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Laboratory Investigation

Biodistribution of copper carboranyltetraphenylporphyrins in rodents bearing an isogeneic or human neoplasm

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Summary

The biodistributions of carborane-containing copper porphyrins, CuTCP and CuTCPH, have been studied previously in mice bearing subcutaneously implanted mammary carcinomas. We now report biodistributions of those porphyrins in Fischer 344 rats bearing intracranial and/or multiple subcutaneous isogeneic 9L gliosarcomas (9LGS). The porphyrin was given either by i.v. infusion or by multiple i.p. injections. When 190 mg CuTCPH/kg body weight was given to the rats by i.v. infusion, median tissue boron concentrations (μg/g) 3 days after the end of infusion were: 64 in subcutaneous tumor, 13 in intracranial tumor, 1 in blood and 3 in brain. When 450 mg CuTCPH/kg body weight was given to the rats by serial i.p. injections, the median concentrations (μg B/g) 4 days after the last injection were: 117 in subcutaneous tumor, 50 in intracranial tumor, 4 in blood, and 4 in brain. CuTCPH biodistribution was also studied in xenografts of the human malignant gliomas U87 and U373, and of the murine EMT-6 mammary carcinoma and the rat 9LGS, each grown subcutaneously in mice with severe combined immunodeficiency (SCIDs). In SCIDs, median boron concentrations (μg/g) 2 days after the last s.c. injection of a total of 190 mg CuTCPH/kg body weight were: 251 in U373, 33 in U87, <0.6 in blood and <0.5 in brain. Because there were such high boron levels in the U373, and because xenografted U373 is similar to spontaneous intracerebral human glioblastoma multiforme (GBM) microscopically, CuTCPH could prove useful as a boron carrier for boron neutron-capture therapy (BNCT) of GBM and of other human malignant gliomas.

Introduction

Boron neutron-capture therapy (BNCT) has been in clinical trials as a postsurgical treatment for adult glioblastoma multiforme (GBM) at Brookhaven National Laboratory (BNL) [1], at the Massachusetts Institute of Technology, and at Helsinki University using i.v.-infused >95% ^{10}B-enriched p-boronophenylalanine (BPA) to concentrate ^{10}B in the tumor pharmacologically. Another compound, ^{10}B-enriched-$Na_2B_{12}H_{11}SH$ (BSH) is being used in Japan and in The Netherlands for BNCT of GBM. BNCT is a two-component cancer treatment based on the interaction of ^{10}B, which is non-radioactive, with thermalized neutrons. High linear-energy-transfer (LET) radiation is produced from the capture of slow neutrons by ^{10}B in the $^{10}B(n,\alpha)^7Li$ reaction, which allows palliation of ^{10}B-rich tumors while sparing from serious damage the contiguous normal tissues containing relatively low concentrations of ^{10}B. For BNCT of malignant brain tumors, it is crucial that there be high concentrations of boron in tumor, relative to those in blood and in normal brain tissues within the neutron-irradiated target volume. BPA yields macroscopically average tumor boron concentrations that are 2–4 times those in normal brain, blood, and other normal tissues. Since radiation doses that can be delivered to the tumor are limited by radiation tolerance thresholds in contiguous normal tissues [2], compounds that yield higher tumor-to-normal tissue boron concentration ratios should enable higher radiation doses to be delivered safely to tumor.

For high therapeutic gain, biodistribution studies in animals should show an average of >30 μg B/g in tumor with $\geq$5 : 1 tumor : normal tissue boron concentration ratios [3]. Requirements will also depend

on the microscopic tissue distribution of boron, preferential concentration of ^{10}B within tumor cells, especially within their nuclei, being most favorable. Although both nickel and copper porphyrins have been studied, copper is preferred because ^{67}Cu and ^{64}Cu are radioisotopes that can be imaged noninvasively by either single-photon emission computed tomography (SPECT) or positron emission tomography (PET), respectively, which should greatly facilitate BNCT treatment planning.

