Pharmacokinetics and Toxicology of Immunotoxins Administered into the Subarachnoid Space in Nonhuman Primates and Rodents

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ABSTRACT

Immunotoxins have been used as possible therapeutic agents in patients with leptomeningeal carcinomatosis. The pharmacokinetics, stability, and toxicity of immunotoxins injected into the intrathecal (I.T.) space were examined in rats and rhesus monkeys. Monoclonal antibodies specific for the human (454A12 and J1) and rat (OX26) transferrin receptors were coupled to recombinant ricin A chain. In monkeys, the maximally tolerated dose of the anti-human transferrin receptor immunotoxin (454A12-rRA) was a dose that yielded a nominal cerebrospinal fluid (CSF) concentration of approximately 1.2 x 10^{-7} M. In rats, the 10% lethal dose (LD_{10}) of the anti-human transferrin receptor immunotoxin was a dose yielding a nominal CSF concentration of 8.8 x 10^{-7} M whereas the LD_{10} of the anti-rat transferrin receptor immunotoxin (OX26-rRA) was a dose yielding a nominal CSF concentration of 1.2 x 10^{-7} M. Thus, the species-relevant antibody resulted in toxicity at a concentration one-seventh that of the immunotoxin with the irrelevant antibody. A comparison of the area under the concentration curve at the LD_{10} for rats with the area under the concentration curve at the maximally tolerated dose in monkeys and humans shows that the species-relevant immunotoxin was a better predictor of the toxic dose of the anti-transferrin receptor immunotoxin in humans than the irrelevant immunotoxin. The pharmacokinetics of the 454A12-rRA immunotoxin within the CSF of monkeys showed a biphasic clearance with an early-phase half-life of 1.4 h and a late phase half-life of 10.9 h. The clearance was 4.4 ml/h or approximately twice the estimated clearance due to bulk flow of CSF. Loss by degradation was ruled out because immunoblot analysis showed that the immunotoxin was stable for up to 24 h after administration. Possible losses in addition to sampling include diffusion into brain tissue and transcapillary permeation. The apparent volume of distribution was 10.1 ml or approximately three-fourths the total CSF volume of the monkey. Dose limiting toxicity corresponded with the selective elimination of Purkinje cells in both rats and monkeys and was manifested clinically as ataxia and lack of coordination. The onset of ataxia in monkeys occurred within 5 days and, in the more mild form, was reversible with time. There was evidence of only minimal inflammation within the CSF, and there were no signs of systemic toxicity. Immunotoxins injected into the subarachnoid space may have potential for treatment of leptomeningeal carcinomatosis.

INTRODUCTION

Meningeal carcinomatosis occurs in 5-20% of all cancer patients, the majority caused either by breast carcinoma or lung carcinoma (1). There are recent indications that the incidence of meningeal carcinomatosis is increasing as patients survive longer with improved systemic therapy. Yet, in two-thirds of cases, it occurs when systemic disease is stable or in complete remission (1-7). These patients have an exceedingly poor prognosis. Patients able to tolerate maximal therapy (i.e., methotrexate and whole-brain irradiation) have a 6-7-month mean survival and less than 15% are alive after 1 year, even with aggressive therapy (7-15). In children, the incidence of CSF

spread of primary CNS tumors such as medulloblastomas and ependymomas can be quite high. As many as 44% of children with medulloblastoma have evidence of spread via CSF pathways, and 31% of children who die from ependymomas have evidence of CSF metastases (16). Malignant gliomas, particularly in the pediatric population, may also have CSF spread. Thus, CSF spread of cancer causes significant morbidity, and the choice of treatments now available is limited.

Monoclonal antibodies coupled to protein toxins such as ricin, called immunotoxins, represent a potent and cell type-selective new class of chemotherapeutic agents (17-19). Immunotoxins can specifically kill a variety of tumor cell types in vitro and in vivo, however, systemic delivery of immunotoxins is hampered by rapid clearance into nontarget tissues and slow transport into tumor tissue. Regional delivery may overcome some of these limitations to immunotoxin delivery, particularly for therapy of CNS cancer, where the blood-brain barrier additionally impedes macromolecule delivery (8, 9, 20-22). In animal models of leptomeningeal carcinomatosis, immunotoxins delivered directly into the CSF prolong survival in tumor-bearing animals (20, 23). These and other studies have demonstrated a potential therapeutic benefit of administration of immunotoxins to the CSF in patients with leptomeningeal carcinomatosis (20, 24, 25).

