# Molecular Targeting and Treatment of Composite EGFR and EGFRvIII-Positive Gliomas Using Boronated Monoclonal Antibodies

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**Abstract**

**Purpose:** The purpose of the present study was to evaluate the anti–epidermal growth factor receptor (EGFR) monoclonal antibody (mAb), cetuximab, (IMC-C225) and the anti-EGFRvIII mAb, L8A4, used in combination as delivery agents for boron neutron capture therapy (BNCT) of a rat glioma composed of a mixture of cells expressing either wild-type ($F98_{EGFR}$) or mutant receptors($F98_{npEGFRvIII}$).

**Experimental Design:** A heavily boronated polyamidoamine dendrimer (BD) was linked by heterobifunctional reagents to produce the boronated mAbs, BD-C225 and BD-L8A4. For *in vivo* biodistribution and therapy studies, a mixture of tumor cells were implanted intracerebrally into Fischer rats. Biodistribution studies were carried out by administering $^{125}$I-labeled bioconjugates via convection-enhanced delivery (CED), and for therapy studies, nonradiolabeled bioconjugates were used for BNCT. This was carried out 14 days after tumor implantation and 24 h after CED at the Massachusetts Institute of Technology nuclear reactor.

**Results:** Following CED of a mixture of $^{125}$I-BD-C225 and $^{125}$I-BD-L8A4 to rats bearing composite tumors, 61.4% of the injected dose per gram (ID/g) was localized in the tumor compared with 30.8% ID/g for $^{125}$I-BD-L8A4 and 34.7% ID/g for $^{125}$I-BD-C225 alone. The corresponding calculated tumor boron values were 24.4 μg/g for rats that received both mAbs, and 12.3 and 13.8 μg/g, respectively, for BD-L8A4 or BD-C225 alone. The mean survival time of animals bearing composite tumors, which received both mAbs, was 55 days ($P < 0.0001$) compared with 36 days for BD-L8A4 and 38 days for BD-C225 alone, which were not significantly different from irradiated controls.

**Conclusions:** Both EGFRvIII and wild-type EGFR tumor cell populations must be targeted using a combination of BD-cetuximab and BD-L8A4. Although *in vitro* C225 recognized both receptors, *in vivo* it was incapable of delivering the requisite amount of $^{10}$B for BNCT of EGFRvIII-expressing gliomas.

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After decades of intensive research, high-grade gliomas are still extremely resistant to all current forms of therapy, including surgery, chemotherapy, and radiotherapy (1). Despite aggressive treatment using combinations of these modalities, the 5-year survival rate of patients diagnosed with glioblastoma multiforme (GBM) in the United States is <1% (2). The single most important advance in the treatment of these tumors over the past 30 years has been the introduction of temozolomide, initially in combination with external beam photon irradiation, and then followed by repetitive cycles of temozolomide alone (3). However, this has only increased the overall median survival by 2.5 months. The failures of surgery, chemotherapy, and radiotherapy to cure patients with high-grade gliomas are due to the inability of these modalities to completely eradicate microinvasive tumor cells within the brain (4). The survival data for patients with multicentric, metastatic brain tumors is almost as dismal (5, 6), and for this reason, the development of new therapeutic approaches to treat both primary and metastatic brain tumors is of the highest importance.

The wild-type epidermal growth factor receptor (EGFR) and its mutant isoforms are now considered prime targets for the

specific delivery of a variety of diagnostic and therapeutic agents (7), including monoclonal antibodies (mAb). EGFR is a 170 kDa transmembrane tyrosine kinase that specifically binds the 53–amino acid peptide ligand, epidermal growth factor and the 50–amino acid autocrine growth factor, transforming growth factor-α (7). The *EGFR* gene is often amplified in human GBMs and other primary brain tumors, but is undetectable or weakly expressed in normal brain. Early studies by Bigner et al. revealed that in a series of 33 human glioma biopsies, 15 showed the amplification of the *EGFR* gene (8). Similar or even higher frequencies of amplification have been observed by others, and this is often associated with increased cell surface receptor expression (9–11).

EGFRvIII, which has an in-frame deletion of exons 2 to 7 of the extracellular domain of the *EGFR* gene (12), is expressed and amplified in up to 57% of GBMs and in 75% of anaplastic astrocytomas (13, 14). Because EGFRvIII seems to be a truly tumor-specific target, several mAbs directed against this receptor have been produced for diagnostic and therapeutic purposes (15). The expression of EGFR in high-grade gliomas is variable, which probably reflects the cellular and molecular genetic heterogeneity of these tumors (16). In a recently reported study of patients with newly diagnosed GBMs, 36% expressed EGFRvIII and 57% were EGFR-positive (14). Because the number of receptors on individual tumor cells could be up to 100 times greater than those expressed on normal glial cells (8), these receptors are of special interest for targeted therapies of brain tumors.

Our own studies have focused on two anti-EGFR mAbs, cetuximab and L8A4 (17–19). Cetuximab is a recombinant, human/mouse chimeric mAb that specifically binds to the extracellular domain of human EGFR and competitively inhibits the binding of epidermal growth factor and other ligands, such as transforming growth factor-α (20). This could result in the inhibition of cell growth, induction of apoptosis, and decreased production of matrix metalloproteinases and vascular endothelial growth factor (21–24). However, the exact molecular mechanisms by which cetuximab exerts its tumoricidal/tumoristatic effects are still unclear (25). Enhancement of the cytotoxic effects of chemotherapeutic agents (26) and the response to ionizing radiation have also been reported (27). L8A4, which specifically recognizes EGFRvIII and not wild-type EGFR, has similar pleiotropic and antitumor effects (28, 29). We have used both cetuximab and L8A4 to deliver boron-10 to genetically engineered F98 rat glioma cells that express either wild-type EGFR or EGFRvIII for boron neutron capture therapy (BNCT) and our results are described in several recent publications (17–19, 30). Briefly summarized, boronated mAb L8A4 was highly effective for BNCT of rats bearing EGFRvIII-expressing gliomas with a 2.8-fold increase in mean survival time (MST) compared with controls that received the bioconjugate alone without neutron irradiation. Similarly, boronated cetuximab was effective as a boron delivery agent for rats bearing EGFR wild-type expressing gliomas with a 1.6-fold increase in MST compared with controls.

