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Enhanced Antitumor Activity and Reduced Toxicity of 1,3-Bis(2-chloroethyl)-1-nitrosourea Administered in Lipid Microspheres to Tumor-bearing Mice

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Stable lipid microspheres (LM) and lipid nanospheres (LN) with average diameters of 200 nm and 50 nm, respectively, were used to encapsulate an lipophilic antitumor agent, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). LM and LN containing BCNU (lipo BCNU and s-lipo BCNU, respectively) were prepared by homogenizing a soybean oil solution of BCNU with egg yolk lecithin, and their antitumor activity via the intravenous route was tested against L1210 leukemia in mice and compared with that of BCNU dissolved in saline. Both lipo-BCNU and s-lipo BCNU showed significantly enhanced antitumor activity with reduced toxicity, when compared with the corresponding doses of BCNU alone. These results suggest that LM and LN may be suitable carriers for lipophilic antitumor agents and may enhance their efficacy.

Key words: Drug delivery system — 1,3-Bis(2-chloroethyl)-1-nitrosourea — Lipid microsphere — Lipid nanosphere — L1210 leukemia

Much attention has been focused on drug delivery systems (DDS) for cancer chemotherapy which aim at the specific targeting of antitumor agents to tumor cells or tumor tissues, thus enhancing the efficacy of chemotherapy as well as reducing its toxicity. For example, microcapsules coated with a monoclonal antibody directed against a tumor-specific antigen are expected to be an extremely effective targeting vehicle for antitumor agents. In fact, there have been numerous studies demonstrating that microcapsules containing chemotherapy agents show an excellent antitumor activity against certain types of experimental tumors.[1–5] However, most of these approaches have been clinically unsuccessful, because the microcapsules were trapped by the reticuloendothelial system before reaching the target tumor cells.[6–8]

We have attempted to improve the pharmacological activity and/or to minimize the toxicity of chemotherapy agents by the incorporation of drugs into lipid microspheres (LM) consisting of egg yolk lecithin and soybean oil.[9] LM show a similar tissue distribution to liposomes, and provide a stable, injectable and safe carrier that can be used for drug delivery.

Most chemotherapy agents are hydrophilic and not hydrophobic. Among them, the nitrosourea agents are one of the few exceptions which are lipophilic.[10–14] They can enter the brain across the blood-brain barrier and have an excellent antitumor activity against brain tumors originating from neuronal tissues. Nitrosourea antitumor agents are also highly effective for malignant lymphoma. Their adverse effects are bone marrow suppression, renal toxicity, and pulmonary toxicity and these are a consequence of drug accumulation during chronic treatment.[10, 11] Among the several nitrosourea agents used clinically, we selected 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), since it is the most lipophilic compound.[12]

In this study, lipo-BCNU was used for treating mice bearing L1210 tumors and the antitumor activity via the intravenous route was compared with that of BCNU dissolved in saline. At the same time, the antitumor activity of a finer lipid emulsion containing BCNU (s-lipo BCNU) was also examined, since smaller liposomes have been reported to accumulate better in tumor sites.[6, 15]

MATERIALS AND METHODS

Tumors L1210 leukemia (ascites) cells were kindly provided by the Japanese Foundation for Cancer Research (Tokyo) and were maintained in this laboratory by weekly intraperitoneal passage in CDF_1 male mice. Ascites containing L1210 cells (1×10^5 ascites cells/0.2 ml) were used for the intraperitoneal inoculation of 6-week-old recipient CDF_1 male mice.

Drugs BCNU was dissolved in 99.5% ethanol and stored at −20°C. BCNU was dissolved in physiological saline immediately before use in the experiments.

Preparation of lipo-BCNU LM containing BCNU were prepared as described previously.[16] Briefly, BCNU (40 mg) was dissolved in 1 g of soybean oil, and 120 mg of egg yolk lecithin was added to the solution. The mixture was homogenized for 5 min at 15,000 rpm with a Polytron type homogenizer. Then 2.58% glycerol aqueous solution was added to the mixture with agitation to make a 10% (w/v) oil-in-water suspension, and further

homogenization was done at 20,000 rpm for 20 min. This mixture was passed through a French Pressure Cell Press (SLM Instruments, Inc., IL), and the procedure was repeated another 5 times to ensure complete emulsification and achieve a final concentration of 4 mg/ml. The average diameter of the LM (lipo-BCNU) thus prepared was 198±34 nm as measured by a Coulter N4 particle analyzer (Coulter, FL). The size varied according to the homogenizing conditions and the lecithin content. LM and lipid nanospheres (LN) without BCNU were prepared in the same manner.