Our study assessed the pharmacokinetics, stability, and toxicity of immunotoxins in the CSF of rats and rhesus monkeys. To address the nonspecific toxicity of immunotoxins in animals and to thoroughly identify the potential of species-specific, antibody-mediated toxicity in an animal model we constructed and studied a human-specific immunotoxin as well as an analogous rat-specific immunotoxin.

MATERIALS AND METHODS

The immunotoxins studied were: (a) 454A12-rRA, an anti-human transferrin receptor monoclonal antibody to the IgG1 isotype (454A12) (26) conjugated to recombinant ricin A chain (rRA); (b) J1-rRA, an IgG2a monoclonal antibody to the human transferrin receptor coupled to rRA (26); and (c) OX26-rRA, an IgG2a monoclonal antibody to the rat transferrin receptor (27) conjugated to rRA. Conjugation of rRA to the antibodies was carried out as described previously (28).

Protein Synthesis Assay

The cytotoxic effects and potency of the anti-rat transferrin receptor immunotoxin were tested on a rat myeloma cell line, Y3-Ag1.2.3 (29) and a rat bladder carcinoma cell line, NBT II (30). The myeloma cell line was maintained in RPMI 1640 containing 10% fetal calf serum, 10 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, 20 μg/ml gentamicin, and 2 mM glutamine; the bladder cancer cell line was maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10 μg/ml gentamicin. The protein synthesis rate was assayed as previously described (24).

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1 The abbreviations used are: CSF, cerebrospinal fluid; MTD, maximally tolerated dose; LD_{10}, 10% lethal dose; AUC, area under the concentration curve; CNS, central nervous system; PBS, phosphate-buffered saline; i.t., intrathecal; p.c., percutaneous; TTR, transferrin receptor; In-DTPA, indium diethyltriaminepentaacetic acid.
In Vivo Studies

**Rats.** Immunotoxins, diluted to various concentrations with PBS, were injected into the CSF of Sprague-Dawley rats either by direct injection into the cisterna magna or by injection through a catheter placed into the i.t. space. For direct injection, animals were anesthetized with i.p. ketamine (Parke Davis, Morris Plains, NJ) (80–85 mg/kg). The total volume injected was 100 µl suspended in PBS with 0.2% bovine serum albumin. This volume was injected slowly, using a 25-gauge needle placed p.c. into the subarachnoid space of the cisterna magna. Injections were performed only after CSF was clearly visualized in the hub of the needle. A nominal concentration achieved in the CSF, and presented in “Results,” was calculated based on a CSF volume in the rat of 0.25 ml (31). The length of survival was recorded as the number of days following injection until death or until animals were sacrificed because of severe toxicity incompatible with humane animal handling. Body weight was measured at intervals of 3 days and compared to that of control animals given injections of PBS.

In an attempt to more carefully control the amount of immunotoxin injected and to permit eventual multiple dosing, a catheter system was developed, using a modification of the technique described by Fuchs et al. (32). Animals were anesthetized with i.p. ketamine (80–85 mg/kg) and xylazine (10 mg/kg). Animals were placed in a rat stereotaxic head frame (Kopf) and their necks flexed at a 90-degree angle. A midline sagittal incision was made from the neck of the monkey was flexed and a 23-gauge butterfly needle was advanced into the subarachnoid space of the monkey. 2.25 kg, were anesthetized with i.m. ketamine (10 mg/kg). The injection procedure and were excluded from study.

**Monkeys.** Nine rhesus monkeys, weighing 4–10 kg, and one cynomologus monkey, 2.25 kg, were anesthetized with i.m. ketamine (10 mg/kg). The occiput and posterior aspects of the neck were shaved and then sterilized with Betadine. The midline of the suboccipital region was then determined using the palpable landmarks of the inion and the arch of C2. The neck of the monkey was flexed and a 23-gauge butterfly needle was advanced through the skin, s.c. tissue and fascia into the cisterna magna. The position of the needle was confirmed by rapid and easy flow of CSF that had typical pulsation with respiration. Immunotoxins were administered over 5 min in a total volume of 1 ml. Gentle barbotage was used during injection to improve mixing within the CSF. Two control animals received instillation of 1 ml normal saline.