BNCT is a chemoradiotherapeutic modality that is based on the selective delivery of a stable isotope, boron-10, to tumor cells, followed by irradiation with low-energy thermal neutrons. The resulting nuclear capture and fission reactions ($^{10}B[n,\alpha]^{7}Li$) yield α particles and $^{7}Li$ nuclei, which have high linear energy transfer and path lengths of approximately one cell diameter. Each component can be manipulated independently, so that the interval between administration of the $^{10}B$-containing agent and neutron irradiation can be adjusted to an optimal time at which the differential between boron concentration levels in normal tissues and tumor are maximized. For BNCT to be successful, there must be the selective accumulation of a sufficient amount of $^{10}B$ (~20 μg/g wt or $10^9$ atoms per cell) in the tumor and low levels in blood and normal brain, and enough thermal neutrons must be delivered to the tumor site. The destructive effects of the high-energy α particles are limited to $^{10}B$-containing cells. In addition to these, low linear energy transfer γ rays and high-energy protons are produced as a result of the capture of thermal neutrons by normal tissue hydrogen and nitrogen atoms. The $n,\gamma$ reaction with H and the $n,p$ reaction with N, together with nontumor uptake of $^{10}B$, determine normal tissue tolerance (31). BNCT has primarily been used to treat patients with high-grade gliomas (31), recurrent malignant meningiomas (32), either cutaneous primaries or cerebral metastases of melanoma (31), and most recently, patients with therapeutically refractory carcinomas of the head and neck (33). Interested readers are referred to several recent reviews (31) and monographs relating to BNCT (34, 35).

Human GBMs exhibit considerable heterogeneity in EGFR expression (8–14), with tumors composed of subpopulations of EGFR wild-type, EGFRvIII, and EGFR-negative cells. In the present study, we have simulated this by using F98 rat gliomas composed of a mixture of EGFR wild-type and EGFRvIII-expressing tumor cells, designated $F98_{EGFR}$ and $F98_{npEGFRvIII}$, respectively. Because the latter had a nonfunctional, i.e., nonphosphorylated receptor, they have no growth advantage over EGFR wild-type–expressing cells and therefore tumors derived from a mixture of cells seem to be stable in their composition. In the present study, we have evaluated the efficacy of boronated cetuximab and L8A4 for BNCT of rats bearing composite tumors, which were produced by implanting equal numbers of $F98_{EGFR}$ and $F98_{npEGFRvIII}$ cells. As described in this report, a significant increase in the survival time of rats bearing composite gliomas could only be shown when boronated cetuximab and L8A4 were used in combination. Although *in vitro* cetuximab recognized both EGFR and $EGFR_{vIII}$ expressing F98 glioma cells; *in vivo*, it could not deliver a therapeutically sufficient amount of $^{10}B$ for successful BNCT. In contrast, each boronated mAb was highly effective as a boron delivery agent for tumors composed of a single population of cells expressing the corresponding receptor.

## Materials and Methods

***Preparation of boronated dendrimers and their linkage to mAbs L8A4 and cetuximab.*** Dendrimers are synthetic polymers with a well-defined globular structure. They are composed of a core molecule, repeat units that have three or more functionalities, and reactive surface groups (36). They are an attractive platform for drug delivery because of their low cytotoxicity and the multiplicity of reactive terminal groups. For these reasons, we have used them as precision macromolecules to heavily boronate mAbs. A fifth-generation polyamidoamine dendrimer, containing 128 reactive terminal amino groups, was boronated with the methylisocyanato polyhedral borane anion $Na(CH_3)_3NB_{10}H_8NCO$ to yield a boronated dendrimer (BD) using a procedure which we have previously described in detail elsewhere (37). In order to site-specifically

link the BD to the $F_c$ region of the mAb, it was reacted with *N*-succinimidyl 3-(2-pyridyldithio) propionate and the resulting product was cleaved with DTT to yield a SH-containing BD. This, in turn, was reacted with KMUH (*N*-[k-maleimidoundecanoic acid]hydrazide) to produce KMUH-BD. Both L8A4 and cetuximab, previously known as C225 (20), were oxidized with $NaIO_4$ and then linked to the hydrazide group of KMUH-BD to yield the bioconjugates BD-L8A4 and BD-C225 (37). This was purified by column chromatography using Sephacryl S-300 and eluted with 0.1 mol/L of phosphate and 0.2 mol/L of NaCl buffers (pH 7.5). Fractions were collected and protein concentrations were determined spectrophotometrically by means of the Coomassie blue protein assay by measuring absorbance at 495 nm using a Beckman DU-6 spectrophotometer (Beckman Instruments, Inc.). Boron was quantified by means of direct current plasma-atomic emission spectroscopy using a Spectraspan VB spectrometer (Applied Research Laboratories), as previously described (38). Fractions containing the highest concentrations of both protein and boron were pooled and used in the studies described in the following section.

***Radioiodination of boronated mAbs.*** Radiolabeling of the boronated mAbs with $^{125}I$ was carried out using IODO-GEN precoated iodination tubes according to the procedure described by the manufacturer (Pierce). Briefly, 10 μL (1.0 mCi) of carrier-free $Na^{125}I$ (ICN Biomedicals, Inc.) and 100 μL of 25 mmol/L phosphate buffer (pH 7.5; 0.1 mol/L NaCl) were added to precoated tubes. After incubation for 6 min at ambient temperature, the activated iodide was removed and added to the mAb or BD-mAb solution (50 μg/50 μL) and then incubated for an additional 6 to 9 min. The radiolabeled bioconjugates were purified on a Bio-Spin 30 TRIS column (Bio-Rad Laboratories) by elution with 25 mmol/L of phosphate buffer (pH 7.5). Protein-bound and free $^{125}I$ were determined by precipitation with trichloroacetic acid and γ-scintillation counting was carried out using a well counter (TM Analytic). The $^{125}I$-labeled native mAb or BD-mAbs were shown to be stable and were not dehalogenated for at least 1 week when kept at 4°C.

***Cell lines and* in vitro *mAb binding assays.*** $F98_{EGFR}$ and $F98_{npEGFRvIII}$ cells were produced by transfecting F98 cells with the human genes encoding either wild-type EGFR (19, 39) or its mutant isoform, EGFRvIII (17). The resulting cell lines, respectively designated $F98_{EGFR}$ and $F98_{npEGFRvIII}$, expressed $\sim 1.2 \times 10^5$ receptor sites of either wild-type EGFR or EGFRvIII. Following ligation, these receptors were only weakly phosphorylated (i.e., nonfunctional or nonphosphorylated). However, these cell lines were ideally suited for our studies because they were nonimmunogenic in syngeneic Fischer rats. In contrast, another set of human wild-type EGFR and EGFRvIII transfectants, which were produced by Dr. Frank Furnari (Ludwig Institute, San Diego, CA), expressed $\sim 1.2 \times 10^6$ functional receptor sites per cell. However, these were immunogenic in Fischer rats, and consequently, could only be propagated in nude rats.