Determination of BCNU content BCNU content was determined by high-performance liquid chromatography (HPLC). The BCNU in LM, LN or aqueous solution was extracted by addition of 100 volumes of methanol, and then aliquots were subjected to HPLC. BCNU was assayed by reverse-phase (RP) HPLC using an LC-8A chromatograph (Shimadzu Corporation, Tokyo) equipped with a octadecylsilica column (Zorbax ODS 5 μm, 4.6 mm ID × 150 mm, Rockland Technologies, Inc., PA) with a solvent system of methanol/distilled water (60:40, v/v) at a mobile phase. The flow rate was 0.5 ml/min. The absorbance of the column eluate at 250 nm was continuously monitored with a UV spectrophotometer (SPD-6AV, Shimadzu). The column temperature was maintained at 60°C. Under these conditions, the retention time of BCNU was 5.17 min and no other compounds were detectable. The concentrations of BCNU were calculated with reference to standard curves.

Animals Male CDF_1 mice, aged 5 weeks, were purchased from Nihon Seibutu Zairyo Kenkyusho (Tokyo) for this study. They were housed in an air-conditioned room immediately after arrival and had free access to diet and water for 1 week. Then they were randomly allocated to groups for the antitumor and survival studies.

***In vivo* antitumor experiment** The *in vivo* antitumor activity was determined using tumor-bearing mice. The percent increase in lifespan (ILS) was calculated by using the formula: (mean survival time of the treated group/mean survival time of the control group) × 100 − 100. Drugs were given according to the experimental schedule and the survival time was monitored. The concentrations of all drugs used *in vivo* experiments were measured by HPLC.

Antitumor experiment 1 The CDF_1 mice were randomly allocated to 12 groups (n=10–11) and L1210 ascites cells (1×10^5/mouse) were implanted intraperitoneally. The first group was the saline control, while the 2nd and 3rd groups were LM and LN controls without BCNU (n=11). The 4th to 6th groups respectively received low (3 mg/kg), medium (10 mg/kg), and high (30 mg/kg) doses of BCNU dissolved in saline. The 7th to 9th groups received lipo-BCNU with the same BCNU doses, and the 10th to 12th groups received s-lipo BCNU. Treatment was initiated on the 2nd day after tumor cell implantation and was given once daily for 5 consecutive days. The agents or vehicles were injected intravenously via the tail vein in a volume of 0.2 ml per 25 g.

Antitumor experiment 2 The experimental conditions used were the same as those in experiment 1, except that treatment (20 mg/kg BCNU) was given once on day 2 after tumor cell implantation.

Antitumor experiment 3 The experimental conditions were the same as those in experiment 1, except that treatment was given 3 times on days 2, 9, and 16.

Toxicity studies Lipo-BCNU or BCNU was administered at a dose of 0.2 ml via the tail vein on days 0, 7, and 14 to normal male CDF_1 mice (6 weeks old and weighing 20–25 g). Groups of 8 mice per dose were monitored over 60 days, deaths were noted and the mean weights of surviving mice were determined. In the other study, a single treatment was given and the number of deaths and the weights of surviving mice were monitored over 14 days.

Statistical analysis The significance of differences between groups was determined by using Student's t test and $P<0.05$ was used as the criterion of statistical significance.

RESULTS

Stability of lipo-BCNU When LM containing BCNU were stored at 4°C, the BCNU content remained almost 100% over 2 weeks (Fig. 1(a)). Even after 3 months, 80% of the BCNU was retained by the LM. On the other hand, the BCNU content of physiological saline decreased with time and was 38.9% after 4 weeks.

At room temperature, the BCNU content of lipo-BCNU was about 80% after 5 days (Fig. 1(b)), while that of aqueous solution was about 3.5%.