CSF samples (±1.5 ml) were taken at specified intervals for pharmacokinetic studies as well as for analysis of CSF glucose, protein, and cell count. Blood chemistry was tested throughout the experiment. Animals were given water ad libitum. Their feedings were restricted for 8 h before administration of immunotoxin or withdrawal of CSF.

Clinical observations included regular weighing of each animal and observation of grooming, appearance, ability to walk and maintain an upright position, facility for recovery after change in position, and ability to use either arms or legs independently to maintain a position or to reach for an object.

**Pharmacokinetics**

In monkeys, a nominal CSF concentration was defined as the dose divided by a CSF volume of 13 ml. The experimentally measured concentrations of immunotoxin in the CSF samples followed a biexponential decrease with time. An equation of the form

\[ C = A \exp(\alpha t) + B \exp(\beta t) \]  

was fit to the data using nonlinear regression analysis based on a finite difference Levenberg-Marquardt algorithm (IMSL, Inc., Houston, TX). The ratio \( A/(A + B) \), denoted \( R \), is the fractional contribution of the early or \( \alpha \) phase at time zero. The half-lives of the early and late (\( \alpha \) and \( \beta \)) phases are equal to \( \ln 2/\alpha \) and \( \ln 2/\beta \). In one animal CSF samples were not collected until 6 h following injection; in this case, the \( \alpha \) phase half-life was set equal to the average of the values of the other six animals. In three animals CSF samples were not collected after 12 h; the \( \beta \) phase half-life in those animals was set equal to the average of the other four animals. The apparent volume of distribution (\( V_d \)) was calculated as the dose divided by the concentration extrapolated to time zero. The AUC is the integral of the concentration curve from time zero to infinity, calculated as \( A/\alpha + B/\beta \). Clearance was calculated as the dose divided by AUC.

**Dose-Response Curves**

The rat toxicity data were analyzed using the PROBIT program in the SAS software (SAS Institute, Cary, NC) and the nonlinear regression program AR in the BMDP software library (BMDP Stastical Software, Los Angeles, CA). Moribund animals that were sacrificed for humane reasons were considered deaths. When no animals survived treatment at a particular dose, a point estimate of the fractional survival was given by one-half an animal divided by the number of animals treated at that dose. The predicted dose-response curves were used to estimate values for LD_{50}.

**Immunotoxin Quantification**

Antibody (rabbit anti-rRA) was coated onto microtiter plate wells. Plates were washed and incubated for 2 h with the CSF samples (diluted at least 1:10 in PBS). Plates were washed, and the detecting enzyme-conjugated-antibody (horse-radish peroxidase-goat anti-mouse IgG) was incubated on the plates for 2 h. Plates were washed, and enzyme substrate o-phenylenediamine (OPD) was added and incubated for 15 min. The reaction was stopped with \( H_2SO_4 \). Well absorbances were read on a microtiter plate reader at 490 nm. The concentrations of the unknown samples were determined from a standard curve of 454A12-rRA in PBS of known concentration processed at the same time. Samples in CSF were diluted 1/10 into PBS to make the buffer more similar to that of the standards. The lower level of detection of the assay was 3.9 ng/ml.

**Immunoblots**

CSF samples taken from rhesus monkeys at 1, 2, 4, 6, 8, 12, and 24 h after immunotoxin administration were run on sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose filter, and stained with goat anti-mouse antibodies linked to avidin-horse-radish peroxidase. After the final rinse the filters were incubated with color indicator and quenched with hydrogen peroxide.

**RESULTS**

**Rats.** Injection of 454A12-rRA into the subarachnoid space of rats at the highest dose tested produced a reproducible neurological deficit. At that dose, the nominal CSF concentration was 3.6 \( \times 10^{-6} \) M. Animals developed symptoms within 3–5 days, initially manifested by extension of the hind limbs and eventually displayed as a dramatic truncal imbalance, wide-based and staggering gait and an inability to change position without falling over. This severe ataxia is consistent with a cerebellar deficit. Included in this ataxia was an inability to maintain adequate alimentation and the animals lost weight. Animals who displayed this severe toxicity were sacrificed.