When the lipophilic carborane-containing tetraphenylporphyrins NiTCPH and CuTCPH were given to mice bearing EMT-6 mammary carcinomas, tumors accumulated more than 80 μg B/g and animals showed little or no porphyrin toxicity [4]. Tumor control by BNCT has been demonstrated recently using ^{10}B-enriched CuTCPH in mice bearing EMT-6 leg tumors [5]. Because BNCT is being used for the experimental treatment of brain malignancies in humans, it is of interest to determine the biodistribution of CuTCPH in gliomas. Rats bearing 9LGS and SCID mice bearing human gliomas were used for the pharmacokinetic studies presented here; in a reference study CuTCPH was also given to SCIDs bearing either an EMT-6 murine mammary carcinomas or a rat 9LGS.

Materials and methods

Porphyrins

CuTCPH and CuTCP (the octamethylester analog of CuTCPH) were synthesized as described [4,6]. The preparation of a 3.7 mg porphyrin/ml solution composed of 9% Cremophor EL (CRM) and 18% propylene glycol (PRG) requires that the porphyrin is first dissolved in tetrahydrofuran (1.5% of the total volume) and heated to 40 °C for 15 min. CRM (9% of total volume) is added and heated to 60 °C for 2 h, allowing most of the tetrahydrofuran to evaporate. After cooling to room temperature, PRG (18% of total volume) is added, followed by saline (71.5% of total volume), which is added dropwise with rapid stirring. The solution is degassed by stirring under vacuum (≈30 mmHg) for 30–60 min and then filtered (Millipore,8 μm).

Animals

Male Fischer rats (Taconic Farms, Germantown, NY) weighing approximately 250 g were implanted with intracerebral and/or multiple subcutaneous 9LGS [7–9]. The 9LGS cells were cultured in Dulbecco's MEM supplemented with 5% fetal bovine serum. For initiation of subcutaneous tumors, 2×10^6 cells in 100 μl of culture medium were implanted in each of up to 4 locations on the dorsal thorax [8,9]. For initiation of intracerebral tumors, 1×10^4 cells in 1 μl of culture medium were injected 5 mm deep in the left frontal lobe through the coronal suture at a point 4 mm lateral to the midline. A 27-gauge needle was fitted with a polyethylene collar and connected via a catheter to a 1 μl Hamilton syringe. Porphyrin injections began 10–13 days after the tumor cell injection. Without treatment, rats with such intracerebral tumors live an average of 22 ± 4 days after tumor initiation [7].

Female SCIDs (T, B and NK cell deficient, (SCID biege) Taconic Farms, Germantown, NY) weighing 20–25 g were implanted s.c. with 9LGS, U373 or U87 cells. 5×10^6 U373 or U87 cells in 100 μl were used for implantation. For initiation of EMT-6 and 9L tumors, single-cell suspensions of 2.5×10^6 cells in 100 μl culture medium were implanted s.c. into SCIDs. CuTCPH administration was begun on day 13 after U87 implantation, on day 27 after U373 implantation, on day 8 after EMT-6 implantation, and on day 16 after 9LGS implantation.

Drug administration

Rats were anesthetized using ketamine/xylazine. Continuous i.v. infusions were carried out with procedures similar to those used to infuse BPA [9]. A cannula made of medical grade silicone tubing was inserted into the anterior facial vein with the tip of the cannula secured near the external jugular vein. Infusions (2–50 h) were carried out following recovery from anesthesia using flexible tethers and calibrated syringe pumps (Harvard Apparatus, South Natick, MA).

Six i.p. injections were given to rats and mice over a period of 2 days. For convenience, 3 i.p. injections were given per day 4 h apart during 8 h each day. Rats were lightly anesthetized with carbon dioxide/oxygen before each injection.

Tissue sampling and euthanasia

Blood was sampled from each rat by retroorbital venous sinus puncture. Tumors were excised aseptically from the anesthetized animal at various times after administration of porphyrin. At the last timepoint, the rats were euthanized by oxygen/carbon dioxide overdose. Liver,

brain, skin, and oral mucosa samples were removed for boron analyses. Two days after the last injection, SCIDs were deeply anesthetized using Halothane inhalation and then euthanized by open-chest transmyocardial aspiration of >0.2 ml of blood from the right ventricle using a 27-gauge needle. During necropsy, tumor, brain, skin and liver samples were removed for boron analyses. Hematoxylin- and eosin-stained sections of >200 mg U87 and U373 tumors were evaluated by a histopathologist blinded to their identity.