At 37°C, the BCNU content decreased rapidly in both preparations. Residual percent of BCNU in LM after incubation for 3 and 24 h was 18.5% and 3.8%, respectively. Also, when lipo-BCNU was incubated for 5 min at 37°C with human serum, more than 90% of BCNU was released from the LM, as determined by Sepharose 4B column chromatography (data not shown).

The particle size of lipo-BCNU was 206±41 nm at 4°C after 2 months. However, at 37°C, the emulsion was destroyed and the oil phase separated from the water phase. Similar results were obtained with s-lipo BCNU.

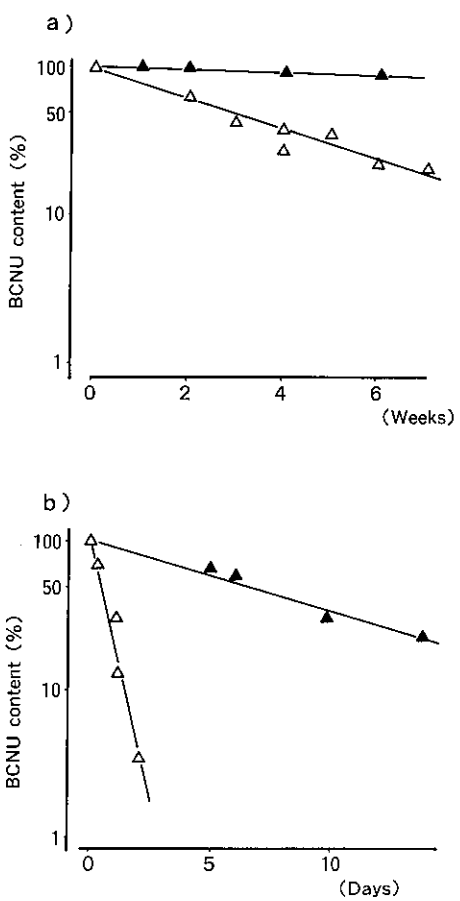
***In vivo* antitumor activity of consecutive administrations of lipo-BCNU in L1210-bearing mice** As shown in Table I, the average lifespan of the saline controls was 7.08±0.27 days (mean±SD), and the LM and LN controls showed a similar lifespan to that of the saline controls. Therefore, LM and LN seemed to have no intrinsic effect on the lifespan of L1210-bearing mice.

Table I. Effect of Consecutive Administrations of BCNU, Lipo-BCNU, or s-Lipo BCNU on the Lifespan of L1210 Tumor-bearing Mice

Preparation	Drug dose (mg/kg)	Survival time (day)	Mean±SD (day)	%ILS
Saline	0	7, 7, 7, 7, 7, 7, 7, 7, 7, 7, 7, 8	7.08±0.27	
LM	0	7, 7, 7, 7, 7, 7, 7, 7, 7, 7, 11	7.36±1.20	4
LN	0	7, 7, 7, 7, 7, 7, 7, 7, 7, 8, 8	7.18±0.38	1
BCNU	3	7, 7, 7, 8, 8, 8, 8, 8, 8, 9	7.8±0.60 *	10
	10	12, 13, 13, 13, 13, 13, 14, 14, 14, 14	13.3±0.64	88
Lipo-BCNU	3	7, 8, 8, 8, 8, 9, 9, 9, 9, 10	8.5±0.81 *	20
	10	16, 17, 17, 18, 18, 18, 18, 19, 19, 19	17.9±0.94 *	152
s-Lipo BCNU	3	9, 9, 9, 9, 9, 9, 9, 9, 9, 10	9.1±0.30	28
	10	17, 17, 17, 17, 18, 18, 19, 19, 20, 24	18.6±2.06	163

CDF_1 mice were inoculated intraperitoneally with L1210 cells on day 0 and were subsequently treated i.v. on days 2–6. The %ILS (percent increase lifespan) was determined as the median survival time of treated animals/the median survival time of control animals.
* $P<0.01$ by Student's t test.

When the average lifespan was compared among the three groups of mice given BCNU at 3 mg/kg/day, the LM and LN groups showed a longer lifespan than that of the saline-treated group.