Histological examination of the brains and spinal cords of animals treated with this dose of immunotoxin showed dramatic cerebellar Purkinje cell loss. At the highest concentrations of 454A12-rRA examined, there was also vacuolization within the cerebellar and spinal cord white matter. These changes did not reflect demyelination and were greatest in those areas in contact with the CSF pathways (Fig. 1, B and C).

The monoclonal antibodies to the human transferrin receptor studied here do not detectably cross-react with either the rat or monkey TTR. Therefore, the Purkinje cell toxicity may be due to non-TTR-mediated uptake of the immunotoxins by Purkinje cells. Native ricin...
A chain (33) and recombinant ricin A chain (data not shown) do not kill Purkinje cells, even at lethal doses, indicating that the antibody plays a role in mediating Purkinje cell sensitivity. Borges et al. (34) have shown that Purkinje cells take up various CSF-borne molecules, including antibodies.

Since the extent to which TIR-mediated toxicity of the immunotoxin causes toxicity to normal cells in vivo was unknown and represents a potential dose-limiting toxicity in humans, we examined the toxicity of the anti-rat TfR-specific monoclonal antibody (OX26) conjugated to rRA. OX26-rRA inhibits protein synthesis by 50% at $3 \times 10^{-10}$ M in the rat myeloma line, Y3-Ag1.2.3, and at $1 \times 10^{-10}$ M in a rat carcinoma cell line, NBT II (data not shown). This potency of OX26-rRA to rat cells is in the range of the potency of 454A12-rRA to human cells (24, 35). In vivo, the OX26-rRA was 7.4-fold more toxic to rats than 454A12-rRA (Fig. 2). OX26 is an antibody of the IgG2a isotype and 454A12 is of the IgG1 isotype. To rule out the difference in isotype as playing a role in toxicity, we coupled J1, an anti-human TIR monoclonal antibody of the IgG2a isotype, to rRA and compared its toxicity to rats to that of 454A12-rRA and OX26-rRA. The toxicity of J1-rRA after injection into the subarachnoid space was less than that of OX26-rRA and was not detectably different from the toxicity of 454A12-rRA (data not shown). The OX26-rRA, therefore, appears to be more toxic to rats due to its capacity to bind to the rat TIR. However, the pattern of toxicity of OX26-rRA was similar to that of 454A12-rRA with ataxia and Purkinje cell loss (Fig. 1, A and B). Thus, expression of TIRs on normal CNS tissues accessible to the subarachnoid space appears to increase animal susceptibility to the toxicity of anti-transferrin receptor immunotoxins. However, the magnitude of this increase is not so great as to eliminate the potential of safe delivery of therapeutic doses of immunotoxins.

**Monkeys.** Injection of 2871 μg of 454A12-rRA, yielding a nominal CSF concentration of $1.2 \times 10^{-6}$ M, produced unacceptable toxicity to primates (Table 1). The animal that received 1400 μg of immunotoxin displayed less severe toxicity and improved during a 6-week observation period. Initially this animal required tube feedings to maintain adequate alimentation but subsequently recovered partially. One animal treated with 909 μg of 454A12-rRA experienced severe toxicity and was euthanized. The other two animals treated with 909 μg displayed ataxia, but it was not as severe as that seen with 1400 or 2871 μg of immunotoxin. The 1400- and 2871-μg-treated animals initially required tube feedings. Within 1 month these animals improved to near normal with only minimal residual ataxia evident. Animals that received 91 and 287 μg of immunotoxin had no evidence of clinical toxicity. They remained normal for as long as 5 months after injection. The MTD of 454A12-rRA in monkeys, i.e., the dose one level below the dose that caused unacceptable or irreversible toxic symptoms was 287 μg. This dose corresponds to a nominal CSF concentration of $1.2 \times 10^{-7}$ M.

Blood chemistries in the treated primates remained stable for up to 4 months following injection. There was an initial mild increase in hepatic enzymes (aspartate aminotransferase/alanine aminotransferase up to 200) during the first 10 days after administration. These changes resolved within 2 weeks after immunotoxin injection and were unrelated to the dose of immunotoxin. Control animals displayed similar mild increases in hepatic enzymes, indicating that these changes were related to the repeated anesthesia used to obtain multiple CSF and blood samples. Decrease in weight (less than 10%) occurred in all animals in the first 2 weeks after administration of immunotoxin.