Boron analyses

Direct current plasma-atomic emission spectroscopy [DCP-AES] (ARL/Fisons Model SS-7) was used (detection limit: 0.1 μg B/ml). Tissue samples (50–130 mg) were digested at 60°C for one hour with sulfuric acid : nitric acid (1 : 1). Triton X-l00 and water were added to give final concentrations of approximately 50 mg tissue/ml, 15% total acid v/v and 5% Triton X-100 v/v. Porphyrin solutions were analyzed for boron by prompt gamma spectroscopy [10].

Results

Rat biodistribution studies

Various methods of porphyrin administration were employed to achieve maximal boron concentrations in 9LGS tumors and minimal concentrations in blood. Boron concentrations in non-tumor bearing rat brain tissues (Table 1), generally between 1 and 10 μg B/g regardless of the protocol used, were similar to those observed in mice [4]. In rats, either i.v. infusions or multiple i.p. injections were used, whereas only multiple i.p. injections were used in mice [4].

Figures 1A and B show arithmetically normalized averaged rat blood and tumor boron concentrations, respectively, from various doses of CuTCP all given by i.v. infusion over various periods of time. Since doses to rats ranged from 50 to 214 mg/kg body weight, the resulting tumor boron concentrations were each normalized to 214 to compare tissue uptake efficiency at varying doses. The standard deviations for blood boron (Figure 1A) were considerably smaller than those for tumor boron (Figure lB). The blood boron curves are, in general, similar to each other. However, at the 0-d and 1-d timepoints, the blood boron from the highest dose rates, which are 25 mg/kg/h (from 50 mg/kg), and 4.5 mg/kg/h (from 214 mg/kg) are higher than those from dose rates ≤3.0 mg/kg/h. It appears (Figure 1B) that the average normalized tumor boron increases with total dose at the early timepoints but not by 2 or 3 days after the end of infusion.

The pharmacokinetics of blood boron concentrations (normalized to 195 mg/kg body weight) from CuTCPH given to rats are shown in Figure 2A. Two doses were given by i.v. infusion (150 and 194 mg/kg) while two were given by serial i.p. injections (195 and 450 mg/kg). The peak blood boron level occurs immediately after the end of i.v. infusion and boron

Table 1. Boron concentrations (μg/g) of various tissues from rats given two different doses of CuTCPH or one dose of CuTCP. Values are median (and range)

Porphyrin	CuTCP	CuTCPH	CuTCPH
Total dose (method of administration)	214 mg/kg body wt. (48-h i.v. infusion)	195 mg/kg body wt. (48-h i.v. infusion)	450 mg/kg body wt. (serial i.p. injections)
Time after administration	3 days	3 days	4 days
9L gliosarcoma, s.c.	41 (33–52)	64 (37–84)	117 (105–120)
9L gliosarcoma, i.c.		13 (8–16)	49 (39–61)
Blood	0.6 (0.3–0.9)	0.8 (0.6–1.0)	3.8 (1.7–8.8)
Brain	1.9 (0.6–4.6)	2.7 (1.5–5.0)	2.0 (1.8–2.1)
Tongue		49 (33-57)	
Parotid gland	124 (76–154)	119 (102–163)	
Lymph gland	300 (158–368)	285 (204–363)	
Extra-orbital lacrimal gland	24 (18–62)	45 (41–54)	
Ears (mainly skin)	16 (15–20)	17 (14–19)	40 (40–46)
Lung	80 (62–108)	80 (74–98)	
Kidney		16 (14–19)	
Spleen	644 (591–680)	860 (820–1000)	1700 (1400–1900)
Liver	406 (378–535)	640 (570–690)	1100 (930–1100)