For *in vitro* binding assays of L8A4, $F98_{EGFR}$ or $F98_{npEGFRvIII}$ cells were seeded into T-150 flasks and cultured for 2 days to establish confluent monolayers. Cells were washed twice with PBS and then harvested by incubating them with 0.5 mmol/L of EDTA at 37°C for 10 min. Following the removal of EDTA, $10^6$ $F98_{npEGFRvIII}$ cells were added to 1.5 mL tubes to which 110 ng of $^{125}I$-radiolabeled L8A4 and varying amounts (0.1 ng-100 μg) of either unmodified cetuximab or L8A4 in 0.1% BSA had been added, and these were incubated at 4°C for 90 min. The cells then were washed thrice with PBS, cell-bound and free radiolabeled antibody were separated by centrifugation, and radioactivity was determined by γ-scintillation counting. *In vitro* binding assays of cetuximab were carried out by adding varying amounts of $^{125}I$-mAb (5-100 ng) to $F98_{EGFR}$, $F98_{npEGFRvIII}$, or receptor-negative F98 parental cells and incubating them at 4°C for 90 min. Cell-bound radioactivity was determined as described above for binding studies with L8A4.

***Evaluation of tumorigenicity of mixed $F98_{EGFR}$ and $F98_{npEGFRvIII}$ gliomas.*** All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996) and our protocol was approved by the Institutional Laboratory Care and Use Committee of The Ohio State University. In order to define the tumorigenicity of composite $F98_{EGFR}$ and $F98_{npEGFRvIII}$ gliomas and to compare this to F98 parental, $F98_{EGFR}$ and $F98_{npEGFRvIII}$ tumors, CD-Fischer rats (Charles River Laboratories) were stereotactically implanted with $10^3$ or $10^5$ F98, $F98_{EGFR}$, or $F98_{npEGFRvIII}$ cells or a 1:1 mixture of $F98_{EGFR}$ and $F98_{EGFRvIII}$ cells into the right caudate nucleus, as previously described (40). Briefly, rats were sedated by the i.p. administration of a mixture of ketamine/xylazine at a dose of 120 mg of ketamine/20 mg xylazine per kilogram of body weight, following which a plastic screw (Arrow Machine Manufacturing, Inc.) was embedded into the skull. Tumor cells at a concentration of $10^3$/10 μL for therapy studies or $10^5$/10 μL cells for biodistribution studies were injected over 10 to 15 s through a central hole in the plastic screw into the right caudate nucleus. The cells were suspended in serum-free DMEM containing 1.2% to 1.4% agarose with a gelling temperature of <30°C. The screw hole was then filled with bone wax immediately after withdrawal of the 27-gauge needle, and the operative field was flushed with betadine before the scalp incision was closed with a single sterilized clip. The rats were observed daily and weighed thrice per week after tumor implantation in order to monitor their clinical status. As determined in previous studies with the F98 glioma model (41–43), the combination of sustained weight loss, ataxia, and periorbital bleeding indicated that death was imminent. Therefore, in order to minimize discomfort, animals displaying these signs were euthanized and survival times were determined from the day of tumor implantation to euthanization plus 1 day.

***Biodistribution of $^{125}I$-BD mAbs.*** Biodistribution studies were carried out in tumor-bearing animals 12 to 14 days following tumor implantation. Animals were divided into five experimental groups of five rats each. Animals in groups 1 to 3 had mixed gliomas, those in group 4 had $F98_{EGFR}$ tumors and group 5 had $F98_{npEGFRvIII}$ gliomas. Rats in group 1 received a mixture of $^{125}I$-labeled BD-C225 and BD-L8A4 (5 μCi/50 μg, 2.5 μCi each), those in group 2 received an equal amount (5 μCi/50 μg) of $^{125}I$-BD-L8A4 alone, and those in groups 3 to 5 received an equal amount of $^{125}I$-BD-C225. Because these bioconjugates do not traverse the blood-brain barrier, they were administered intracerebrally by means of convection-enhanced delivery (CED). This technique completely bypasses the blood-brain barrier, maximizes delivery to the tumor, and minimizes uptake by extracranial organs and blood (44). As we have previously described (45), a plastic cannula was inserted into the entry port of the screw and then advanced 5 mm below the dura into the tumor. CED of BD-L8A4 was carried out as described in detail elsewhere (18). For biodistribution studies, rats received 5 μCi/10 μL of $^{125}I$-labeled mAbs delivered for 30 min at rate of 0.33 μL/min and were euthanized 24 h later. Tumor, normal brain, blood, and other tissue samples were taken, weighed, and then radioactivity was determined by means of γ-scintillation counting using a well counter. These were counted along with triplicate samples of the injectate in order to correct for the decay of the isotope before γ counting and the percentage of injected dose per gram (% ID/g) was calculated.

***Therapy experiments and dosimetry.*** BNCT was done 14 days following stereotactic implantation of a 1:1 mixture of $F98_{EGFR}$ and $F98_{npEGFRvIII}$ glioma cells (500 of each cell type). Approximately 2 weeks later (10-12 days), rats were transported to the Nuclear Reactor Laboratory at the Massachusetts Institute of Technology for neutron irradiation. Animals were randomized on the basis of weight into experimental groups of 10 animals each as follows: group 1, CED of BD-C225 + BD-L8A4 and BNCT; group 2, CED of BD-L8A4 and BNCT; group 3 CED of BD-C225 and BNCT; group 4, CED of BD-C225 in rats bearing $F98_{npEGFRvIII}$ glioma and BNCT; and group 5, irradiated controls; group 6 untreated controls. BNCT was initiated 24 h after CED of 10 μL of BD-mAb (40 μg of $^{10}B$/750 μg of mAb). All irradiated rats were anesthetized with a mixture of ketamine and xylazine. BNCT was carried out at the MITR-II reactor in the M011 irradiation facility, which produces a beam of thermal neutrons of high purity and intensity with no measurable fast neutron component. Two rats at a time were positioned in a $^6Li$-enriched polyethylene box that provided