Organs of sacrificed animals other than the brain appeared normal by gross and histological examination. Brain histology was remarkable for dramatic Purkinje cell loss (Fig. 3). This appeared to be
Immunoblot analysis of the CSF samples of immunotoxin shows that the immunotoxin conjugate remains stable in the CSF up to 24 h after injection. There was no evidence of reduction of the disulfide bond linking the recombinant ricin A chain and monoclonal antibody 454A12 up to 24 h after treatment (Fig. 5).

**DISCUSSION**

Immunotoxins have potential for the treatment of leptomeningeal spread of cancer (20, 21, 23). We have examined the pharmacology and toxicity of a monoclonal antibody against the human transferrin receptor (454A12) linked to recombinant ricin A chain (rRA) after injection into the subarachnoid space in rats and monkeys.

Antibodies to the transferrin receptor were chosen because transferrin receptors are found on many types of tumors and thus a single immunotoxin could potentially be used to treat a variety of tumors (25, 37-41). In the normal adult brain, transferrin receptors are located primarily on the blood vessel endothelium (25, 27, 40, 42-47). Administration i.t. should minimize contact with normal transferrin receptor-bearing cells.

Pathological examination of the brain and spinal cord in these animals revealed a loss of Purkinje cells, as well as minor white matter changes such as vacuolation. Histopathology did not show any evidence of vasculitis in the brain, spinal cord, choroid plexus, or venous sinuses in the rat or the rhesus monkey. These histological changes were clearly dose related and corresponded well to the clinical symptoms of ataxia that occurred in these animals. There was no evidence of cerebral toxicity and no evidence of ependymitis or ventriculitis. Previous studies in guinea pigs and rats indicated a similar toxicity after i.t. administration of immunotoxins (33).

The clearance of 454A12-rRA immunotoxin from monkey CSF averaged 4.4 ml/h. Of this, approximately 1 ml/h may represent loss by sampling. The remainder is still greater than the estimated clearance by bulk CSF flow. The steady-state rate of CSF production in rhesus monkeys, measured by ventriculocisternal perfusion of 131I-albumin in anesthetized animals, is 1.8 ml/h (48). CSF production measured by the same method but using blue dextran as the marker ($M_t = 2 \times 10^5$) is 1.7 ml/h (49). Clearance of albumin and blue dextran...
presumably equals the rate of bulk CSF flow because of the low capillary permeability and tissue diffusivity of these compounds and their inertness in binding to structures in contact with the CSF. The clearances of \[^{[H]}\text{Hinulin (M, 1300–1800)}] and \[^{[14C]}\text{Inulin (M, 2500–3500)}] are 2.6 and 2.3 ml/h, respectively (48). The significantly larger clearance of inulin compared to albumin and dextran is attributed to diffusional loss of the smaller molecular weight compound into brain tissue and/or transcapillary permeation. Bulk CSF flow thus accounts for approximately one half of the clearance of 454A12-rRA. The other half probably represents loss to brain tissue or across capillaries. The toxicity to the Purkinje cells and vacuolization in the cerebellum and spinal cord at doses that yielded a nominal CSF concentration above \(3.9 \times 10^{-7}\) M suggest that the immunotoxin is taken up in these areas of the brain.

The clearance of 454A12-rRA is also somewhat larger than the reported clearance of other macromolecular drugs which have been administered directly into the CSF of monkeys. Riccardi et al. (50) measured the clearance of *Escherichia coli* l-asparaginase and 111In-DTPA administered simultaneously via an Ommaya reservoir into the ventricles of fully awake rhesus monkeys. The clearance of l-asparaginase was approximately 2 ml/h and was significantly more rapid than the clearance of the extracellular marker 111In-DTPA. The researchers could not determine whether the additional loss of l-asparaginase above that of bulk CSF flow (111In-DTPA clearance) was due to active transport into brain capillaries or uptake by brain cells. Collins et al. (51) administered recombinant α-interferon intraventricularly and measured a clearance of 3 ml/h. Hertler et al. (21) conducted studies of an immunotoxin made from an anti-CD7 monoclonal IgG2a antibody conjugated to deglycosylated ricin purified from castor beans. From data given in that paper, we calculated a clearance from CSF of approximately 2.5 ml/h. What might account for the somewhat larger clearance of 454A12-rRA compared with that of other macromolecules? We considered degradation of the conjugate as one possibility, but Western blot analysis of the conjugate indicates that most of the conjugate remains intact. Uptake into brain tissue and capillary losses are other possibilities.

