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Boron-Containing Nucleosides as Potential Delivery Agents for Neutron Capture Therapy of Brain Tumors

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ABSTRACT

The purpose of the present study was to evaluate both *in vitro* and *in vivo* a series of boron-containing nucleosides that potentially could be used as delivery agents for neutron capture therapy. The rationale for their synthesis was based on the fact that proliferating neoplastic cells have increased requirements for nucleic acid precursors, and, therefore, they should preferentially localize in the tumor. A series of 3-carboranlyalkyl thymidine analogs has been synthesized and a subset, designated N4, N5, and N7, and the corresponding 3-dihydroxypropyl derivatives, designated N4–2OH, N5–2OH, and N7–2OH, have been selected for evaluation. Using these compounds as substrates for recombinant human thymidine kinase-1 and the mitochondrial isoenzyme thymidine kinase-2, the highest phosphorylation levels relative to thymidine were seen with N5 and the corresponding dihydroxypropyl analog N5–2OH. In contrast, N4, N4-OH, N7, and N7-OH had substantially lower phosphorylation levels. To compare compounds with high and low thymidine kinase-1 substrate activity, N5 and N7 and the corresponding dihydroxypropyl derivatives were selected for evaluation of their cellular toxicity, uptake and retention by the F98 rat glioma, human MRA melanoma, and murine L929 cell lines, all of which are thymidine kinase-1(+), and a mutant L929 cell line that is thymidine kinase-1(−). N5–2OH was the least toxic (IC_{50}, 43–70 μM), and N7 and N7–2OH were the most toxic (IC_{50}, 18–49 μM). The highest boron uptake was seen with N7–2OH by the MRA 27 melanoma and L929 wild-type (wt) cell lines. The highest retention was seen with L929 (wt) cells, and this ranged from 29% for N5–2OH to 46% for N7. Based on the *in vitro* toxicity and uptake data, N5–2OH was selected for *in vivo* biodistribution studies either in rats bearing intracerebral implants of the F98 glioma or in mice bearing either s.c. or intracerebral implants of L929 (wt) tumors. At 2.5 hours after convection-enhanced delivery, the boron values for the F98 glioma and normal brain were 16.2 ± 2.3 and 2.2 μg/g, respectively, and the tumor to brain ratio was 8.5. Boron values at 4 hours after convection-enhanced delivery of N5–2OH to mice bearing intracerebral implants of L929 (wt) or L929 thymidine kinase-1(−) tumors were 39.8 ± 10.8 and 12.4 ± 1.6 μg/g, respectively, and the corresponding normal brain values were 4.4 and 1.6 μg/g, thereby indicating that there was selective retention by the thymidine kinase-1(+) tumors. Based on these favorable *in vitro* and *in vivo* data, neutron capture therapy studies will be initiated using N5–2OH in combination with two non-cell cycle dependent boron delivery agents, boronophenylalanine and sodium borocaptate.

INTRODUCTION

Despite decades of intensive research, high-grade gliomas still are extremely resistant to all of the current forms of therapy, including surgery, chemotherapy, and radiotherapy (1). The failure of these modalities to cure patients with glioblastoma multiforme is because of their inability to completely eradicate microinvasive tumor cells within the brain (2, 3). Molecular targeting strategies to treat a variety of human cancers, including brain tumors, currently are under intensive investigation (4). Boron neutron capture therapy is one of these strategies (5, 6). It is based on the nuclear capture reaction that occurs when boron-10, which is a non-radioactive constituent of natural elemental boron, is irradiated with low-energy thermal neutrons to yield high-linear energy transfer α-particles (^{4}He) and recoiling lithium-7 nuclei. In order for boron neutron capture therapy to be successful, a sufficient number of ^{10}B atoms must be selectively delivered to neoplastic cells, and enough thermal neutrons must be absorbed by them to sustain a lethal $^{10}B(n,\alpha)^7Li$ capture reaction. These requirements are discussed in detail in several recent reviews and monographs (7–9).

To date, two low molecular weight boron-containing drugs have been used clinically for boron neutron capture therapy of brain tumors, sodium undecahydromercapto-*closo*-dodecaborate ($Na_2B_{12}H_{11}SH$ or "borocaptate;" ref. 10, 11) and *p*-dihydroxyborylphenylalanine (boronophenylalanine; ref. 11–15). Both of these drugs are nontoxic at the doses at which they are being used clinically for boron neutron capture therapy of glioblastomas and melanomas. Several other classes of low molecular weight boron-containing drugs are under development, including porphyrins (16) and nucleosides. The rationale for the synthesis of the latter (17–19) is based on the fact that neoplastic cells have increased requirements for nucleic acid precursors. This is because of their higher proliferative rates compared with normal cells and the involvement of salvage pathways designed to reuse nucleosides, which are precursors of essential cellular biochemical building blocks such as DNA and RNA. Additional support for the synthesis of boronated nucleosides was based on the fact that thymidine isosteres 5-bromo-2′-deoxyuridine and 5-iodo-2′-deoxyuridine are both effective substrates for thymidine kinase and comparable with thymidine itself (20). Both analogs have been used to assess the proliferative activity of malignant tumors and to determine what fraction of cells is in S phase. The 5-bromo-2′-deoxyuridine labeling indices have been used by the late Dr. Takao Hoshino *et al.* (21) at the University of California (San Francisco) to assess the proliferative activities of low- and high-grade gliomas and to serve as a prognostic factor to predict the survival of glioma patients (22).

As summarized in several reviews (5, 23–25), the design and synthesis of boronated nucleosides have involved a number of research groups. The efforts of Lesnikowski *et al.* (25, 26) and Nemoto *et al.* (27, 28) have focused mainly on carboranyl and dihydroxyboryl derivatives of deoxyuridine and thymidine and, to a lesser extent, on the corresponding nucleoside bases. Sood *et al.* (29) and Burnham *et al.* (30) have synthesized cyanoborane derivatives of a variety of nucleosides. Lesnikowski *et al.* (26) synthesized the first ^{10}B-enriched nucleoside, 5-(dihydroxyboryl)-2′-deoxyuridine, and cellular uptake studies revealed that ~15–17% of the thymidine in DNA was replaced by it. The minimum requirement for the selective uptake of a boronated nucleoside by malignant *versus* benign cells is that one or

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more phosphorylating enzymes with substrate specificity for the nucleoside has (or have) elevated activity levels in malignant cells.