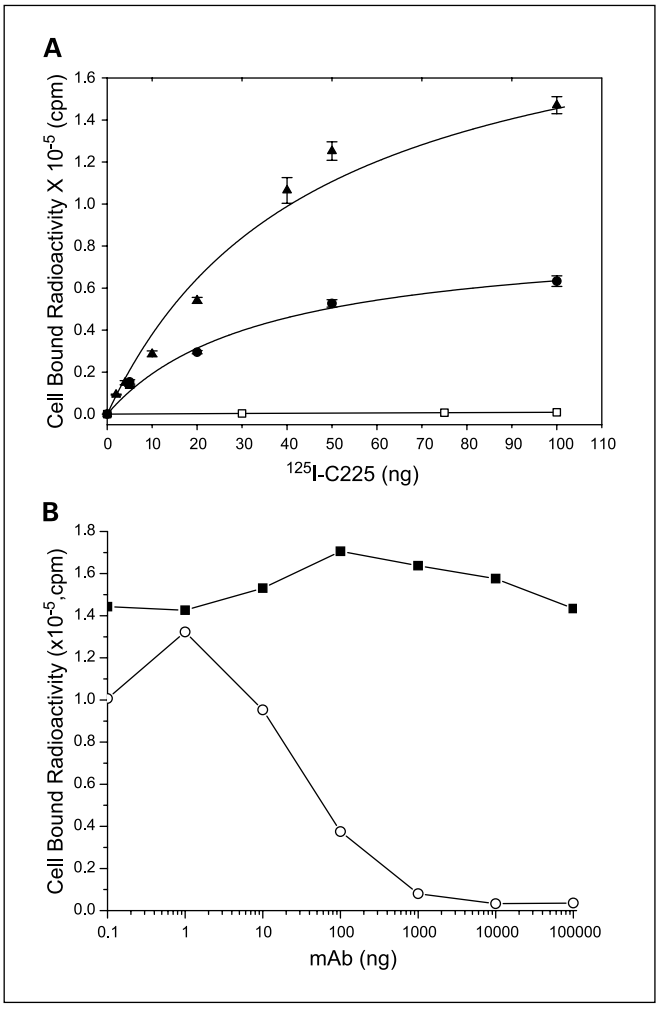


**Fig. 1.** Antibody binding assays. *A,* cellular binding of cetuximab. Varying amounts of $^{125}$I-C225 were incubated at 4°C for 90 min with $F98_{EGFR}$ glioma cells expressing wild-type EGFRs (▲), $F98_{npEGFRvIII}$ cells expressing EGFRvIII receptors (●), and parental receptor – negative $F98_{WT}$ cells (□). Cell-bound radioactivity was determined by γ-scintillation counting. *B,* competitive binding assays. Varying amounts of unlabeled L8A4 (○) or cetuximab (■) were added to $F98_{npEGFRvIII}$ cells tubes containing 110 ng of $^{125}$I-labeled L8A4. Cell-bound radioactivity was determined by γ-scintillation counting.

whole-body shielding from the thermal neutrons during an irradiation. The animals' heads were aligned in the middle of a 13 × 2 $cm^2$ aperture, machined in the box lid, which served as the beam delimiter. Four fission counters, located at the periphery of the 15 cm circular field automatically controlled beam delivery and provided real-time data on the relative neutron fluence during an irradiation.

Dosimetric measurements were done, as previously described (46), using bare gold foils and a graphite-walled ionization chamber (V = 0.1 $cm^3$) flushed with reagent grade carbon dioxide on both dead rats and phantoms made from type 6 nylon. The measured dose rates in the brain (2.2% nitrogen by weight), normalized to the reactor operating at a power of 5 MW, were 18.5 cGy/min for photons, 7.7 cGy/min for thermal neutrons from the nitrogen capture reaction ($^{14}$N [n, p] $^{14}$C) and 3.4 cGy/min/μg $^{10}$B in tissues. For dosimetric calculations, boron concentrations were determined in tumor normal brain, liver, and blood in a separate group of animals 24 h after CED of BD-L8A4 and BD-C225. Animal irradiations were done with the reactor operating at a power between 4.0 and 4.8 MW. These took between 6.9 and 8.6 min to deliver a thermal neutron fluence of 2.64 × $10^{12}$ n.$cm^{-2}$ to complete previous dose prescriptions (46). After the completion of BNCT, the animals were held at the Massachusetts Institute of Technology for ~3 days to allow induced radioactivity to decay before they were returned to The Ohio State University in Columbus, OH for clinical monitoring.

***Monitoring of clinical status and neuropathologic evaluation.*** All animals were weighed thrice a week and their clinical status was evaluated at the same time. Once the animals had progressively growing tumors, as evidenced by the combination of sustained weight loss, ataxia, and periorbital hemorrhage they were euthanized in order to minimize discomfort. Survival times were determined by adding 1 day to the time between tumor implantation and euthanization. The brains of all animals in the therapy studies were removed after death, fixed in 10% buffered formalin, and then cut coronally at the level of the optic chiasm and 2 mm anterior and posterior to it. Selected tissue sections through the tumor were embedded in paraffin, cut at 4 μm, stained with H&E and examined microscopically. The tumor size index was determined by measuring with calipers the tumor's greatest cross-sectional diameter in 2-mm coronal sections of the brain under a dissecting microscope. A semiquantitative grading scale ranging from 0 to 4 was used. Each section was scored as follows: 0, no tumor; 1, very small (i.e., microscopic, <1 mm); 2, small (~1-3 mm); 3, large (~4-7 mm); and 4, massive (>8 mm).

***Statistical evaluation of survival data.*** The MST, SE, and median survival time were calculated for each group using the Kaplan-Meier method whereas Kaplan-Meier and Cox survival curves were also plotted (47, 48). The hypotheses involved comparing the survival curves of animals that received either the combination or the individual, boronated mAbs to irradiated controls. We first fit a Cox proportional hazards regression model to the data, and the assumption of proportional hazards was assessed. Because this assumption was not violated, long-range tests were done for these comparisons using a Bonferroni method of adjustment for the multiple comparisons. Because there are four groups, each of the six pairwise comparisons were tested using a significance level of $\alpha = 0.05/6 = 0.0083$.

