Intrathecal Chemotherapy with 1,3-Bis(2-chloroethyl)-1-nitrosourea Encapsulated into Hybrid Liposomes for Meningeal Gliomatosis: An Experimental Study

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ABSTRACT

1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), one of the nitrosoureas, is effective against malignant glioma. To develop its use in intrathecal chemotherapy, we encapsulated BCNU in hybrid liposomes composed of dimyristoylphosphatidylcholine and micellar surfactants (Tween 20) and dissolved it in artificial cerebrospinal fluid (lipo-BCNU). We then studied the toxicity of hybrid liposomes and cellular proliferation inhibition of lipo-BCNU in vitro. We found that 3 mM hybrid liposomes did not affect the viability of human endothelial cells and that lipo-BCNU inhibited the proliferation of human glioma cell lines U-105MG, U-251MG, and U-373MG, and rat glioma cell lines C6 and 9L in a concentration-dependent fashion. Wistar rats that were administered lipo-BCNU intracisternally showed no weight loss, neurological symptoms, or histological changes of the brain and spinal cord. A Wistar rat model of meningeal gliomatosis was established by intracisternal inoculation of 0.1 ml cell suspension containing 1 x 10⁶ or 5 x 10⁶ viable C6 glioma cells. Two days after inoculation, lipo-BCNU (BCNU, 2.5 mg/kg) was administered intracisternally. When 1 x 10⁶ glioma cells were inoculated (experiments 1 and 2), the median survival times were 24.5 and 26 days in the control groups and 32 and 45 days in the lipo-BCNU-treated groups, respectively. When 5 x 10⁶ glioma cells were inoculated (experiments 3–6), the median survival times were 17–29.5 days in the control groups and 23–44 days in the treated groups, respectively. Significantly prolonged survival was obtained in three of six experimental groups. After the administration of 1 ml lipo-BCNU (BCNU, 4.67 mM) or 1 ml BCNU solubilized with 5% dextrose/water (BCNU, 4.67 mM) into the cisterna magna of dogs, the cisterna magna cerebrospinal fluid was sampled, and the BCNU concentrations were assayed by high-performance liquid chromatography. The half-life of the lipo-BCNU was longer than that of BCNU with 5% dextrose/water. These results suggest that the intrathecal administration of lipo-BCNU may be possible for the treatment of meningeal gliomatosis.

INTRODUCTION

Meningeal gliomatosis is an uncommon but serious complication of cerebral gliomas (1). Against meningeal gliomatosis, the effectiveness of radiation therapy and systemic chemotherapy appears to be limited. Although drugs such as methotrexate (2, 3), 1-β-D-arabinofuranosylcytosine (2–4), N,N',N''-triethylenetriphosphoramide (2, 5, 6), and neocarzinostatin (7) have been administered intrathecally, they have had insufficient therapeutic effects. We investigated intrathecal chemotherapy using ACNU,3 water-soluble nitrosourea, the drug of first choice in the treatment of malignant glioma in Japan. We found that, in dogs, CSF distribution of ACNU was achieved by ventriculoembran perfusion methods without serious toxicity (8), and we conducted clinical trials (9, 10). Among primary central nervous system tumors, medulloblastoma and primitive neuroectodermal tumors responded to the therapy; astrocytic tumors responded poorly. To improve these results, we investigated the intrathecal chemotherapy with BCNU, one of the nitrosoureas, the i.v. administration of which is effective for glioma (11, 12). However, BCNU is not suitable for intrathecal injection because of its lipophilicity and low aqueous solubility. To overcome these disadvantages, we encapsulated BCNU in hybrid liposomes composed of DMPC and Tween 20 for intrathecal administration. The hybrid liposomes we prepared have some characteristics that distinguish them from liposomes (13–16). The preparation is simple, without organic solvents that appear to be toxic for the central nervous system. In addition, these hybrid liposomes are uniform in size and stable for more than 2 weeks. Furthermore, we expected that the half-life of BCNU in the CSF would be prolonged by encapsulation into hybrid liposomes. In this study, we evaluated the toxicity of hybrid liposomes and cellular proliferation inhibition of lipo-BCNU in vitro and then used rats to evaluate the toxicity and antitumor activity and dogs to evaluate the pharmacokinetics of intrathecally injected lipo-BCNU.