In mammalian cells, there are four principal kinases of the deoxyribonucleoside salvage pathway: (*a*) cytosolic thymidine kinase-1; (*b*) mitochondrial thymidine kinase-2; (*c*) 2′-deoxycytidine kinase; and (*d*) 2′-deoxyguanosine kinase. Thymidine kinase-1 and thymidine kinase-2 catalyze the transfer of a γ-phosphate group from ATP to the 5′-hydroxyl groups of the respective nucleosides. The expression of thymidine kinase-1 is regulated tightly during the cell cycle, and the active enzyme is found only in S-phase cells (31, 32). Thymidine kinase-1 is widely distributed and expressed in all of the proliferating neoplastic cells, but it is virtually absent in normal cells (33, 34). Unlike thymidine kinase-1, 2′-deoxycytidine kinase does not seem to be strictly cell-cycle regulated, and it is expressed by a wide variety of cell types (35–38). Various malignant tumors have been shown to have a 2- to 5-fold increase of 2′-deoxycytidine kinase levels compared with the corresponding normal tissues (38–40). At present, it is not known whether a subset of malignant tumors has elevated levels of thymidine kinase-2 or 2′-deoxyguanosine kinase activity. These enzymes are localized predominantly in the mitochondria and seem to be equally active at low to moderate levels in proliferating and nonproliferating cells (31). Therefore, thymidine kinase-1 and 2′-deoxycytidine kinase seem to be the primary targets of boronated nucleosides (39, 40), and knowledge of their substrate specificity has been indispensable for the rational design of boron-containing nucleoside derivatives (41–43). Recently, we have synthesized and have carried out a preliminary biochemical evaluation of a small library of boronated thymidine analogs, which revealed that they were excellent substrates of thymidine kinase-1 (44).

The purpose of the present study was to evaluate the *in vitro* cellular toxicity, retention, and uptake of a small subset of these borononucleosides described in a companion paper (45), and to evaluate the *in vivo* uptake and persistence of the most promising one in brain tumor-bearing rats and mice after administration by either convection-enhanced delivery or direct intratumoral injection. The data presented in this report suggest that one of these nucleosides, designated N5–2OH, is a promising new boron delivery agent for neutron capture therapy.

MATERIALS AND METHODS

Evaluation of Boron-Containing Nucleosides in Phosphorylation Transfer Assays. Fig. 1 summarizes a selection of boron-containing nucleosides, the synthesis of which has been described recently in detail by us (44). These were evaluated in a phosphorylation transfer assay as described previously (44). Briefly, the assay reaction mixture contained 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 125 mM KCl, 10 mM DTT, 0.5 mg/ml BSA, 1 mM ATP with 0.3 μM [γ-^{32}P]-ATP (Amersham Pharmacia Biotech, Arlington Heights, IL), and 10 or 100 μM of the boronated thymidine analog. The reactions were initiated by the addition of purified recombinant thymidine kinase-1 or thymidine kinase-2, and the DMSO concentration was kept constant at 1%.

Tumor Models. The F98 rat glioma model has been described by us in detail elsewhere (46), and its biological behavior and lack of response to therapy closely simulates that of human high-grade brain tumors. After intracerebral implantation into syngeneic Fisher rats, it forms a progressively growing infiltrative tumor. The MRA 27 melanoma was derived from a primary cutaneous melanoma of a 60-year-old white male and has been propagated *in vitro* since 1986 and *in vivo* as an intracerebral tumor in nude rats since 1991 (47). After implantation, it forms an expanding intracerebral mass, and in contrast to the F98 glioma, it does not have an infiltrative pattern of growth. Both F98 glioma and MRA27 melanoma cells were propagated *in vitro* in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY), penicillin (100 units/ml), streptomycin (100 μg/ml), and 2 mM glutamine.

The L929 (American Type Culture Collection, Manassas, VA) cell line was one of the first to be established in continuous culture, and clone 929 was the first cloned strain that was developed. The parent L strain was derived from normal s.c. areolar and adipose tissue from a C3H/An mouse, and clone 929, which is thymidine kinase-1(+), was from the 95th subculture generation of the parent strain. Its thymidine kinase-1(−) counterpart (American Type Culture Collection) was derived from the wild-type (wt) cell line L929. Both can be propagated *in vitro* as continuous cell lines or *in vivo* as tumors in nude mice.

Thymidine Kinase-1 and 2′-Deoxycytidine Kinase Assays. Studies were performed to detect the phosphorylation of [methyl-^{3}H]-thymidine using total protein extracted from the F98 glioma, MRA 27 melanoma, and L929 cell lines. As described in more detail elsewhere (40, 48), 10 μg of total protein extracts were added to a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 2 mM DTT, 5 mM $MgCl_2$, 10 mM NaF, 5 mM ATP, 0.5 mg/ml BSA and a mixture of 10 μM thymidine, 1 μM [methyl-^{3}H]-thymidine, 1.5 mM dCyd for assessment of thymidine kinase-1 activity or 10 μM dCyd, and a mixture of 1 μM [methyl-^{3}H]-dCyd, 1.5 mM thymidine for determination of 2′-deoxycytidine kinase activity. The reaction mixture was incubated at 37°C, and 10 μl were applied to DEAE-filter membranes (Millipore, Bellerica, MA) at a time interval of 0, 10, 20, and 30 minutes. The filters were washed three times, 5 minutes each, with 5 mM ammonium formate, and the radioactivity was determined in a scintillation counter.

Determination of *In vitro* Toxicity of Borononucleosides. To be useful as boron delivery agents, the candidate nucleosides must be nontoxic at concentrations that are high enough to deliver a sufficient amount (~20 μg/g tumor) of boron-10 to sustain a $^{10}B(n,\alpha)^7Li$ capture reaction at the cellular level (5–7). Therefore, the first step in evaluating boron compounds as possible delivery agents for neutron capture therapy is to determine their *in vitro* toxicity, and this was carried out as described previously (49). Briefly, F98 glioma, MRA 27 melanoma, L929 (wt), or thymidine kinase-1(−) cells were dispensed into 96-well, microculture plates (Corning Inc., Corning, NY) at a concentration of 10^4 cells/100 μl of supplemented DMEM. The plates were incubated at 37°C in a humidified CO_2 incubator for 24–48 hours before the addition of a representative set of borononucleosides, N5, N5–20H, N7, and N7–20H, which were solubilized in DMSO, the final concentration of which was <1%. The concentrations of the nucleosides ranged from 12.5 to 150 μM. The plates were incubated for an additional 24 hours, after which the cells were fixed for 1–2

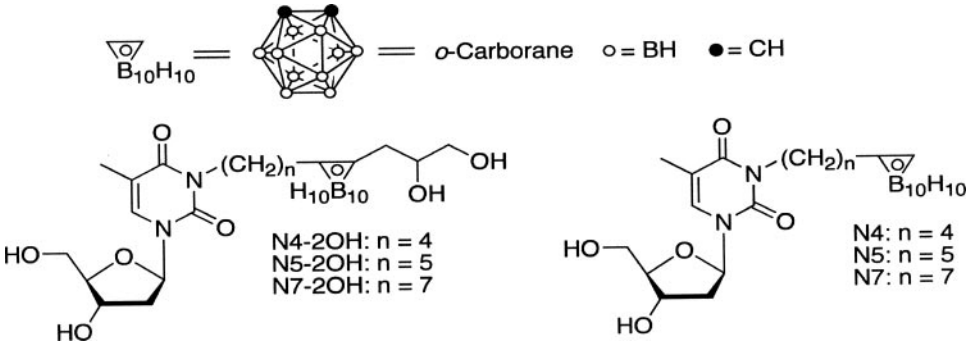

Fig. 1. Chemical structures of 3-(carboranyl-alkyl)- and 3-(dihydroxypropyl carboranyl-alkyl) thymidine analogs that have been synthesized (43).

hours at 4°C by the addition of cold trichloroacetic acid, then washed with distilled water, and air dried. After this, 100 μl of 0.4% sulforhodamine B (Sigma-Aldrich, St. Louis, MO), dissolved in 1% acetic acid, were added to each well, and after a 20-minute incubation at ambient temperature, the supernatants were decanted, the wells were washed five times with 1% acetic acid, air dried, and the bound protein was solubilized by the addition of unbuffered Trizma base (Sigma Chemical Co., St. Louis, MO). The plates were read immediately in an ELISA plate reader (Dynex Technologies MRX-ELISA Microplate Reader, Chantilly, VA) at an absorbance of 490 nm, and the percentage absorbance relative to the controls was calculated, and from these data, the concentration required to produce a 50% reduction in viability (IC_{50}) was determined.