## Results

**In vitro *binding activity of BD-C225 and L8A4.*** As shown in Fig. 1A, cetuximab was reactive with both $F98_{EGFR}$ and $F98_{npEGFRvIII}$ cells, but not F98 parental cells. As shown in

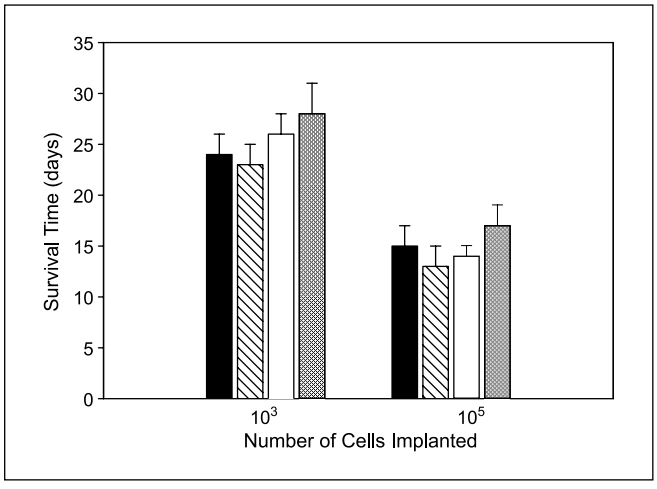


**Fig. 2.** Tumorigenicity of homotypic and composite F98 gliomas. Fischer rats received either $10^3$ or $10^5$ F98 glioma cells intracerebrally ($F98_{WT}$, ■; $F98_{npEGFRvIII}$, ▨; $F98_{EGFR}$, □; and a mixture of $F98_{EGFR}$ and $F98_{npEGFRvIII}$, ▧). MST ± SD are indicated for groups of five animals each. There were no statistically significant differences in MSTs of animals containing either homotypic or composite tumors.

**Table 1.** Radiolocalization of $^{125}$I-BD-L8A4 and BD-C225 either alone or in combination in $F98_{EGFR}$ and $F98_{npEGFRvIII}$ glioma bearing rats 24 h following intracerebral administration by CED

| $^{125}$I-mAb/tumor composition* | % ID/g † | | | | | Tumor/brain ratio ‡ |
|---|---|---|---|---|---|---|
| | Tumor | Brain ipsilateral | Brain contralateral | Liver | Blood | |
| L8A4 + C225/$F98_{EGFR}$ + $F98_{npEGFRvIII}$ | 61.4 ± 8.1 | 6.1 ± 3.1 | 2.4 ± 1.7 | 0.31 ± 0.12 | 0.17 ± 0.02 | 9.9 |
| L8A4/$F98_{EGFR}$ + $F98_{npEGFRvIII}$ | 30.8 ± 7.5 | 5.9 ± 2.9 | 2.2 ± 1.5 | 0.41 ± 0.14 | 0.25 ± 0.04 | 5.2 |
| C225/$F98_{EGFR}$ + $F98_{npEGFRvIII}$ | 34.7 ± 4.3 | 4.1 ± 0.6 | 1.4 ± 0.4 | 0.19 ± 0.09 | 0.13 ± 0.08 | 8.4 |
| C225/$F98_{EGFR}$ | 53.3 ± 6.6 | 5.3 ± 2.2 | 1.2 ± 0.2 | 0.15 ± 0.04 | 0.22 ± 0.07 | 10.0 |
| C225/$F98_{npEGFRvIII}$ | 20.3 ± 3.6 | 4.8 ± 1.7 | 1.0 ± 0.5 | 0.41 ± 0.26 | 0.19 ± 0.07 | 4.2 |
| L8A4/$F98_{npEGFRvIII}$§ | 60.1 ± 10.8 | 6.2 ± 1.2 | 2.0 ± 0.5 | 0.19 ± 0.05 | 0.05 ± 0.01 | 9.6 |
| C225/$F98_{WT}$§ | 13.2 ± 1.6 | 5.6 ± 2.5 | 2.4 ± 2.2 | 0.17 ± 0.10 | 0.36 ± 0.07 | 2.4 |
| L8A4/$F98_{WT}$§ | 14.6 ± 2.7 | 2.9 ± 1.2 | 1.2 ± 0.5 | 0.22 ± 0.08 | 0.05 ± 0.01 | 5.6 |

*Each animal received 5 μCi of $^{125}$I-labeled BD-L8A4 or BD-C225 intracerebrally by CED.
† Percentage of injected dose was based on the amount recovered relative to that administered.
‡ This ratio is based on tumor uptake versus ipsilateral (tumor-bearing) cerebral hemisphere.
§Previously published data (24, 25).

Fig. 1B, unlabeled mAb L8A4 competitively inhibited the binding of $^{125}$I-labeled L8A4 to $F98_{npEGFRvIII}$ cells and increasing its concentration eventually almost completely blocked the binding of the radiolabeled mAb. In contrast, cetuximab, even up to a concentration of 100 μg/mL, did not inhibit the binding of $^{125}$I-labeled L8A4, which indicated that L8A4 and cetuximab recognized different epitopes of the EGFRvIII molecule.

**In vivo *tumorigenicity of mixed $F98_{EGFR}$ and $F98_{npEGFRvIII}$ gliomas.*** The tumorigenicity of gliomas composed of a 1:1 mixture of $F98_{EGFR}$ and $F98_{npEGFRvIII}$ cells was compared with those consisting entirely of either $F98_{EGFR}$ cells or $F98_{npEGFRvIII}$ cells. All rats died following implantation of a 1:1 mixture of $10^3$ or $10^5$ $F98_{EGFR}$ and $F98_{npEGFRvIII}$ cells. As shown in Fig. 2, the corresponding MST ± SD were 28 ± 3 days and 17 ± 1 days for animals bearing composite tumors, compared with 26 ± 4 days and 15 ± 2 days, respectively, for $10^3$ and $10^5$ $F98_{EGFR}$ cells and 23 ± 2 and 13 ± 2 for corresponding numbers of $F98_{npEGFRvIII}$ cells. As determined by the Wilcox-Gehan rank sum test (48), the MSTs of animals bearing tumors composed of a mixed population of cells were not statistically different ($P = 0.1$) from those groups bearing tumors composed of single population.