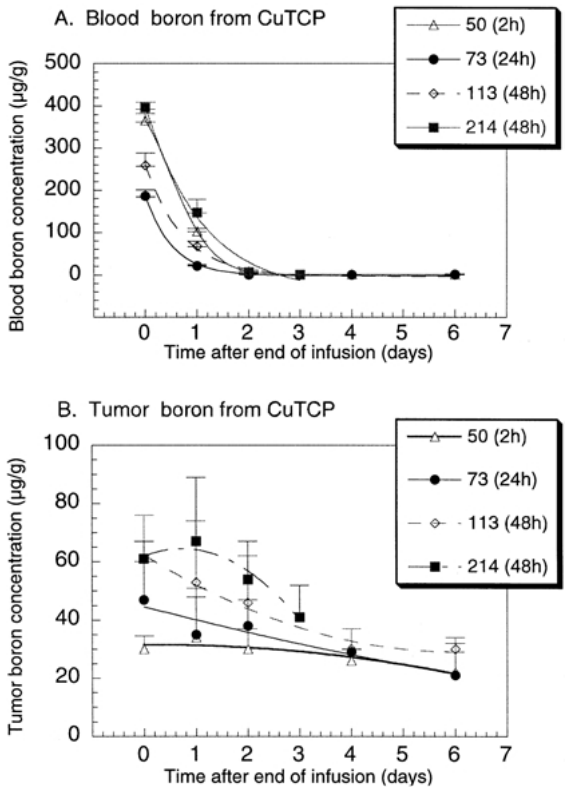

Figure 1. Average blood (A) and 9LGS tumor (B) boron concentrations arithmetically normalized to a dose of 214 mg CuTCP/kg body weight. Legend: Actual doses in mg CuTCP/kg body weight and the period of i.v. infusion are in parentheses.

kinetics appear to follow a first-order exponential function. However, after i.p. injections, the peak blood boron level occurs approximately 1 day after the last i.p. injection and boron kinetics are better described by a second-order exponential function.

The CuTCPH-derived tumor boron pharmacokinetics are shown in Figure 2B. When comparing at similar porphyrin doses, the absolute amounts of tumor boron appear to be higher from CuTCPH than from CuTCP when each is given by i.v. infusion. And even higher boron concentrations occur in liver and spleen from either porphyrin (Table 1). As are differences in tumor boron values from CuTCPH and CuTCP, boron concentrations in liver and spleen are about 50% higher from CuTCPH than from CuTCP. However, these differences are proportional to the difference in the percent boron of each porphyrin as CuTCPH has 42% more boron than does CuTCP.

The increase in tumor : blood boron concentration ratio with time is demonstrated in Figure 3, which shows both tumor and blood boron concentrations

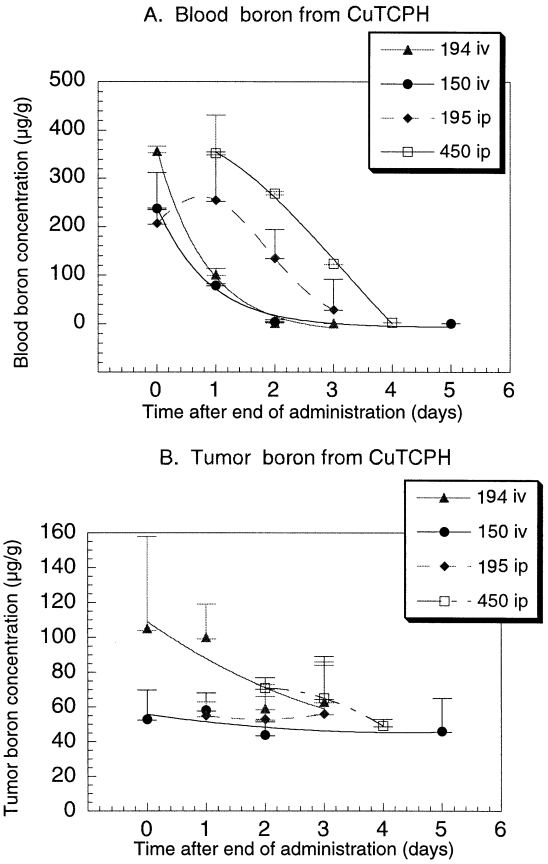

Figure 2. Average blood (A) and 9LGS tumor (B) boron concentrations arithmetically normalized to a dose of 195 mg CuTCPH/kg body weight. Legend: Actual doses in mg CuTCPH/kg body weight followed by the mode of administration (i.p. or i.v.) given over a 48 h period.

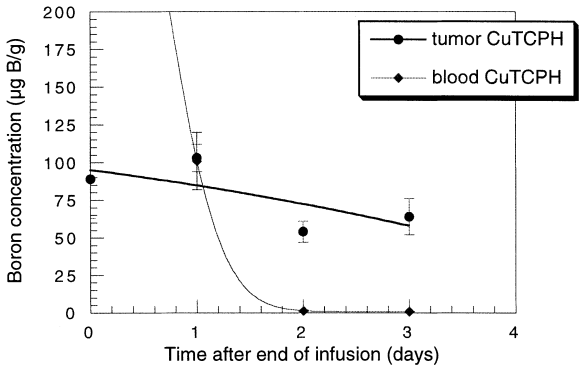

Figure 3. Average 9LGS tumor and blood boron concentrations in rats after the end of 50 h i.v infusions of CuTCPH at a total dose of 95 mg/kg body weight CuTCPH.

together for the 195 mg/kg body weight dose. The blood boron is shown to decrease exponentially with time, while tumor boron decreases almost linearly considerably more slowly with time after the end of infusion.