Determination of *In vitro* Cellular Uptake and Retention of Borononucleosides. Cellular uptake of the boron compounds in sufficient amounts is an essential requirement for boron neutron capture therapy, and the procedure that we have used to determine this has been described in detail elsewhere (48). Briefly, T150 flasks containing supplemented DMEM were seeded with 5×10^6 to 10^7 F98 glioma, MRA 27 melanoma, or L929 cells and incubated for 24–48 hours at 37°C. After this, the medium was replaced with DMEM containing 17.5 μM of the test nucleoside, which had been solubilized in DMSO, or 500 μM of boronophenylalanine (Katchem, Prague, Czech Republic), which was used as a reference standard, and the cells were incubated for an additional 24 hours at 37°C. After this, the medium was decanted, the cells were washed twice with PBS (pH 7.4), disaggregated by trypsinization, counted, sedimented, and boron uptake was determined by direct current plasma-atomic emission spectroscopy, as described previously (50). Cellular retention of boron was determined by propagating the cells, which had been initially cultured in the presence of the nucleosides, for an additional 12 hours in compound-free medium, after which they were washed and harvested. Percentage retention was determined by dividing the amount of boron, determined after incubation in boron-free medium, by the value that was obtained after 24 hours incubation.

Tumor Models and *In vivo* Delivery of Bononucleosides. All of the animal research was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University. 10^5 F98 glioma cells (10^5) in 10 μl of serum-free DMEM containing 1.4% agarose with a low-gelling temperature (<30°C) were implanted stereotactically into the brains of Fisher (Charles River Laboratories, Wilmington, MA). Cells were injected over 10–15 seconds through a central hole in a plastic screw (Arrow Machine Manufacturing Inc., Richmond, VA) into the right caudate nucleus, using a procedure described in detail elsewhere (51). Fourteen days after intracerebral implantation, rats were anesthetized with katamine/xylazine and then placed in a stereotactic headframe (David Kopf Instruments, Tujunga, CA). Using the same stereotactic coordinates as those that had been used to implant the tumor, an infusion cannula was inserted into the entry port of the plastic screw, which had been embedded into the calvarium, and the cannula was advanced into the tumor. Convection-enhanced delivery was carried out, as described recently by us (52), using 10 μl of a solution containing 75 μg of boron in the chemical form of either N5–2OH, solubilized in 50% DMSO, or boronophenylalanine-fructose complex, solubilized in PBS. These were administered by convection-enhanced delivery using a Harvard syringe pump (Harvard Instruments, Cambridge, MA) at a rate of 0.33 μl/minutes for 30 minutes. Rats were divided into two groups of four animals each. Group 1 received N5–2OH by convection-enhanced delivery, and Group 2 received boronophenylalanine. The animals were euthanized at 1 and 2.5 hours after convection-enhanced delivery, the tumor, normal tumor bearing (ipsilateral), and nontumor bearing (contralateral) cerebral hemispheres were removed, and blood was collected for boron determination by direct current plasma-atomic emission spectroscopy (50).

To evaluate the tissue effects after intracerebral infusion, a group of four rats received 100 μl of 750 μg N5–2OH solubilized in 50% DMSO, which was administered intracerebrally by osmotic pumps (Alzet model #2001D, Direct Corp., Cupertino, CA) over 72 hours. Clinically, the rats tolerated this well, and although there was a small (<10%) loss in weight, this was regained within 3 days after administration. Animals were euthanized 1 week after administration of N5–2OH, and their brains were removed and fixed in 10% buffered formalin, after which coronal sections were cut ~2 mm rostal and caudal to the site of infusion. These were embedded in paraffin, and 4-μ sections were cut and stained with hematoxylin and eosin (H&E).

To have a tumor model in which the cells were identical in all of the characteristics except their expression of thymidine kinase-1, L929 (wt) and thymidine kinase-1(−) tumors were established in NIH nude/nude mice by implanting 10^6 cells s.c. or intracerebrally by a free-hand technique into the right cerebral hemisphere. Two weeks later, the s.c. tumors had attained a weight of 0.3 to 1.1 g, and the intracerebral tumors weighed 80–130 mg. Mice bearing s.c. tumors were injected intratumorally over 2 minutes with either boronophenylalanine or 15 μl of N5–20H (50 μg of boron) solubilized in 70% DMSO, which was shown previously to be nontoxic when injected into the brain. This was followed by a second injection 2 hours later. To inhibit *de novo* synthesis of DNA, 5-fluoro-2′-deoxyridine (Sigma Chemical Co.) was administered concomitantly with N5–2OH. Animals were euthanized at 4 and 6 hours after the first intratumoral injection, and tumor, blood, and normal tissue samples were taken. Alternatively, 10 μl of N5–2OH (50 μg boron solubilized in 70% DMSO) were administered intracerebrally by convection-enhanced delivery at a rate of 0.33 μl/minutes to mice bearing intracerebral implants of L929 (wt) or thymidine kinase-1(−) cells. Two hours later animals were euthanized, tissue samples were taken, and boron concentrations were determined by direct current plasma-atomic emission spectroscopy.

***In vivo* Proliferative Activity of F98 Glioma and L929 Tumors.** To obtain an estimate of the potential maximum *in vivo* uptake of nucleosides, studies were carried out in animals bearing either thymidine kinase-1(wt) or thymidine kinase-1(−) L929 tumors or F98 gliomas, using a standardized labeling procedure with 5-bromo-2′-deoxyuridine (Zymed Laboratories, Inc., South San Francisco, CA; ref. 53). L929 cells were implanted s.c. into the dorsum of NIH nude mice, and F98 glioma cells were implanted intracerebrally into the caudate nucleus of syngeneic Fisher rats. Approximately 2 weeks later, when tumors had attained a sufficient size, animals were injected i.p. with a mixture of 5-bromo-2′-deoxyuridine and 5-fluoro-2′-deoxyridine at 2-hour intervals for a total of six injections for F98 glioma and eight injections for L929 tumor-bearing animals. Two animals were euthanized 30 minutes after each injection, the tumors were excised, fixed in ethanol, embedded in paraffin, and then 4-μ sections were cut. These were immunostained for 5-bromo-2′-deoxyuridine(+) cells using a commercially available kit (Zymed Laboratories Inc.), counterstained with H&E, and the percentage 5-bromo-2′-deoxyuridine(+) cells was determined by counting 3–5 medium-power microscopic fields.