MATERIALS AND METHODS

Hybrid Liposomes and Encapsulation of BCNU. BCNU was provided kindly by Bristol-Myers Squibb Co. (Evansville, IN). Artificial CSF was prepared as follows: 34.77 g NaCl, 1.08 g KCl, 0.920 g CaCl2·2H2O, 0.545 g MgCl2·6H2O, 0.880 g MgSO4·7H2O, 0.385 g NaH2PO4·2H2O, and 3.0 g glucose were dissolved in 980 ml distilled water as a 5% stock solution. A vial of artificial CSF (108 ml) consisted of the 5% stock solution (21.6 ml), distilled water (85.1 ml), and 0.1 N HCl (1.3 ml). Immediately before use, 8.4% NaHCO3 (2.53 ml) was added to a vial of the artificial CSF, which had a pH of 7.35. The components of artificial CSF were 142.02 meq/liter Na+, 2.89 meq/liter K+, 2.50 meq/liter Ca2+, 2.49 meq/liter Mg2+, 126.28 meq/liter Cl−, 1.42 meq/liter SO42−, 0.49 meq/liter H2PO4−, and 22.89 meq/liter HCO3−. Hybrid liposomes were prepared by dissolving both phospholipids and micellar surfactants (PEG) in the buffer solution with sonication (13–16). In this experiment, the hybrid liposomes were made of 3 mM DMPC as phospholipids and 0.33 mM Tween 20 as PEG surfactants sonicated in artificial CSF in an ultrasonicator (Branson model B2200; Yamato Scientific, Inc., Tokyo, Japan) for 5 min at 45°C in an atmosphere of nitrogen and filtered in a sterile manner through a 0.45 μm Millipore filter. The clear stock solution of hybrid liposomes encapsulating BCNU (lipo-BCNU) was prepared by dissolving DMPC, Tween 20, and BCNU in artificial CSF with sonication in various concentrations (Fig. 1). The average hydrodynamic diameter of lipo-BCNU thus prepared was about 80 nm by dynamic light-scattering measurement (14). These hybrid liposomes are uniform in size and stable for more than 2 weeks (Fig. 2).

Glioma Cells. Human glioma cell lines U-105MG, U-251MG, U-373MG (17–19); rat glioma cell line C6 (20); and rat gliosarcoma cell line 9L (21), cultured in our laboratory, were used. The glioma cells were cultured in tissue culture flasks (Corning Glass, Corning, NY) in RPMI 1640 medium containing penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM glutamine, and 10% FBS in a humidified atmosphere of 5% CO2 at 37°C. The medium was changed every 3 days, and the cells were subcultured before they became confluent.

MTT Assay. We modified methods described by Mosmann (22) and Nikkhah et al. (23). MTT (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS at a concentration of 2 mg/ml and 50 μl were added to each microculture well. After 4 h incubation at 37°C, the culture plates were
Fig. 1. Hybrid liposomes were prepared by dissolving both phospholipids and micellar surfactants (PEG) in the buffer solution with sonication. The clear stock solution of hybrid liposomes encapsulating BCNU (lipo-BCNU) was prepared by dissolving 3 mM DMPC, 0.33 mM Tween 20, and BCNU in various concentrations in artificial CSF with sonication for 5 min at 45°C in an atmosphere of nitrogen.

centrifuged at 1000 rpm for 10 min, and the medium supernatant was removed. Acid-isopropanol (100 μl; 0.04 N HCl in isopropanol) was added to each well, and plates were shaken for dissolving. After sufficient solubilization of the MTT-formazan product, absorbance at 570 nm was measured with a Bio-Rad model 2550 EIA reader (Hercules, CA). The percentage of control was calculated as a percentage of absorbance at 570 nm of the treated cells compared to absorbance at 570 nm of the control (untreated) cells. The P value was determined by the unpaired t test.