***Biodistribution of $^{125}$I-labeled BD-mAbs in glioma-bearing rats.*** Biodistribution of the bioconjugate following CED of $^{125}$I-BD-C225 and $^{125}$I-BD-L8A4 in combination or individually was determined by γ-scintillation counting in rats bearing either composite or single receptor–expressing tumors. As shown in Table 1, 61.4% ID/g was localized in rats bearing composite tumors at 24 h following CED of BD-C225 and BD-L8A4. In contrast, following CED of BD-C225, 34.7.7% ID/g was detected in composite tumors compared with 30.8% ID/g for BD-L8A4. Using the Wilcoxon rank sum test to calculate exact $P$ values, the differences in uptake of BD-C225 and BD-L8A4 individually or in combination were highly significant ($P = 0.005$). Twenty-four hours following CED of BD-C225, 53.3% ID/g was localized in $F98_{EGFR}$ gliomas compared with 20.3% ID/g in $F98_{npEGFRvIII}$ gliomas. Similarly, following CED of BD-L8A4 to $F98_{npEGFRvIII}$ glioma–bearing rats, 60.1% ID/g was detected in the tumor. Normal brain uptake of the bioconjugates ranged from 4.1% to 6.2% ID/g in the tumor-bearing (i.e., ipsilateral) cerebral hemisphere compared with 1.0% to 2.4% ID/g for the non–tumor-bearing (i.e., contralateral) cerebral hemisphere. Very low levels ranging from 0.05% to 0.41% ID/g were detected in the liver and blood. Tumor to normal brain ratios of 10.0 and 9.6, respectively, were seen following administration of BD-C225 or BD-L8A4 in animals that had homotypic $F98_{EGFR}$ or $F98_{npEGFRvIII}$ gliomas. The tumor to normal brain ratio was 9.9 for animals bearing composite tumors that received both mAbs.

**Table 2.** Boron concentrations and physical radiation doses delivered to tumor, brain, and blood in rats bearing composite gliomas

| Group | Boron concentrations (μg/g)* | | | Absorbed dose (Gy) † | | |
|---|---|---|---|---|---|---|
| | Tumor | Brain ‡ | Blood | Tumor | Brain | Blood |
| BD-L8A4 + BD-C225 | 24.4 | 2.4 | <0.5 | 7.3 | 2.4 | 1.9 |
| BD-L8A4 | 12.3 | 2.4 | <0.5 | 4.5 | 2.4 | 1.9 |
| BD-C225 | 13.8 | 1.6 | <0.5 | 4.9 | 2.2 | 1.9 |

*Boron content was calculated from radiolocalization data shown in Table 1. Rats received BD-L8A4 or BD-C225 containing 40 μg of $^{10}$B intracerebrally by means of CED.
† Physical dose estimates include contributions from γ photons, and the $^{14}N(n,p)^{14}N$ and $^{10}B(n,\alpha)^{7}Li$ capture reactions.
‡ Boron concentrations for the tumor-bearing cerebral hemisphere.

**Table 3.** Survival times of rats bearing either composite or homogeneous $F98_{EGFR}$ and $F98_{npEGFRvIII}$ gliomas following CED of mAbs BD-L8A4 and BD-C225 either alone or in combination

| Group | n* | Tumor | Survival times (d)* | | | Increased life span (%)† | |
|---|---|---|---|---|---|---|---|
| | | | Range | Mean ± SE | Median | Mean | Median |
| BD-L8A4 + BD-C225 | 10 | $F98_{EGFR}$ + $F98_{npEGFRvIII}$ | 37-87 | 55 ± 5 | 51 | 97 | 89 |
| BD-L8A4 | 10 | $F98_{EGFR}$ + $F98_{npEGFRvIII}$ | 30-42 | 36 ± 1 | 35 | 29 | 30 |
| BD-C225 | 10 | $F98_{EGFR}$ + $F98_{npEGFRvIII}$ | 29-49 | 38 ± 2 | 37 | 36 | 37 |
| BD-C225 | 10 | $F98_{npEGFRvIII}$ | 30-47 | 37 ± 2 | 36 | 32 | 33 |
| BD-C225‡ | 11 | $F98_{EGFR}$ | 39-88 | 55 ± 4 | 50 | 107 | 92 |
| BD-L8A4‡ | 11 | $F98_{npEGFRvIII}$ | 41 to >180 | 70 ± 11 | 58 | 168 | 123 |
| Irradiated controls | 10 | $F98_{EGFR}$ + $F98_{npEGFRvIII}$ | 29-40 | 34 ± 1 | 33 | 21 | 22 |
| Untreated controls | 9 | $F98_{EGFR}$ + $F98_{npEGFRvIII}$ | 25-34 | 28 ± 1 | 27 | — | — |

*Number of animals per group.
†Percentage of increased life span was defined relative to mean and median survival times of untreated controls.
‡Previously published data (24, 25).

Tumor uptake of BD-C225 and BD-L8A4 was 13.2% and 14.6% ID/g, respectively, in rats bearing receptor-negative F98 wild-type gliomas (Table 1). This could either be considered background or nonspecific uptake. The amounts detected in normal brain, liver, and blood were equivalent to those observed in animals bearing receptor-positive gliomas.

***Tissue boron concentrations and dosimetry.*** Boron concentrations in composite tumor and selected normal tissues and the calculated physical doses delivered to them are summarized in Table 2. The tumor boron concentration in rats that received the combination of BD-C225 and BD-L8A4 was 24.4 μg/g compared with 13.8 and 12.3 μg/g, respectively, following CED of either BD-C225 or BD-L8A4. Boron levels in the ipsilateral tumor-bearing cerebral hemisphere were 1.6 to 2.4 μg/g and were in the undetectable range (<0.5 μg/g) in blood and other normal tissues. Dosimetric calculations were based on mean boron concentrations of tumor, brain, and blood at 24 h following CED of mAbs. Based on these total boron concentrations, the mean absorbed dose delivered to mixed gliomas was 7.3 Gy following CED of both BD-C225 and BD-L8A4, 4.9 Gy following CED of BD-C225, and 4.5 Gy following CED of BD-L8A4 (Table 2). The normal brain doses were 2.2 to 2.4 Gy. All doses in this report were expressed as the physical absorbed dose. No attempt was made to apply biological weighting factors, which would have significantly increased the calculated dose delivered to the tumor.