Subcellular Localization of Boron-10 by Means of Secondary Ion Mass Spectrometry. Secondary ion mass spectrometry has been recognized as a powerful tool for subcellular boron analysis in boron neutron capture therapy (54–57). The human T98G glioblastoma cell line was selected for evaluation of subcellular boron localization and the delivery characteristics of the nucleoside, N4 (see Fig. 1). T98G glioma cells were grown on sterilized silicon wafer pieces, as described recently by us (56). The cells were exposed to 50 μM of N4 (dissolved in 0.5% DMSO) for 30 minutes or 8 hours, after which the samples were cryogenically prepared by a freeze-fracture method (57). After this, the samples were freeze-dried at −80°C for 24 hours. The fractured cells on silicon substrates were photographed with a reflected light microscope and coated with a thin layer of Au/Pd alloy before ion microscopic analysis. A CAMECA IMS-3F ion microscope (Paris, France), a direct-imaging secondary ion mass spectrometer, which has a spatial resolution of 0.5 μm, was used in the present studies. This was operated in the positive secondary ion imaging mode with samples biased to +4500 V, as described in detail elsewhere (54, 56). Energy and mass-filtered secondary ion images were magnified and projected on a single microchannel plate/phosphor screen image detection assembly and recorded using a (Photometrics, Ltd., Tucson, AZ, model CH220) charged-coupled liquid-cooled camera and digitized to 14 bits/pixel with a Photometrics camera controller. In each secondary ion mass spectrometry imaging analysis, which contained up to eight individual cells, secondary ion images of ^{39}K, ^{23}Na, ^{11}B, ^{12}C, and ^{40}Ca were recorded. In general, the image integration time for ^{39}K and ^{23}Na was 0.4 seconds. The ^{11}B, ^{12}C, and ^{40}Ca images were recorded for 2 minutes. Computer image processing was done using DIP Station (Hayden Image Processing Group, Boulder, CO) on a Macintosh Quadra 840AV. Subcellular quantification of ^{39}K, ^{23}Na, and ^{11}B signals in each cell was done using relative sensitivity factors to the cell matrix ^{12}C image in the same spatial registration (54). The absolute dry weight concentrations, obtained by using this method, were converted into wet weight concentrations by assuming 85% water content uniformly distributed throughout the cell.

Table 1 *Phosphorylation of boronated nucleosides and 3-dihydroypropyl thymidine analogs*

	TK1*		TK2		TK1*		TK2
Compound	10 μM	5 μM	100 μM	Compound	10 μM	5 μM	100 μM
Thd†	1	1	1	Thd	1	1	1
N4	0.13 ± 0.04	0.17 ± 0.02	0.003	N4-2OH	0.21 ± 0.01	0.21 ± 0.06	0.003
N5	0.41 ± 0.06	0.57 ± 0.04	0.001	N5-2OH	0.41 ± 0.05	0.39 ± 0.04	0.002
N7	0.11 ± 0.03	0.15 ± 0.02	<0.001	N7-2OH	0.13 ± 0.07	0.13 ± 0.08	<0.001

Abbreviations: Tk, thymidine kinase; Thd, thymidine.
* Values are expressed as relative to that of Thd. Values represent the mean of three experiments ± SD.
† The values for Thd were arbitrarily set to 1. The final DMSO concentration in the enzyme assays was 1%.

Table 2 *TK1 and dCK activity of tumor cell lines and normal brain**

	Activity (pmol/mg/min)†	
Cell line	TK1	dCK
F98 glioma	356 ± 18	43 ± 5
MRA 27 melanoma	444 ± 21	38 ± 5
L929 TK1(+)	228 ± 16	30 ± 2
L929 TK1(−)	9 ± 3	29 ± 3
Normal brain (cortex)	≤5	≤10

Abbreviations: Tk, thymidine kinase; dCK, 2′-deoxycytidine kinase; Thd, thymidine.
* The phosphorylation of [methyl-^{3}H]Thd and [methyl-^{3}H]dCyd was determined using total protein extracts from the cell lines indicated.
† The data represent the means of three separate experiments; ±SD.

RESULTS

Phosphorylation of Boron-Containing Thymidine Analogs by Thymidine Kinase-1. The thymidine kinase-1 substrate characteristics of 3-(carboranylalkyl)thymidine analogs, designated N4, N5, and N7, and the corresponding 3-(dihydroxypropylcarboranylalkyl) thymidine derivatives, N4–2OH, N5–2OH, and N7–2OH, were screened in phosphorylation transfer assays as substrates for the recombinant human thymidine kinase-1 and thymidine kinase-2 enzymes. Phosphorylation data are summarized in Table 1, and values have been expressed relative to that of thymidine, which was arbitrarily set at 1. Among the carboranyl alkyl compounds, N5 had the highest value (0.41 ± 0.06), and among the dihydroxypropylcarboranylalkyl analogs, N5–2OH had the highest value (0.41 ± 0.05). As reported previously, compounds with a pentylene spacer (N-5, N5–2OH) showed higher phosphorylation rates than those with ethylene, propylene, butylene, hexylene, and heptylene spacers (44), thereby indicating the importance of spacer length for the interaction of the borononucleoside with the substrate-binding domain of thymidine kinase-1. The data obtained also showed that neither of the compounds was effectively phosphorylated by thymidine kinase-2. Previously, we found that these compounds were not substrates for *Drosophila melanogaster* multifunctional deoxynucleoside kinase, as well as recombinant human 2′-deoxycytidine kinase and 2′-deoxyguanosine kinase (data not shown). As reported by Al-Madhoun *et al.* (45) in a companion paper, N5 and N5–2OH were the best substrates for thymidine kinase-1, with K_{cat}/K_m values of 27% and 36% relative to that of thymidine, supporting the data obtained with the thymidine kinase-1 phosphoryl transfer assays, summarized in Table 1.

***In vitro* Toxicity, Cellular Uptake, and Retention of Borononucleosides.** Initially, thymidine kinase-1 and 2′-deoxycytidine kinase activities of the F98 glioma, MRA 27 melanoma, L929(wt), and the L929 thymidine kinase-1(−) cell lines were determined and compared with that of normal brain tissue to be able to put the *in vitro* toxicity data into an appropriate context (Tables 2 and 3). Thymidine kinase-1 activities ranged from 228 pmol/mg/minutes for L929 (wt) to 444 pmol/mg/minutes for MRA 27. Thymidine kinase-1 activity was 25 times lower in L929 thymidine kinase-1(−) cells compared with that of L929 (wt) cells. As expected, normal brain tissue showed the lowest thymidine kinase activity (<5 pmol/mg/minutes). The 2′-deoxycytidine kinase activity of the cell lines was 10 to 15% of that of the corresponding thymidine kinase-1 values. However, higher activity for 2′-deoxycytidine kinase (3–5-fold) can be observed in malignant cells compared with normal tissue extracts.