Assessment of Toxicity of Hybrid Liposomes in Vitro. Endothelial cells from human umbilical vein and culture medium E-GM were obtained from Kurabo, Inc. (Osaka, Japan). After harvesting, the cells were sorted by the trypan blue exclusion method, and 1 X 10⁴ viable cells in 100 μl of the culture medium were incubated in 96-well microtiter plates. After 24 h, 3 mM hybrid liposomes not encapsulating BCNU, 3 mM phosphatidylcholine, and 0.33 mM Tween 20 in 10 μl artificial CSF were added to each well, and the plates were incubated again. After 48 h, the number of viable cells was checked using the MTT assay.

Assessment of Cytotoxicity of Lipo-BCNU in Vitro. Human glioma cell lines U-1O5MG, U-251MG, U-373MG; rat glioma cell line C6; and rat gliosarcoma cell line 9L were incubated in the medium that contained lipo-BCNU for 48 h. BCNU concentrations in the medium were 93.4, 233.5, 467.1, and 934.3 μM. Cell viability was tested using the MTT assay.

Assessment of in Vivo Toxicity of Intrathecal Hybrid Liposomes and Lipo-BCNU. Male Wistar rats, weighing approximately 180 g and obtained from Japan SLC (Shizuoka, Japan), were used in the in vivo experiments. The rats were maintained in a specific pathogen-free environment in the Laboratory Animal Research Center of Kumamoto University Medical School and fed sterile laboratory pellets and water. The rats were anesthetized by i.p. injection of sodium pentobarbital at a dose of 50 mg/kg. Hybrid liposomes not encapsulating BCNU or lipo-BCNU (0.1 ml) at BCNU concentrations of 5.25, 10.51, and 21.02 mM (the doses of BCNU were 0.625, 1.25, and 2.5 mg/kg body weight, respectively) were injected percutaneously into the cisterna magna. The rats were checked daily for changes in body weight and for neurological symptoms. At 23 days after the injection, the rats were killed by i.p. injection of sodium pentobarbital at a fatal dose, and their brains and spinal cords were removed, fixed in 10% formalin, and embedded in paraffin. Representative sections were stained with H&E and the Klüver-Barrera stain and inspected under a light microscope.

Meningeal Gliomatosis Model. Cultured C6 glioma cells were harvested, and the cell suspension was centrifuged at 800 rpm for 5 min; the supernatant

Fig. 2. Electron micrograph of hybrid liposomes composed of DMPC and PEG surfactants obtained by means of cryotransfer technique (JEM-2000, Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan). They are uniform in size, and the diameter is about 80 nm.
Intrathecal chemotherapy with liposome-encapsulated BCNU. The proliferation of human endothelial cells exposed for 48 h to 3 mM hybrid liposomes not encapsulating BCNU, 0.33 mM Tween 20, or 3 mM DMPC in the artificial CSF was not inhibited. The controls consisted of untreated endothelial cells. The P value was determined by unpaired t test. Data presented are means; bars, SDs.

Assessment of in Vivo Antitumor Activity of Intrathecal Lipo-BCNU. Two days after tumor inoculation, the rats were divided randomly into two groups (control group and treated group). Lipo-BCNU (0.1 ml) at a BCNU concentration of 21.02 mM (the dose of BCNU was 2.5 mg/kg body weight) was injected percutaneously into the cisterna magna of rats in the treated group. All rats were checked daily for 60 days, and changes in body weight and neurological symptoms were recorded. The life span of treated rats was compared to that of the controls and the median life span as percentage of control was determined. The P value was determined by the generalized Wilcoxon test. If there were long-term survivors (more than 60 days) without microscopic evidence of tumor in the control group, they were excluded from the experiment, and the same number of long-term survivors in the treated group was also excluded.