***Responses following BNCT.*** Survival data following BNCT are summarized in Table 3, and Kaplan-Meier and Cox survival plots are shown in Fig. 3A and B, respectively. Untreated control rats had a MST ± SE of 28 ± 1 days compared with a modest increase of 34 ± 1 days for the irradiated controls. Animals that received CED of both B-C225 and BD-L8A4 had a MST of 55 ± 5 days (range, 37-87 days) compared with 38 ± 2 days (range, 29-49 days) for animals that received CED of BD-C225 and 36± 1 days (range, 30-42) for animals that received CED of BD-L8A4 alone.

The survival curves for animals that received CED for both B-C225 and BD-L8A4 were compared with those for animals that received CED of BD-C225 ($P = 0.0025$) and CED of BD-L8A4 ($P = 0.0002$) alone via pairwise log-rank tests. Both tests were statistically significant, indicating that those animals receiving CED of both B-C225 and BD-L8A4 experienced greater survival than those that received CED of BD-C225 and CED of BD-L8A4 alone. Almost identical mean tumor size indices (3.7-3.8) were observed among the different BNCT treatment groups indicating that all animals had approximately the same size tumors at the time of death. The corresponding percentage of increased life spans were 97% for the combination of BD-C225 and BD-L8A4 versus 36% for CED of BD-C225 alone and 29% for CED of L8A4. Histopathologic examination of representative sections of brains taken from animals of each group showed actively growing tumors, which as previously described (17), were histologically indistinguishable from one another.

In contrast, the differences in MSTs of animals bearing composite tumors, which had received either BD-C225 or BD-L8A4 alone were not significantly different from each other or from irradiated controls (37, 38, and 34 days, respectively). As previously reported, the best survival data in animals bearing homotypic tumors were obtained with BD-L8A4 in $F98_{npEGFRvIII}$ glioma–bearing rats (18), and with BD-C225 in rats bearing $F98_{EGFR}$ gliomas (19).

## Discussion

The purpose of the present study was to determine if a rat glioma, which was composed of two populations of F98 glioma cells, one expressing wild-type EGFR and the other EGFRvIII, could be treated as effectively by means of neutron capture therapy using two boronated anti-EGFR mAbs, cetuximab and L8A4, versus either cetuximab or L8A4 alone. Although *in vitro* binding data indicated that cetuximab recognized both receptors, *in vivo* biodistribution studies in F98 glioma–bearing rats revealed that the combination of mAbs resulted in almost twice as much $^{125}I$ localized in the composite tumors compared with $^{125}I$-BD-cetuximab or $^{125}I$-BD-L8A4 alone. BNCT was carried out 24 h following CED of BD-C225 and BD-L8A4 in rats bearing composite tumors. The MST of animals that received both boronated mAbs was 55 days compared with MSTs of 36 and 38 days for animals that received either one or the other boronated mAb. These data clearly indicated that in order to effectively treat composite tumors, it was essential that there be molecular targeting of *both* receptors. Cetuximab, which recognized both wild-type EGFR and EGFRvIII *in vitro*, did not deliver a sufficient $^{10}B$ payload to achieve a therapeutic effect.

Studies by Cavenee and colleagues have shown that U87 EGFRvIII gene–transfected human glioma cells had a significant growth advantage over U87 transfectants that expressed normal levels of wild-type EGFR (49). Even if only 1% of the starting population was EGFRvIII-positive, these cells eventually outgrew EGFR wild-type cells and the resulting tumors were composed almost entirely of the former (50). However, these transfectants had functionally active receptors. In contrast, the EGFRvIII transfectants that we used in the present study had a nonfunctional receptor that was not autophosphorylated following activation. This meant that they had no growth advantage over EGFR wild-type cells, which was highly advantageous because otherwise, there would have been a constantly changing ratio of EGFRvIII and wild-type EGFR cells. We have previously shown (17) that the tumor volume doubling times for EGFRvIII and F98 gliomas were equivalent (50.8 ± 4.8 and 52.0 ± 3.0 h, respectively). This, together with the stability of receptor expression over a period of years, the *in vitro* binding of cetuximab and L8A4 to cells expressing the corresponding receptors, and the data that we have obtained in previous (17, 18, 39), as well as in the present study, establish the suitability of the $F98_{EGFR}$ and $F98_{npEGFRvIII}$ cell lines for targeting studies. As reported by Aldape et al. (51), human GBMs could contain a mixture of cells expressing both EGFRvIII (ΔEGFR) and amplified wild-type EGFR. Similarly, Nishikawa et al. observed that 95% of ΔEGFR-positive gliomas also contained cells that expressed wild-type EGFR (52). However, in both studies, no cells were identified that coexpressed both receptors, and to the best of our knowledge, this has not been reported by anyone.[9] Therefore, the model that we have used also simulated human GBMs in this important aspect. On the other hand, the glioma model used in these studies would not be useful for experiments with EGFR-targeting agents, such as the low molecular weight receptor tyrosine kinase inhibitors, gefitinib and erlotonib, whose tumoricidal activity is dependent on biologically functional receptors (53, 54).

Turning to our biodistribution data, following the administration of a mixture of $^{125}$I-BD-L8A4 and $^{125}$I-BD-C225, 61.4% ID/g localized in composite tumors, whereas in contrast, when a single mAb was used, this was reduced by almost half. As we have previously reported (18), when $^{125}$I-BD-L8A4 was administered to animals bearing $F98_{npEGFRvIII}$ tumors or $^{125}$I-BD-C225 to rats bearing $F98_{EGFR}$ gliomas (19), the amount of radioactivity localized in the tumors was almost equivalent (60.1% and 53.3% ID/g, respectively) to that observed in the present study (61.4% ID/g; Table 1). The data from the biodistribution studies were predictive of those obtained in the therapy studies. The greatest prolongation in survival time of animals bearing composite tumors was seen following the administration of boronated cetuximab *and* L8A4 with a MST of 55 ± 5 days and a range of 37 to 87 days. Almost identical survival data were obtained in rats bearing EGFR-expressing tumors that had received boronated cetuximab (Table 3; ref. 25). The most therapeutically effective targeting was observed in EGFRvIII glioma–bearing rats that had received boronated L8A4 (MST, 70 ± 11 days; range, 41 to >180 days; Table 3; ref. 24).