There was a substantial difference in 9LGS boron concentrations depending on the location of the tumor. Three days after rats were infused with a total dose of approximately 195 CuTCPH mg/kg body weight, subcutaneous tumors contained an average of 63 μg B/g while intracerebral tumors in the same rat contained an average of only 13 μg B/g (Table 1).

The 200 μg CuTCP/gbw and the 150 μg CuTCPH/gbw doses did not cause significant alterations in hematologic indices or in plasma levels of commonly measured enzyme levels compared with those in control rats that received similar infusions of solvent only. At the 450 mg/kg dose, CuTCPH did not cause any abnormal behavioral or physical changes in rats.

SCID mouse biodistribution studies

In all SCID groups, the boron concentration in normal tissues were similar, e.g. blood, brain, skin (ear) and liver (Table 2). Two days after the last injection, the median boron concentrations in blood and brain were less than 1 μg B/g, those in skin ranged from 9 to 15 μg B/g, and those in liver ranged from 500 to 800 μg B/g in all four groups. The boron concentrations in subcutaneous 9LGS in rats and mice were similar, with medians of 54 and 66 μg B/g, respectively, at total doses of 195 μg CuTCPH/gbw. Two days after the end of porphyrin administration, the median subcutaneous 9LGS weights in the rats (330 mg) and in the SCIDs (320 mg) were almost identical.

The subcutaneously implanted human xenograft U373 showed an astonishingly high median boron concentration of 251 μg B/g after a dose of 190 μg CuTCPH/gbw. The U373 tumors weighed a median of 54 mg, even less than did the EMT-6. In contrast, the U87 xenograft had a median boron concentration of 33 μg B/g and a median weight of 101 mg. The physical appearance of the two tumors were quite different; the U373 tumor looked macroscopically much more like a typical human GBM. It was soft and its cut surfaces were generally light brown, tinged with yellow, most likely because of tumor necrosis, in some irregularly and poorly demarcated areas.

Macroscopically, the U87 tumor was firm in consistency and in marked contrast to those of U373, its cut surfaces were uniformly off-white in color with no trace of yellow discoloration. Histopathological examination (hematoxylin/eosin staining) of the U87 glioma by light microscopy showed a thinly encapsulated, densely cellular, sarcomatous neoplasm with moderate nuclear pleomorphism, several mitotic figures per microscopic field at 400× magnification, without extraordinary endothelial proliferation in its uniformly distributed blood vessels. Its H & E appearance is not unlike densely cellular non-necrotic zones of some human astrocytomas. One submillimeter-diameter zone of fresh necrosis was noted in the center of a tumor section. In contrast, the microscopic appearance of H & E-stained sections of the U373 tumor showed large, irregularly outlined zones of tumor necrosis. The viable portions of the tumor, approximately one-half of its volume, showed more nuclear pleomorphism than those of the U87 tumor, as well as extraordinary endothelial hyperplasia of its thick-walled blood vessels. The occasional appearance of a palisade or garland of small tumor nuclei at the periphery of a zone of tumor tissue necrosis

Table 2. Boron concentration (μg/g) of various tissues from SCID mice bearing one of four types of tumors given CuTCPH by serial i.p. injections over a 32-h period at 2 days after the last injection

	U373	U87	EMT-6	9LGS
CuTCPH dose (μg/gbw)	190	190	190	190
Number of mice	6	7	10	10
Tumor	250 (220–570)	33 (26–69)	66 (29–83)	100 (70–135)
Blood	0.5 (0.3–0.6)	0 (0–0.1)	0.3 (0.2–0.4)	0.2 (0.2–0.3)
Cerebrum	0.6 (0.2–1.9)	0.2 (0–0.5)	0.4 (0.2–0.6)	0.2 (0.1–0.3)
Skin	15 (8–15)	13 (4.7–18)	13 (8.0–14)	9.0 (5.6–12)
Liver	810 (660–940)	751 (630–1100)	620 (460–690)	530 (360–630)

contributed to an overall appearance similar to that of human GBM.