In vitro toxicity and uptake studies were carried out using the F98 glioma, MRA 27 melanoma, L929(wt), and L929 thymidine kinase-1(−) cell lines. Boronophenylalanine, which was used as a reference standard, was the least toxic of the agents that were tested with an IC_{50} of >25 mM against all of the cell lines, whereas, in contrast, all of the tested nucleosides showed moderate toxicity against the four cell lines (Table 3). N7 and N7–2OH were the most toxic with IC_{50} values ranging from 18 μM with L929 thymidine kinase-1(−) cells to 49 μM with F98 glioma cells. N5–2OH was the least toxic of the candidate nucleosides with IC_{50} values ranging from 43 to 70 μM, depending on the cell line.

Uptake and retention data are summarized in Table 4. Cellular boron concentrations, expressed as μg B/10^9 cells, were the lowest for boronophenylalanine, although its molar concentration in tissue culture medium was 28.5 times greater than that of the test nucleosides. The highest boron uptake was seen with N7–2OH (256.4 ± 10 μg) with the MRA 27 melanoma and the lowest with N5 (20.5 ± 6.0 μg) with the F98 glioma. As expected, among the cell lines tested, the lowest uptake was observed with thymidine kinase-1(−) L929 cells (4.8–12.9 μg), which ranged from 3 to 19% of those obtained with L929 (wt) cells (69.4 ± 11.8 to 160.1 ± 17.0 μg). The lowest percentage retention was observed with the F98 glioma, and this was 0% at 12 hours, and the highest values were obtained with L929 (wt) cells, which ranged from 29% for N5–2OH to 46% for N7. Boronophenylalanine retention was 0% at 12 hours for all of the cell lines except the L929 (wt) cell line, which had retained 8% of its boron at 24 hours after incubation in boron-free medium.

***In vivo* Proliferative Activity of F98 Glioma and L929 (wt) Cells.** The *in vivo* uptake of 5-bromo-2′-deoxyuridine, after 2–6 intratumoral injections, was determined by immunostaining and then enumerating the percentage positive cells per 100 cells counted. The percentage positive F98 glioma and L929 cells at 4 hours after the second injection were 8 ± 3% and 13 ± 3%, respectively (Table 5).

Table 3 *Cytotoxicity (IC_{50}) of boronated nucleosides*

Test agent	Cell lines tested*,†			
	F98	MRA 27	L929 (wt)	L929/TK1(−)
BPA	>25mM	>25mM	>25mM	>25mM
N5	48 μM	58 μM	24 μM	24 μM
N5-2OH	70 μM	58 μM	43 μM	58 μM
N7	27 μM	36 μM	23 μM	18 μM
N7-2OH	49 μM	64 μM	22 μM	24 μM

Abbreviations: TK, thymidine kinase; BPA, boronophenylalanine.
* Cells were incubated in the presence of varying concentrations of the test agent for 24 hrs at 37°, following which viability was determined by the sulforhodamine B microcytotoxicity assay (49).
† The data represent that concentration of the test agent required to produce a 50% reduction in viability (IC_{50}), as determined by spectrophotometric absorbance relative to control wells not containing the test agent. The IC_{50} was calculated by means regression analysis using SigmaPlot with six replicates per concentration of the test agent.

Table 4 *In vitro uptake and retention of boronated nucleosides*

Test agent*	F98 glioma		MRA 27		L929 (wt)		L929 TK1(−)	
	Uptake*	% Retention†	Uptake	% Retention	Uptake	% Retention	Uptake	% Retention
BPA	6.6 ± 1.0	0	16.6 ± 3.4	0	15.6 ± 0.9	8	11.8 ± 1.8	0
N5	20.5 ± 6.0	0	60.0 ± 11.9	4.3	69.4 ± 11.0	45	12.9 ± 7.5	0
N5-2OH	25.7 ± 6.0	0	169.0 ± 44.0	22	92.0 ± 20.0	29	9.3 ± 2.7	0
N7	46.0 ± 3.0	0	202.8 ± 72.0	27	91.1 ± 12.0	46	6.0 ± 2.4	0
N7-2OH	37.0 ± 4.0	0	256.4 ± 10.0	32	160.1 ± 17.0	29	4.8 ± 1.5	0

Abbreviations: TK, thymidine kinase; BPA, boronophenylalamine; DCP-AES, direct current plasma-atomic emission spectroscopy.

* Cells were incubated in the presence of 500 μM of BPA or 17.5 μM of the test nucleoside for 24 hrs at 37°C. After this, they were harvested, and boron content was determined by means of DCP-AES and expressed as μg B/10^9 cells. Data represent the means of three replicates; ±SD.

† Cellular retention of boron was determined by propagating the cells, which initially had been cultured in the presence of the nucleosides, for an additional 12 h in compound-free medium, after which they were washed, harvested, and boron content was determined. Values are expressed as a percentage of that determined after 24-h incubation with the nucleoside.

Table 5 *In vivo uptake of BUdR by TK1(+) L929 and F98 glioma cells*

Time after injection (h)	No. of injections	Percent-labeled cells*	
		L929 (wt)	F98
4	2	13 ± 3	8 ± 3
6	3	22 ± 5	12 ± 2
8	4	35 ± 6	17 ± 6
10	5	43 ± 6	20 ± 3
12	6	45 ± 7	22 ± 5

Abbreviations: BUdR, 5-bromo-2′-deoxyuridine; TK, thymidine kinase.

* L929 TK1(−) cells were uniformly negative at all time intervals.

This increased to 20 ± 3% and 43 ± 6% at 10 hours, and almost identical values were recorded at 12 hours. As expected, L929 thymidine kinase-1(−) cells were uniformly negative at all of the time intervals. From these data, the length of the cell cycle, the duration of S-phase, the labeling index, and the growth fraction were calculated using a standard procedure (53). For the F98 glioma, these were 18.9 hours (8%) and 6.9 hours (22%), respectively. The corresponding values for L929 (wt) cells were 17.2 (13%) hours and 5.2 hours (45%), respectively. These data suggest that sustained delivery for at least 12 hours and possibly longer may be required to obtain maximum *in vivo* uptake of the boronated nucleoside. The thymidine kinase-1 positivity of the L929 (wt) cells was confirmed by immunostaining with an antithymidine kinase-1 antibody and an avidin-biotin detection system. L929 (wt) cells were strongly positive, and thymidine kinase-1(−) cells were negative (data not shown). Based on these data, *in vivo* uptake studies with N5–2OH were initiated.