Intrathecal Pharmacokinetics of Lipo-BCNU in Dog. Two adult mongrel dogs weighing 11.8 and 12.1 kg were anesthetized with ketamine and pentobarbital and placed in the sphinx position. Anesthesia was maintained with a mixture of nitrous oxide, oxygen, and halothane. One dog received a bolus injection of 1 ml lipo-BCNU (BCNU concentration, 4.67 mM; BCNU dose, 1 mg/body) into the cisterna magna, and CSF samples were withdrawn from the cisterna magna at 5, 10, 30, and 45 min postinjection. BCNU solubilized with 5% dextrose/water as described by Levin et al. (25) and diluted in artificial CSF at BCNU concentration of 4.67 mM was administered by bolus injection into the cisterna magna of the other dog (BCNU dose; 1 mg/body), and the CSF was sampled in the same way. The CSF samples were frozen immediately. The BCNU concentrations were assayed by reverse-phase high-performance liquid chromatography using a LC10A system (Shimazu Co., Tokyo, Japan) equipped with an octadecylsilica column (Cosmosil SC18-AR 5 μm, 4.6 mm inside diameter × 150 mm; Nacalai Tesque, Inc., Kyoto, Japan) with a solvent system of acetonitrile and 0.1% acetic acid in the mobile phase.

Fig. 3. Absorbance at 570 nm of human endothelial cells by MTT assay. The proliferation of human endothelial cells exposed for 48 h to 3 mM hybrid liposomes not encapsulating BCNU, 0.33 mM Tween 20, or 3 mM DMPC in the artificial CSF was not inhibited. The controls consisted of untreated endothelial cells. The P value was determined by unpaired t test. Data presented are means; bars, SDs.

Fig. 4. Growth rate of glioma cell lines exposed to lipo-BCNU for 48 h. BCNU concentrations in the medium were 93.4, 233.5, 467.1, and 934.3 μM. The proliferation of human glioma cell lines U-105MG, U-251MG, and U-373MG, and of rat glioma cell lines C6 and 9L, was inhibited in a concentration-dependent fashion. Plotted areas, growth rate of glioma cell lines exposed to 3 mM hybrid liposomes not encapsulating BCNU for 48 h. Data presented are means; bars, SDs.
Intrathecal chemotherapy with liposome-encapsulated BCNU

Toxicity of Hybrid Liposomes. Neither 3 mM hybrid liposomes not encapsulating BCNU, 3 mM DMPC, or 0.33 mM Tween 20 in artificial CSF damaged the viability of human endothelial cells. The absorbances at 570 nm of human endothelial cells exposed to 3 mM hybrid liposomes not encapsulating BCNU and 0.33 mM Tween 20 for 48 h were 0.471 ± 0.014 and 0.416 ± 0.014, respectively (for the control it was 0.432 ± 0.027). For endothelial cells exposed to 3 mM phosphatidylcholine for 48 h, the absorbance was 0.576 ± 0.004 (for the control it was 0.465 ± 0.027; Fig. 3). Hybrid liposomes did not affect the growth of human endothelial cells.

Cytotoxicity of Lipo-BCNU in Vitro. The percentages of control C6 glioma cells exposed to lipo-BCNU at BCNU concentrations of 93.4, 233.5, 467.1, and 934.3 μM in the medium for 48 h were decreased to 76, 70, 49, and 24%, respectively. The half-life of BCNU in the liposomal preparations when in cell culture medium was 35.7 min (date was not shown). Similarly, lipo-BCNU inhibited the proliferation of U-105MG, U-251MG, U-373MG, and 9L cells in a concentration-dependent fashion (Fig. 4).

Toxicity of Hybrid Liposomes and Lipo-BCNU in Vivo. Neither weight loss nor neurological changes were observed in the rats that had received injections of hybrid liposomes or lipo-BCNU at BCNU doses of 0.625, 1.25, and 2.5 mg/kg into the cisterna magna (Fig. 5). Microscopically, no ependymitis, periventriculitis, meningitis, vasculitis, or demyelination was observed.

