells relative to normal neuronal and supportive tissue cell populations. To be useful to the surgeon resecting the tumor, or as an adjuvant to surgical therapy, the compound must then either become visible or cytotoxic or both. Hematoporphyrin derivative has been the most intensively studied photosensitizing agent and is known to accumulate selectively in some brain tumors relative to normal brain.15 Because it is not lipophilic and has a high affinity for serum proteins, HpD is not efficiently distributed to tumor cells where the blood-brain barrier is intact. Clinical studies to date have not demonstrated efficacy for its use in brain-tumor therapy.6,12

In contrast, the lipophilic properties of cationic compounds enable them to cross the intact blood-brain barrier.16 Their delocalized positive charge, combined with the hyperpolarized (more negative) mitochondrial membrane potential of certain tumor cell types, results in the selective accumulation and retention of these compounds by some tumors.6,20 Once selectively retained by the tumor, some of these compounds can be made to fluoresce and/or to kill the cell as a result of exposure to light at the appropriate wavelength.14-16

Meningiomas

Meningiomas are neoplasms derived from the arachnoid villi. In the normal state, these cells are responsible for the resorption of cerebrospinal fluid from the subarachnoid space into the venous sinuses. Meningiomas are usually benign, well-circumscribed masses that indent rather than invade brain or spinal cord; nonetheless, malignant and invasive forms exist. We studied three different meningioma cell cultures established from operative specimens. Of these, two were histologically and clinically benign (Meningiomas A and B) and one was malignant (Meningioma C). The benign and malignant forms of this tumor behaved similarly for both uptake and retention (Table 1 and Fig. 3). Initial uptakes, as well as retention of 3H-TPP, were similar to the WI-38 cells that were used as a negative control. This suggests that neither the benign nor malignant meningiomas tested have hyperpolarized mitochondrial membrane potentials and that lipophilic cationic compounds would not be useful for localizing and/or treating these tumors.

Human Malignant Gliomas

Malignant glial tumors pose a difficult problem for the neurological specialist attempting to treat these tumors medically or surgically.17 The tumor/normal brain parenchymal margins are difficult to differentiate at surgery, making it nearly impossible to safely resect these tumors completely. There is evidence that radical excision of the gross component of malignant gliomas may offer prolonged survival, with concomitant improvement in functional capacity.1 A compound that could create a visible demarcation between tumor and normal brain tissue would allow for safer resection up to that margin, leaving only those cells that comprise the invading tumor "fingers" and "islands." If the same or a different compound was also capable of selective cell killing after exposure to light of the appropriate wavelength, then intraoperative treatment of the tumor bed could be performed after the initial gross resection.
In vitro uptake of $^3$H-TPP by CNS tumor cells

![Dequalinium Clonogenic Assay](image)

**Fig. 9.** Photograph of 3-hour and continuous-exposure dequalinium clonogenic assays. Multi-well plates for each cell type tested are shown with corresponding concentration of dequalinium ($\mu$M) for each well.

Increased intratumoral levels of the lipophilic cationic compound rhodamine-123 have been demonstrated after intravenous injection in a malignant glioma model in rats, demonstrating potential uptake of these compounds by glial tumors. $^5,^{16}$

The three human malignant gliomas evaluated in this study were of pathological grade III/III and demonstrated variable selective uptake and retention of $^3$H-TPP. Figure 4 shows that the degree of selective retention of Gliomas 1 and 2 is intermediate between the positive control MCF-7 and negative controls CV-1 and WI-38, but that Glioma 3 behaves similarly to WI-38 in this regard. The variability of uptake and retention characteristics between these three histologically similar tumors suggests that some, but not all, malignant gliomas may be amenable to this type of therapy. A rapid *in vitro* method to identify susceptible tumors at the time of surgery, similar to that described here, or perhaps based upon flow cytometric measurements of uptake of a fluorescent dye, could be developed if further studies indicate efficacy for these drugs in the treatment of tumor patients.

The rat C6 glioma cells demonstrated strong uptake and retention characteristics for $^3$H-TPP (Figs. 2 and 5) and is long established in an *in vivo* model.

**Human Malignant Neurofibrosarcoma**

The human malignant neurofibrosarcoma cells also exhibited excellent uptake and retention characteristics for $^3$H-TPP (Figs. 2 and 5). Recently, human neurofibrosarcoma has been grown successfully in the nude mouse subrenal capsule and has been used as a model for other experimental treatment protocols. $^13$ Neurofibrosarcoma is a fatal tumor refractory to current modalities of therapy and is associated with a 5-year survival rate of less than 20%. $^{18}$

The development of an effective treatment for neurofibrosarcoma would be beneficial for neurofibromatosis patients as well as sporadic cases of neurofibrosarcoma in the general population. The promising uptake and retention characteristics of the neurofibrosarcoma cells suggest the need for further *in vitro* and *in vivo* studies to investigate the efficacy of novel lipophilic cationic compounds for tumor treatment.

**Neonatal and Cortical Cell Preparations**

The neonatal rat Schwann and cortical cell preparations demonstrated a strong affinity for $^3$H-TPP. The cortical cell preparation reflects the retention characteristics of a mixed population of cells. The behavior of the tissue culture preparation of neonatal cells implies that lipophilic cationic compounds may be selectively retained by populations of normal cells. This observation is important as it demonstrates the potential risks of neurotoxicity with these compounds and the need to proceed with animal studies to differentiate uptake and retention characteristics between normal and tumor tissues. Beckman, *et al.*, $^3$ studied the differential retention characteristics of the lipophilic cationic compound rhodamine-123 by glioma and normal brain tissue of the rat *in vivo*. They concluded that rhodamine-123 is taken up and retained by the glioma cells, but not by the normal brain tissue. Further studies are needed to clarify this issue.

**Clonogenic Assay**

Dequalinium is a lipophilic compound which contains two delocalized positive charges and has previ-
ously been shown to be selectively toxic to carcinoma cells (Fig. 1 lower). The fact that dequalinium was more toxic to the C6 glioma, neurofibrosarcoma, and MCF-7 cells than to the negative control WI-38 cells at both the 3-hour and continuous-exposure times (Figs. 7, 8, and 9) supports the \(^3\)H-TPP uptake and retention data, and implies that other compounds of this class may also be effective against these tumor cell lines.

We have demonstrated that several CNS tumors, including human glioma, human neurofibrosarcoma, and the rat C-6 glioma strongly retain the lipophilic cationic compound \(^3\)H-TPP and that they have increased susceptibility to the cytotoxic action of the lipophilic cationic compound dequalinium. Further studies are indicated to assess the in vitro distribution and effects that this class of compounds has on tumor growth and animal survival.

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References


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