The total CSF volume in monkeys is approximately 13 ml. The apparent volume of distribution of 454A12-rRA was 10.1 ml, larger than values found from studies by Riccardi et al. (50), Collins et al. (51), and Hertler et al. (21) of approximately 4, 6, and 6 ml, respectively. Immunotoxin in this study was administered by cisternal puncture with barbotage so that the calculated \(V_d\) probably represents initial mixing of 454A12-rRA into more of the total CSF volume.

An especially important question is the extent to which toxicity to normal cells will limit the therapeutic potential of immunotoxins that are directed to receptors expressed on normal cells. As monoclonal antibodies are often species specific, it may be difficult to determine the potential toxicity, qualitatively and quantitatively, in other species. This problem likely resulted in unexpected toxicity in previous clinical trials of immunotoxins (52). To obtain a better predictor of the potential toxicity to humans, we utilized a monoclonal antibody specific to the rat transferrin receptor (OX26) (27) coupled to rRA and compared the toxicity of the anti-rat TfR immunotoxin with that of the anti-human TfR immunotoxin and to an isotype-matched control. The anti-rat TfR immunotoxin in rats was 7 times more toxic than the anti-human TfR immunotoxin (Fig. 2). Binding of the anti-rat transferrin receptor antibody to normal rat cells represents additional toxicity that is not detected by tests with the anti-human TfR antibody. Thus, preclinical pharmacology in animals with the appropriately species-matched antibody may be necessary to predict the toxic dose in clinical trials.

It has been recently proposed by Collins et al. (51) that the AUC of plasma kinetic data be used as a guide for dose escalation schedules in Phase I clinical trials. They examined data from clinical trials of 16 anticancer agents and found that the AUC of plasma concentrations in mice at the LD₅₀ was a substantially better predictor of toxicity (AUC at the MTD) in humans than dose/m². It appears, from the data in our study, that the AUC of species-specific immunotoxins administered into the CSF may also be a good predictor of interspecies toxicity (Table 3). The AUC at the LD₅₀ of 454A12-rRA in rats is estimated as 980 nm × h. This calculation is based on assumptions that CSF bulk flow in the rat is 0.75%/min of CSF volume (31, 53) and that, like 454A12-rRA clearance in monkeys, one half of the clearance is by bulk CSF flow and the other half is by loss to brain tissue or capillaries. The AUC for monkeys at the MTD was 360 nm × h. The AUC of OX26-rRA in rats is only about 130 nm × h. The higher value of AUC of the 454A12-rRA conjugate in rats and monkeys compared to the OX26 conjugate in rats probably reflects the lack of cross-reactivity of the 454A12 antibody with transferrin receptors on normal cells in the rat and monkey. Prelimi-
nary assessment of a Phase I clinical trial of 454A12-rRA in humans indicates that the AUC at dose-limiting toxicity was approximately 70 nm h. \(^2\) Thus, the AUC at the LD\(_{10}\) in rats using the rat-specific immunotoxin, OX26-rRA, was a much better predictor of the AUC of 454A12-rRA at the MTD in patients compared with the AUC of 454A12-rRA found at the LD\(_{10}\) in rats or the MTD in monkeys (Table 3).

When 454A12-rRA was tested by a clonogenic assay on human K562 leukemia cells, a 2.8-log kill was achieved when the tumor cells were exposed to a 1.7 \(\times 10^{-10}\) m concentration for 2.7 h and a greater than 4.7-log kill was achieved when the cells were exposed to 5 \(\times 10^{-10}\) m for the same duration. These conditions correspond to AUC values of 0.5 and 1.4 nm h (35). Thus, a potentially large therapeutic window exists between effective killing of tumor cells and toxicity to the normal brain tissue.

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