***In vivo* Uptake of N5–2OH and Boronophenylalanine in F98 and L929 Tumor-Bearing Animals.** The tumor, normal brain, and blood values for N5–2OH in F98 glioma bearing rats at 2.5 hours after convection-enhanced delivery are summarized in Table 6. Maximum uptake was observed at 1 hour, at which time the tumor boron concentrations for N5–2OH and boronophenylalanine were 40.7 ± 11.3 and 68.3 ± 17.9 μg/g, respectively, and the corresponding values for normal brain were 4.8 and 19.0 μg/g. At 2.5 hours after convection-enhanced delivery, the tumor boron values for N5–2OH and boronophenylalanine were 16.2 ± 2.3 and 24.2 ± 7.7 μg/g, respectively, and the normal brain values had fallen to 2.2 and 14.5 μg/g, respectively. The corresponding tumor to brain ratios at 2.5 hours for N5–2OH and boronophenylalanine were 16.8 and 1.8. The former was the highest T/Br ratio that we have ever observed, even when boronophenylalanine was administered via the internal carotid artery with blood-brain barrier disruption (51). Blood boron concentrations were <0.5 μg/ml for N5–2OH and 3.5 μg/ml for boronophenylalanine. These data indicate that there was specific retention of the nucleoside in the tumor and that it had cleared from surrounding normal brain. Microscopic examination of the brains of non-tumor-bearing rats that had received N5–2OH by convection-enhanced delivery revealed small foci of necrosis in proximity to the cannula tip but no inflammatory cell infiltrates. These results suggest that intracerebral infusion of 750 μg of N5–2OH in 50% DMSO had minimal toxicity. Uptake values for L929 (wt) and thymidine kinase-1(−) s.c. tumors at 4 hours after intratumoral injection were 22.8 ± 5.0 and 8.4 ± 3.6 μg/g, respectively, and these had fallen to 14.8 ± 10.5 μg/g and 4.5 ± 1.8 μg/g at 6 hours (Fig. 2). In contrast, there was no detectable boron in samples of liver, blood, or skin. The L929(wt) to L929 thymidine kinase-1(−) tumor boron ratios at 4 and 6 hours were 2.7 and 3.3, respectively, thereby demonstrating that thymidine kinase-1 enzyme activity was an important determinant for the uptake of N5–2OH. On the other hand, uptake of boronophenylalanine in mice bearing s.c. tumors was similar for L929 (wt) and thymidine kinase-1(−) tumors (4.0 ± 2.1 and 5.8 ± 3.1 μg/g, respectively), indicating that this was not influenced by thymidine kinase-1 expression.

The biodistribution of N5–2OH after convection-enhanced delivery to nude mice bearing intracerebral implants of L929 (wt) or L929 thymidine kinase-1(−) tumors is summarized in Table 7. Tumor and normal brain boron concentrations for L929 (wt) tumor-bearing mice were 81.6 ± 20.4 and 3.3 ± 3.2 μg/g, respectively, at 2 hours and 39.8 ± 10.8 and 4.4 ± 1.5 μg/g at 4 hours. The corresponding tumor boron values in mice bearing L929 thymidine kinase-1(−) tumors were 34% lower at 2 hours and 69% lower at 4 hours, whereas, on the other hand, normal brain boron values were similar. These data indicate that thymidine kinase-1 activity was a significant determinant for the enhanced uptake/retention of boron by thymidine kinase-1 positive (wt) tumors.

Subcellular Localization of Boron by Means of Secondary Ion Mass Spectrometry. The subcellular localization of boron as determined by secondary ion mass spectrometry in human T98G glioblastoma cells is summarized in Table 8. Higher amounts of boron were seen in the cytoplasm compared with the nucleus, at 0.5 and 2 hours

Table 6 *Biodistribution of boron in rats bearing i.c. implants of the F98 glioma after CED of N5-2OH and BPA**

Agent†	Time (h)	Boron concentration (μg/g)‡				Tumor/brain ratio§
		Tumor	Ipsilateral brain	Contralateral brain	Blood	
N5-2OH	1.0	40.7 ± 11.3	4.8 ± 3.9	<0.5	<0.5	8.5
N5-2OH	2.5	16.2 ± 2.3	2.2 ± 2.1	<0.5	<0.5	7.4
BPA	1.0	68.3 ± 17.9	19.0 ± 13.6	4.2 ± 1.9	4.2 ± 3.9	3.6
BPA	2.5	24.2 ± 7.7	14.5 ± 7.6	7.5 ± 4.1	3.5 ± 2.0	1.7

Abbreviations: i.c., intracerebral; CED, convection-enhanced delivery; BPA, boronophenylalamine; DCP-AES, direct current plasma-atomic emission spectroscopy.

* F98 glioma cells (10^5) were stereotactically implanted into the right cerebral hemisphere of Fisher rats.

† Fourteen days later, CED was carried out using 10 μl of a solution containing 78 μg of boron in the chemical form of either N5-2OH, solubilized in 50% DMSO, or BPA-fructose complex, solubilized in PBS, and these were administered by CED at a rate of 0.33 μl/min for 30 min.

‡ Boron concentrations were determined by DCP-AES. Values represent the mean of 4 rats; ±SD.

§ The tumor to brain ratio was based on tumor uptake versus ipsilateral (tumor-bearing) cerebral hemisphere and was calculated for data from each individual animal and not simply by dividing the mean tumor boron concentration by the mean ipsilateral normal brain concentration.

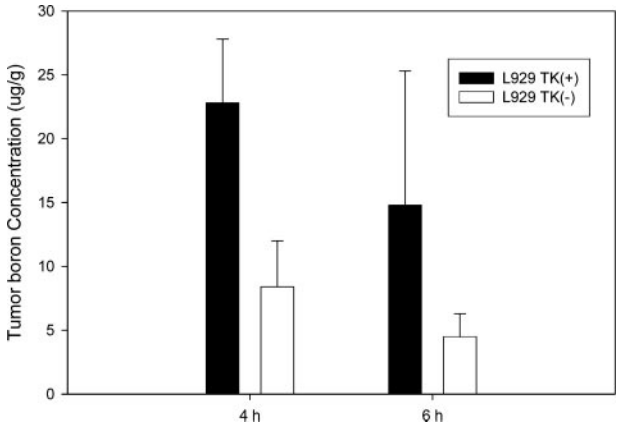

Fig. 2. Tumor boron uptake after intratumoral injection of N5–2OH into mice bearing s.c. implants of L929 tumors. Mice received two injections of N5–2OH containing 50 μg of boron in a 15 μl solution of 70% DMSO, administered intratumoral injection over 2 minutes at 0 and 2 hours. Animals were euthanized at 4 and 6 hours after the first intratumoral injection, and boron concentrations were determined by direct current plasma-atomic emission spectroscopy. *Bars*, ±SD. (*TK*, thymidine kinase)

Table 7 *Biodistribution of boron in nude mice bearing i.c. L929 (wt) or TK1(−) tumors after CED of N5-2OH*

Tumor*	Time† (h)	Boron concentration (μg/g)‡ Tumor	Brain	Blood	Tumor/brain ratio§
L929 (wt)	2	81.6 ± 20.4	3.3 ± 3.2	<0.5	24.7
L929 TK1 (−)	2	53.6 ± 16.6	2.5 ± 1.3	<0.5	21.4
L929 (wt)	4	39.8 ± 10.8	4.4 ± 1.5	<0.5	9.1
L929 TK1 (−)	4	12.4 ± 1.6	1.6 ± 1.1	<0.5	9.8

Abbreviations: i.c., intracerebral; Tk, thymidine kinase; CED, convection-enhanced delivery; DCP-AES, direct current plasma-atomic emission spectroscopy.