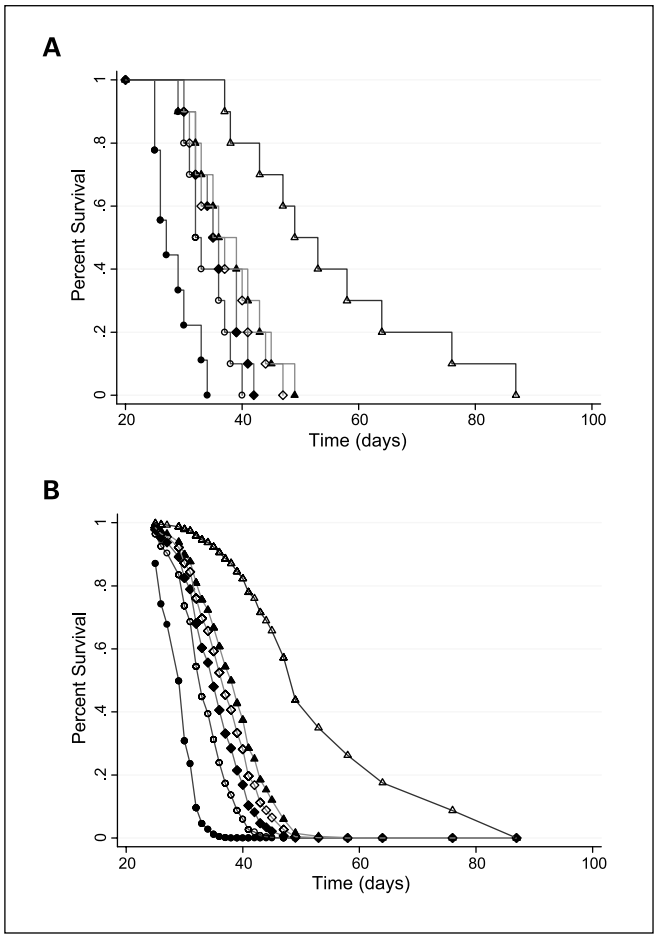


**Fig. 3.** *A,* Kaplan-Meier survival plots for rats bearing composite gliomas. Survival times were plotted for untreated animals (●), irradiated controls (○), and animals that received BD-L8A4 (◆) or BD-C225 (▲) alone or in combination (△). Survival plot of rats bearing $F98_{npEGFRvIII}$ gliomas that received BD-C225 (◇). *B,* Cox survival plots for rats bearing composite gliomas. Survival times were plotted for untreated animals (●), irradiated controls (○), and animals that received BD-L8A4 (◆) or BD-C225 (▲) alone or in combination (△). Survival plot of rats bearing $F98_{npEGFRvIII}$ gliomas that received BD-C225 (◇).

In a recently published report (19), we had concluded that cetuximab would be the best choice as a boron delivery agent for several reasons. First, it has been shown to be clinically active against a variety of EGFR(+) tumors, including colon cancer metastatic to the liver and recurrent squamous cell carcinoma of the head and neck (26, 27, 55). Second, it has been approved by the Food and Drug Administration for clinical use. And third, its production by ImClone has been scaled-up to meet its expanding clinical needs. We also had indicated that at least as far as its use as a boron delivery agent for BNCT, that clinically, it would have to be used in combination with a drug such as boronophenylalanine, which has been widely used for BNCT of both GBMs and extracranial tumors (31). Our present data fully supports this caveat, and if boronated cetuximab were ever to be used clinically as a boron delivery agent for neutron capture therapy, it should be used in combination with boronophenylalanine, or other low molecular weight boron-containing drugs that might become available in the future. Interestingly, cetuximab has also been shown to be active against tumors that are EGFR(-). It has recently been reported

[9] C. David James, personal communication.

that the *in vitro* responsiveness of a panel of human glioma cell lines to X-irradiation, temozolomide, and cetuximab occurred independently of EGFR expression, and that the latter was not essential for a cytotoxic effect (56). It was suggested that there may have been "cross-talk" between toxicity mechanisms that resulted in the death of EGFR-negative cells. Because the tumoricidal activity of BNCT is dependent only on the production of α particles and not whether the receptor is functional or nonfunctional. It could be argued that based on probabilistic considerations, α particles produced as a result of the $^{10}B(n,\alpha)^{7}Li$ capture reaction could, in some instances, have discharged their energy in adjacent cells, whose receptor type was different from that which was being specifically targeted. However, our data do not support a role for a significant bystander effect (57) in the composite, EGFR/EGFRvIII tumor model that was used in the present study.

In a recently published commentary (58) on the role of BNCT, one of the authors (R.F. Barth) made some specific suggestions relating to the future clinical use of this modality for the treatment of both GBMs and extracranial tumors. One major point, which is supported by our own recently published experimental data (17, 18) and the present report, is that EGFR-targeting, boronated mAbs are *not* a stand-alone delivery system, but rather components of a mixture of low and high molecular weight boron delivery agents that would be used in combination. The present results support this approach because only the combination of BD-C225 and L8A4 were effective in treating composite tumors. Finally, we would like to make some comment relating to how BNCT of the F98 glioma compares to another approach for treating this tumor. Rousseau et al. have recently reported (59) that intracerebral administration of carboplatin by CED to F98 glioma–bearing rats, in combination with 6 MV of photon irradiation, resulted in a MST of 97 days. The central nervous system toxicity of CED of cisplatin and carboplatin in this combination is currently being evaluated, and once it has been completed, it should be possible to make a more definitive statement about the potential clinical applicability of this approach. Nevertheless, these survival data were superior to those that we have obtained with boronated mAbs alone in the present and previous studies (17, 18), and is comparable to BNCT survival data that we have reported following intracarotid administration of boronophenylalanine and sodium borocaptate combined with blood-brain barrier disruption (41, 42). How then might boronated mAbs be used for molecular targeting of EGFR for BNCT of brain tumors? One very important advantage of BNCT is the ability to selectively deliver high linear energy transfer radiation to the level of individual cancer cells, whereas concomitantly sparing normal cells in the same radiation field. In contrast, photon irradiation is a physically, rather than biologically, targeted therapeutic modality, and tumor and normal cells in the field of radiation would receive an equal dose. Therefore, one could envision using BNCT in combination with photon irradiation to deliver an added radiation dose to the tumor with little additional dose delivered to the normal brain. Our own previously reported studies on the combination of BNCT with X-irradiation support this approach (43), and promising preliminary clinical results have recently been reported by Kawabata et al. (60). Because BNCT has proven to be effective for the treatment of a variety of extracranial tumors, and most recently, aggressive, therapeutically refractory meningiomas (32), this should provide sufficient impetus for further development of this therapeutic modality.

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