Antitumor Activity of Lipo-BCNU in Vivo. In this study, six separate experiments were performed. In experiments 1 and 2, 1 × 10⁶ viable C6 cells were inoculated; in experiments 3–6, 5 × 10⁶ cells were inoculated. In the terminal stage, almost all rats showed weight loss. Microscopically, the tumor appeared as a few layers or as a continuous multicellular mantle on the surface of the brain and spinal cord (Fig. 6). As shown in Table 1, in experiments 1 and 2, the median survival rates were 24.5 and 26 days in the control groups and 32 and 44.5 days in the treated groups, respectively. The median life spans (% control) were 130 and 171%, respectively. Similarly, in experiments 3–6, the median survival of the treated rats was longer than of the controls. There was a significant difference between the control and treated groups in experiments 2 (P < 0.01), 5 (P < 0.05), and 6 (P < 0.05). The survival curves for rats in experiments 2 and 6 are shown in Fig. 7.

Intrathecal Pharmacokinetics of Lipo-BCNU in Dog. When 1 ml lipo-BCNU (BCNU concentration, 4.67 mM; BCNU dose, 1 mg/body) was administered into the cisterna magna by bolus injection, the BCNU concentrations of cisterna magna CSF were 217.6, 56.5, and...
### Table 1 Effect of intrathecal administration of lipo-BCNU against meningeal gliomatosis

<table>
<thead>
<tr>
<th>Experiment (C6 cells)</th>
<th>Group</th>
<th>n*</th>
<th>Median survival (days)</th>
<th>Median life span (% control)</th>
<th>P value (Wilcoxon test)</th>
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<tbody>
<tr>
<td>1</td>
<td>1 x 10⁶</td>
<td>Control</td>
<td>6</td>
<td>24.5</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>7</td>
<td>32.0</td>
<td>171</td>
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<tr>
<td>2</td>
<td>1 x 10⁶</td>
<td>Control</td>
<td>8</td>
<td>26.0</td>
<td>108</td>
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<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>8</td>
<td>44.5</td>
<td>135</td>
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<tr>
<td>3</td>
<td>5 x 10⁶</td>
<td>Control</td>
<td>11</td>
<td>29.5</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>10</td>
<td>32.0</td>
<td>116</td>
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<tr>
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<td>Treated</td>
<td>11</td>
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<td>Treated</td>
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<td>6</td>
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<td>Control</td>
<td>15</td>
<td>27.0</td>
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<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>16</td>
<td>31.5</td>
<td>135</td>
</tr>
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a, number of rats.  
b, NS, not significant.

6.5 μM at 5, 10, and 30 min after the injection, respectively. Forty-five min postinjection, no BCNU was detected in the CSF. The BCNU concentration decreased exponentially; the half-life was 12.1 min ($\tau^2 = 0.96$). When BCNU solubilized with 5% dextrose/water and diluted with artificial CSF (BCNU concentration, 4.67 μM; BCNU dose, 1 mg/body) was administered into the cisterna magna by bolus injection, the BCNU concentrations of the cisterna magna CSF were 162.1, 14.4, and 1.4 μM at 7.5, 13.6, and 20 min postinjection respectively. BCNU was not detected at 30 and 45 min after the injection. The BCNU concentration decreased exponentially; the half-life was 4.2 min ($\tau^2 = 1.00$; Fig. 8).

### DISCUSSION

In patients with meningeal gliomatosis, radiotherapy is effective for the treatment of bulky metastases of the spinal cord, but it cannot control widespread disease. Most types of systemic chemotherapy do not provide an effective drug concentration in the CSF and, although some drugs have been administered intrathecally, they failed to produce sufficient therapeutic effects (2–7, 9, 10).

To overcome these problems, we investigated the possibility of intrathecal chemotherapy with BCNU. BCNU has the following pharmacological characteristics: (a) it is a highly lipophilic drug with a log $P$ (octanol/water partition coefficient) of 1.5; (b) its capillary transfer constant is high, and it crosses the blood-brain barrier easily; and (c) its time course of action is short. Levin and Levin (25) reported that BCNU can be solubilized in 5% dextrose/water at 60°C, thus making it available for intrathecal chemotherapy. Ueoka et al. (14) have recently produced specific hybrid liposomes composed of vesicular and micellar molecules, the physical properties of which (sizes, membrane fluidity, phase transition, and hydrophobility) could be controlled by changing their composition. We encapsulated BCNU in these hybrid liposomes and dissolved it in artificial CSF to make intrathecal administration possible and to improve the CSF pharmacokinetics.