* Either L929 (wt) or TK1(−) L929 cells (10^6) were implanted into right cerebral hemisphere of nude mice. The mice were studied 2 weeks after implantation. The tumor weights ranged from 80 to 130 mg.

† N5-2OH containing 50 μg of boron in 50% DMSO 10 μl were CED infused into the intracerebral tumors for a rate of 0.33 μl/min administered by CED, and then mice were euthanized 2 and 4 hours after infusion.

‡ Boron concentrations were determined by DCP-AES (μg/g tissue). Values represent the mean of four mice; ±SD.

§ The tumor to brain ratio was based on tumor uptake versus ipsilateral (tumor-bearing) cerebral hemisphere and was calculated for data from each individual animal and not simply by dividing the mean tumor boron concentration by the mean ipsilateral normal brain concentration.

after incubation with N4. Equivalent amounts of boron were seen in the nucleus and cytoplasm at 8 hours, suggesting that there was no preferential nuclear uptake of N4. However, these results cannot be extrapolated to N5–2OH. A similar distribution has been observed with boronophenylalanine (56), suggesting that this compound equilibrated between the nucleus and cytoplasm. As shown in Fig. 3, an optical photomicrograph of several freeze-dried cells, the cytoplasmic volumes of the tumor cells were small, and the nuclei had many small nucleoli. Ion microscopic images of intracellular distributions of ^{39}K, ^{40}Ca, and ^{11}B, respectively, from the same cells as those shown in the optical image, are shown in Fig. 3, *B–D*. The level of brightness within the ion microscopic image was directly proportional to the concentration of the isotope. The ^{39}K image indicated that it was distributed relatively homogeneously throughout the cell. As similar distribution also was observed for ^{23}Na images (data not shown). Intracellular ^{39}K and ^{23}Na concentrations were ~170 mM and 15 mM, respectively, in human T98G glioblastoma cells. The ^{40}Ca images revealed a heterogeneous cytoplasmic distribution and much lower concentrations of total calcium in the nucleus of each cell. The ^{11}B images from the same cells revealed that it was distributed throughout the cell, but that nuclei had slightly higher ^{11}B intensities than the perinuclear cytoplasmic regions, which had high calcium signals. A cell-to-cell variability in ^{11}B uptake also was evident. The nuclear uptake of ^{11}B from N4-treated cells was significantly ($P < 0.01$) higher after an 8-hour exposure compared with that after 30 minutes or 2-hour exposures (data not shown).

DISCUSSION

The rationale for the synthesis of boron-containing nucleosides as delivery agents for neutron capture therapy are 2-fold. First, because the constituent cells of brain tumors are proliferating, they should take nucleosides up in amounts far exceeding normal, non-proliferating glial cells and neurons. Second, after phosphorylation they can be potentially incorporated into DNA. However, even if the nucleosides only were localized intracellulary rather than incorporated into DNA, they still would be attractive boron delivery agents. Since the mid-1990s, more than 40 N-3 substituted thymidine analogs, boronated and non-boronated, have been synthesized by applying concepts originally developed by Soloway *et al.* (5, 23) and subsequently pursued by Lunato *et al.* (43) and Tjarks *et al.* (24, 44, 58). These carboranyl thymidine derivatives have undergone an in-depth enzymatic evalu-

Table 8 *Cellular uptake and retention of N4 by T98G human glioblastoma cells*

Time* (h)	Boron concentration (μg/10^9 cells) Nucleus	Cytoplasm	No. of cells analyzed
0.5	311 ± 9	383 ± 12	37
2	326 ± 11	400 ± 14	40
8	415 ± 12	409 ± 12	41

* Cells were cultured in medium containing 50 μM of N4.

† Boron concentrations were determined by secondary ion mass spectrometry.

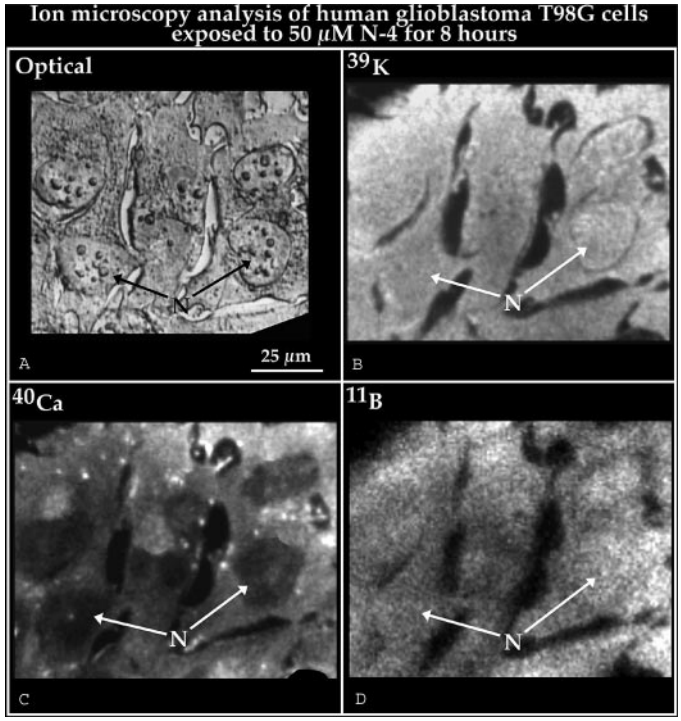

Fig. 3. Subcellular localization of ^{11}B, ^{39}K, and ^{40}Ca, as determined by secondary ion mass spectrometry. The composite photo was from human glioblastoma cell line T98G, which had been exposed to 50 μM of N4 for 8 hours. *Top left panel* (*A*), an optical photomicrograph of several frozen, freeze-dried cells. Cell nuclei with many small nucleoli are discernible. The position of nuclei (*N*) in two cells is indicated by *arrows*. The remaining micrographs (*B–D*) show ion microscopic images revealing intracellular distributions of ^{39}K, ^{40}Ca, and ^{11}B, respectively, from the same cells shown in the optical image.

ation, as reported by us in a companion publication (45). N5 and N5–2OH were the best substrates for thymidine kinase-1, and both showed partial competitive inhibition of thymidine phosphorylation by thymidine kinase-1. Neither compound was a substrate of human recombinant thymidine phosphorylases, thereby indicating potential *in vivo* stability (45). They all exhibited high phosphorylation rates with thymidine kinase-1, the therapeutically relevant isoform of the enzyme, but not with thymidine kinase-2. Of these, N5 and N5–2OH appeared to have the highest phosphorylation rates among all of the compounds tested (45). Based on these data, N5 and N5–2OH were selected for *in vitro* and *in vivo* evaluation as potential boron delivery agents for neutron capture therapy. N4, N4–2OH, N7, and N7–2OH were also selected for a comparative evaluation.