Discussion

In contrast to the clinically tested compounds BPA and BSH, the half-life of boron in tumors from both CuTCP and CuTCPH is in the order of days instead of hours. The blood boron clears considerably more rapidly than does tumor boron, which causes the tumor : blood boron concentration ratio to increase with time, particularly during the first few days after the end of infusion (Figure 3). In the study of CuTCP, the infusion times were varied from 2 to 48 h resulting in a difference in normalized tumor boron uptake only in the early timepoints (<1 day after the end of infusion).

The subcutaneous 9LGS tumor concentrated boron at least 2–5 times more avidly than did the intracerebral 9LGS tumor. This is most likely due to differences between the tumor blood perfusion rates and the vascular beds of 9LGS tumors growing in those different locations. When the total dose of CuTCPH was increased to 450 mg/kg body weight given by serial i.p. injections, the intracranial tumor boron concentration increased to an average of about 50 μg B/g at 4 days after the last injection, when the blood boron concentration was only 5 μg B/g. At that time the subcutaneous tumor had 114 μg B/g. In contrast, both BPA and BSSB ($Na_4B_{12}H_{11}SSB_{12}H_{11}$, dimer of BSH), which are water-soluble, showed no significant differences between boron concentrations in intracerebral and subcutaneous 9LGS tumors [7,8]. This is probably due to the lower molecular weights of BSSB and BPA, which are less than 500 daltons, so that the blood-brain barrier does not restrict access as it does with CuTCP and CuTCPH, which although are lipophilic, have molecular weights greater than 1300 Da.

In mice given the same porphyrin dose, the EMT-6 tumor accumulated about twice as much boron from CuTCPH as from CuTCP. Even when the percentage of boron is taken into account (31.7% vs. 22.3%), CuTCPH delivered significantly more boron. Such differences in uptake between the two porphyrins were not observed in the 9L gliosarcoma. The pharmacokinetics of blood boron in the rat and in the mouse were similar when i.p. injections were used.

The clearance rates in both models are dependent on the volume of administered solution. Larger infusion and injection volumes reduce the rate of elimination of boron from blood. The upper limit for i.v. infusion volume appears to be approximately 9 ml per day (about 0.045 ml/gbw/day) in 220 g rats. Intraperitoneal injections were used because a larger volume of porphyrin solution was needed in order to give the larger dose, 400 mg/kg, which required about 14 ml per day. Giving a greater volume, whether i.p. or i.v., keeps the boron concentration in blood above a therapeutically maximum allowable level for a longer period of time prior to irradiation, during which the tumor would continue to grow unchecked.

The average boron concentrations in EMT-6 tumors were different in the BALB/c and the SCID mice. This may be related to the significant difference in tumor weights in these two mouse strains. In BALB/c mice, a median of 60 μg B/g was found in tumors weighing a median of 420 mg and in SCID mice, a median of 104 μg B/g was found in tumors weighing a median of 200 mg. As was reported in the rat 9LGS tumor and in the murine KHJJ tumor [6], larger tumors often have a larger percentage of necrotic tissue than smaller tumors. This may explain why, on the average, a 50% higher boron concentration was found in tumors growing in SCID mice than in the same tumors growing in BALB/c mice weighing twice as much.

In summary, CuTCPH has high affinity not only for murine mammary carcinomas, but also for the 9L gliosarcoma in rats. Intracranially implanted 9LGS tumors accumulate about one-half to one-fourth the amount of boron as do subcutaneously implanted tumors. But when the total dose of CuTCPH was doubled (to 450 mg/kg body weight), therapeutic amounts of boron were delivered to the intracranial tumor, with little, if any observable toxicity. The human GBM-like xenograft U373 took up an unprecedented median of 250 μg B/g at a total dose of 180 mg/kg body weight in SCID mice. In contrast another human glioma xenograft U87 took up only a median of 33 μg B/g. Because the boron concentrations in xenografted 9L and EMT-6 tumors in SCID mice were similar to those found in their respective species of origin, perhaps CuTCPH will be taken up by some human GBMs to the remarkable extent that it is taken up in the xenografted human GBM-like U373 tumors.

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