As a drug-delivery system in cancer therapy, liposomes and other lipid-based microspheres have been investigated with the aim of increasing efficacy, decreasing toxicity, or increasing the ease of administration (26). Many antitumor agents have been investigated, and BCNU and other nitrosoureas were found to exhibit increased antitumor activity in certain situations. Ritter and Rutman (27) reported that the effects of a low dose of BCNU on L1210 leukemia can be potentiated by the simultaneous administration of dipalmitoylphosphatidylcholine multilamellar liposome vesicles. Inaba et al. (28) reported that the inhibitory effect of 1-(2-chloroethyl)-3-(4-methylcyclclohexyl)-1-nitrosourea liposomes on the lung metastasis, induced by i.v.- or i.m.-planted Lewis lung carcinoma, was greater than that of the free drug. Takenaga et al. (29) found that lipid microspheres consisting of egg yolk lecithin and soybean oil and containing BCNU exhibited not only enhanced activity against L1210 leukemia in mice but also reduced toxicity. These findings show that it is reasonable to encapsulate lipophilic drugs such as BCNU in liposomes.

The intra-CSF administration of liposome-encapsulated drugs has been reported (30–32), and clinical trials are underway (33). Kim et al. (31, 32) showed that in rats and monkeys, liposomes containing...
1-β-arabinofuranosylcytosine was capable of maintaining a cyto-
toxic concentration of free drug in the CSF for weeks. Their clinical 
Phase 1 study showed that seven of nine patients with neoplastic 
meningoits attained a complete cytological response upon intraven-
tricular or intralumbar administration.

Our hybrid liposomes were not toxic to human endothelial cells in 
vitro, and in rats, intracisternally administered lipo-BCNU did not 
cause any neurological symptoms or histological changes in the brain 
and spinal cord. Levin et al. (34) observed no pathological changes in 
a dog that had received 8 weeks of 2 mg/week BCNU by intraven-
tricular administration and another dog treated with 5 mg/week for 8 
weeks. However, a dog that received 5 mg/week BCNU for 4 weeks 
exhibited partial obliteration of the aqueduct and hydrocephalus due 
to ventriculitis. Nagatani et al. (35) found that rats given ACNU
intracisternally at more than 3.0 mg/kg lost body weight progress-
vively; at more than 6.0 mg/kg, ACNU was fatal in 80% of the rats.
In our experiments, the administration of lipo-BCNU at a BCNU dose 
of 2.5 mg/kg did not result in weight loss; nor were there any 
neurological symptoms or pathological changes in the brains and 
spinal cords of rats. Because it was not possible to encapsulate more 
than 21.02 mm (2.5 mg/kg) BCNU in the hybrid liposomes because of 
capacity limitations, we decided that the optimal dose of BCNU was 
2.5 mg/kg.

With regard to cytotoxicity in vitro, lipo-BCNU inhibited the pro-
liferation of glioma cells in a concentration-dependent fashion. We 
used the MTT assay to test antitumor activity in vitro. In a study that 
used a number of rat and human glioma cell lines (36, 37), there was 
a close correlation in chemosensitivity between the clonogenic assay 
and the MTT assay. In three of our six experiments, the intracerebral injection of lipo-BCNU (BCNU, 2.5 mg/kg) significantly prolonged the median survival of rats with meningial gliomatosis. There is a possibility that some immune effect enhanced antitumor activity, because C6 glioma cells were suspended in medium containing 10% 
FBS. However, considering the chemoresistance of C6 glioma (38), our findings suggest that lipo-BCNU is effective in the treatment of 
meningial gliomatosis.

The intrathecal half-life of lipo-BCNU was longer than that of 
BCNU solubilized with 5% dextrose/water. This is another advantage of 
intrathecally injected lipo-BCNU.

In summary, we demonstrated that in rats, intrathecal chemotherapy 
with lipo-BCNU is feasible to guard against subarachnoid dissemi-

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