In contrast to cytoreductive chemotherapeutic agents, ^{10}B delivery agents should have low cytotoxicity, and among the compounds tested, N5–2OH had the lowest. However, the IC_{50} values obtained with the F98 glioma and MRA 27 melanoma did not correlate with thymidine kinase-1 substrate activity (Table 1), thymidine kinase enzyme levels (Table 2), or with nucleoside uptake values (Table 4). It is noteworthy that a substantial difference in the *in vitro* IC_{50} values of L929 wt (43 μM) and L929 thymidine kinase-1(−) cells (58 μM) was observed only with N5–2OH, which can be attributed to its enhanced metabolic activity, compared with that of the other borononucleosides. Similar differences of *in vitro* IC_{50} values of N5–2OH with L929 (wt) and L929 thymidine kinase-1(−) cells also have been observed by us using a the dimethylthiazal tetrazolium assay, where the thymidine kinase-1(−) cells were approximately 2 times more resistant than L929 (wt) cells (45). In general, the moderate toxicity of all of the nucleosides that were evaluated appears to have been related to their lipophilicity rather than to nucleoside metabolism. In previous studies involving carboranyl polyamines, carborane-dependent lipophilicity has been identified by us as the source of substantial *in vitro* and *in vivo* toxicity (59, 60). The cause of this toxicity is still unknown, but it could be reduced partially by the attachment of additional hydrophilic groups to the polyamines. The toxicity of the carboranyl nucleosides may have a similar biochemical basis. *In vitro* uptake and retention was significantly higher for the carboranyl nucleosides than for boronophenylalanine. In addition, a significant difference in uptake/retention between L929 (wt) and L929 thymidine kinase-1(−) cells was observed only for the nucleosides but not for boronophenylalanine, suggesting that nucleoside-specific metabolic processes were partially responsible for their uptake. Because the thymidine kinase-1 activity of F98 glioma cells was relatively high, their low uptake and retention of carboranyl nucleosides compared with MRA 27 melanoma and L929 (wt) cells suggest that other factors, such as nucleoside/nucleotide influx and efflux, also may play important roles in the uptake and retention of these compounds.

The physicochemical properties of a drug are a critical determinant of its ability to traverse the blood-brain barrier, diffuse within the brain, and reach specific targets within the central nervous system (CNS; ref. 61, 62). One of the best physicochemical indicators of diffusion within the CNS is the octanol/water partition coefficient (log *P*; ref. 63, 64). The optimal log *P* for drugs designed to target the CNS should be ~2 (61, 62). As described by us in detail in a companion paper (45), the lipophilicity of N4, N5, and N7 and the corresponding dihydroxypropyl derivatives was assessed by determining their log *P*s by a chromatographic method. Only the log *P* of N5–2OH was close to the optimal for penetrating the brain (45). In addition, it had the best overall enzymatic properties with a K_{cat}/K_m of 36% relative to that of thymidine (45). Furthermore, N5–2OH was not a substrate of recombinant human thymidine phosphorylase, and its corresponding 5′-monophosphate was not a substrate of 5′-deoxynucleotidase 1, which suggests that it should be stable *in vivo*. Based on these data, N5–2OH appears to be an excellent candidate for targeting the CNS.

One of the major questions that must be addressed is "How can the delivery of boron-containing agents to brain tumors be improved?" Factors that may influence ^{10}B compound delivery to brain tumors after systemic administration include the following: (*a*) the plasma concentration profile of the ^{10}B containing drug, which depends on the amount and route of administration; (*b*) permeability of the blood-brain barrier to the ^{10}B-containing agent; (*c*) blood flow within the tumor; and (*d*) lipophilicity of the drug (62). The two chemotherapeutic agents that have been the most effective for treating brain tumors (and, in reality, these are not very effective), bis-chloroethylnitrosourea and cyclohexylchloroethylnitrosourea, have high octanol/water partition coefficients and correspondingly high permeability values. In general, a high steady-state blood concentration will maximize brain uptake, whereas rapid clearance will reduce it, except in the case of intra-arterial drug administration, where, for example, high tumor uptake of both borocaptate and boronophenylalanine has been observed by us after intracarotid administration (51, 65).

A pilot study by us showed that after six i.p. doses of N5–2OH to nude mice bearing s.c. implants of the L929 (wt) tumors, there were undetectable quantities (*i.e.*, <0.5 μg/g) of boron within the tumor. For this reason, we carried out direct intratumoral injection, which would maximize tumor uptake and markedly reduce uptake of N5–2OH by other tissues and organs. Kassis *et al.* (66–68) and Sahu *et al.* (69) have carried out extensive studies on direct intracerebral administration of ^{123}I- and ^{125}I-radiolabeled 5-iodo-2′-deoxyuridine for the diagnosis and treatment of brain tumors and leptomeningeal metastases (70). The direct intratumoral (67), intracerebral (68, 69), or intrathecal (70) routes have proven to be the most effective for delivering the maximum amounts of ^{125}I-5-iodo-2′-deoxyuridine to the tumor, and it was superior to systemic administration (66). For many therapeutic agents, however, slow diffusion within the brain tumor and brain around the tumor severely limits drug distribution after direct intratumoral injection. This could be circumvented using convection-enhanced delivery (71–75) or (as it has been alternatively referred to "microdialysis") (76), by which agents are directly infused into the brain to increase their uptake and distribution. Under normal physiological conditions, interstitial fluids move through the brain by both convection and diffusion. Diffusion of a drug in tissue depends on its molecular weight, ionic charge, and its concentration gradient within normal tissue and the tumor (61, 62). Unlike diffusion, however, convection or "bulk" flow results from a pressure gradient that is independent of the molecular weight of the substance. Convection-enhanced delivery potentially can improve the targeting of both low and high molecular weight molecules to the CNS by applying a pressure gradient to establish bulk flow during interstitial infusion. The volume of distribution is a linear function of the volume of the infusate. Convection-enhanced delivery has been used to efficiently deliver low molecular weight agents (74), drugs (72, 75), and high molecular weight toxins (73) to large regions of the brain without significant functional or structural damage to the brain. Furthermore, it can produce more homogenous dispersion of the agent at higher concentrations than otherwise would be attainable. We have used convection-enhanced delivery for the direct intratumoral injection of boronated epidermal growth factor to rats bearing F98 gliomas that have been genetically engineered to express the epidermal growth factor receptor. Convection-enhanced delivery almost quadrupled the volume of distribution of the boronated epidermal growth factor (52). As shown in the present study, convection-enhanced delivery of N5–2OH to F98 glioma-bearing rats resulted in a T/Br of 7.4 compared with 1.7 for boronophenylalanine. This strongly supports our view that convection-enhanced delivery may be the optimal way to

deliver borononucleosides to CNS tumors, and studies with nanoparticle and liposomal formulation are under investigation currently in our laboratories.

Finally, we would like to make a concluding comment about how we envision boronated nucleosides would be used as a boron delivery agent for neutron capture therapy. Because the uptake of nucleosides is cell cycle dependent, they will be taken up only during S phase of the cell cycle, at which time DNA is being replicated. Even under the best of circumstances, only a relatively small percentage of tumor cells would be in S phase over a short time interval, such as 6–12 hours, and, therefore, a more sustained delivery may be necessary (78). Furthermore, the nucleosides would be used in combination with noncell cycle dependent agents such as boronophenylalanine and sodium borocaptate, which would be administered systemically. In this way, both proliferating and nonproliferating cells would be targeted. Studies to test this hypothesis are planned.

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