With the medium BCNU dose (10 mg/kg/day), the lifespan of the lipo-BCNU group was significantly longer than that of the BCNU-treated group. Treatment with s-lipo BCNU produced a longer lifespan than lipo-BCNU, though the difference between the two was not statistically significant.

The high dose of BCNU (30 mg/kg) appeared to be highly toxic to mice. In the BCNU-treated group, the mice rapidly lost weight and began to die on day 12. The lifespan was not prolonged when compared with that of the medium-dose BCNU group (data not shown). Successive treatment with lipo-BCNU and s-lipo BCNU was also toxic. Mice began to die on day 16 and day 17, respectively.

***In vivo* antitumor activity of a single dose of lipo-BCNU in L1210-bearing mice** In experiment 2, BCNU (20 mg/kg) was administered once on day 2 to avoid the toxicity seen in experiment 1, and the lifespan was compared among groups given BCNU, lipo-BCNU, and s-lipo BCNU. As shown in Table II, the lifespan was 8.3±0.46 days in saline controls, while BCNU at a single dose of 20 mg/kg prolonged the lifespan by 49.4%. Treatment with lipo-BCNU and s-lipo BCNU produced a significantly longer lifespan (74.7% and 84.3%, respectively) than treatment with BCNU.

***In vivo* antitumor activity of intermittent administration of lipo-BCNU in L1210-bearing mice** Intermittent treatment with BCNU on days 2, 9, and 16 after tumor

Fig. 1. The content of BCNU. Four mg/ml of lipo-BCNU (▲) or BCNU aqueous solution (△) was prepared, and stored at 4°C (a) or at room temperature (b), then the contents of BCNU was determined by HPLC. Values are the mean % of two or three assays.

Table II. Effect of a Single Dose of BCNU, Lipo-BCNU, or s-Lipo BCNU on the Lifespan of L1210 Tumor-bearing Mice

Preparation	Drug dose (mg/kg)	Survival time (day)	Mean±SD (day)	%ILS
Saline	0	8, 8, 8, 8, 8, 8, 8, 9, 9, 9,	8.3±0.46	
BCNU	20	11, 12, 12, 12, 12, 12, 13, 13, 13, 14	12.4±0.8	49.4
Lipo-BCNU	20	14, 14, 14, 14, 14, 14, 15, 15, 15, 16	14.5±0.67	74.7
s-Lipo BCNU	20	13, 14, 14, 15, 15, 15, 16, 16, 17, 18	15.3±1.42	84.3

($*$) ($*$)

CDF_1 mice (10 animals/group) were inoculated intraperitoneally with 10^5 L1210 cells on day 0 and were subsequently treated i.v. on day 2. The %ILS (percent increase lifespan) was determined as the median survival time of treated animals/the median survival time of control animals. Average diameter of s-lipo BCNU and lipo-BCNU particles was 50 nm and 200 nm, respectively.
Significant difference ($*$ $P<0.01$) by Student's t test. All BCNU-treated groups showed significantly better survival than the vehicle(saline)-treated group ($P<0.01$).

Table III. Effect of Intermittent Treatments with BCNU, Lipo-BCNU, or s-Lipo BCNU on the Lifespan of L1210 Tumor-bearing Mice

Preparation	Drug dose (mg/kg)	Survival time (day)	Mean±SD (day)	%ILS	Survivors/tested on day 60
Saline	0	7, 8, 8, 8, 8, 8, 8, 8, 8, 8, 8	7.91±0.29		0/11
LM	0	8, 8, 8, 8, 8, 8, 8, 8, 8, 8, 9	8.09±0.29	2	0/11
LN	0	8, 8, 8, 8, 8, 8, 8, 8, 8, 8, 8	8±0	1	0/11
BCNU	10	9, 10, 10, 10, 10, 10, 10, 10, 11, 14, 15	10.8±1.80	36.5	0/11
Lipo-BCNU	10	12, 12, 12, 13, 13, 13, 14, 14, 14, 14	13.1±0.83	65.6	0/10
s-Lipo BCNU	10	11, 11, 12, 12, 13, 13, 13, 13, 14, 16	12.8±1.4	61.8	0/10
BCNU	20	13, 13, 13, 15, 16, 16, 16, 16, 18, 20	15.6±2.15	97.2	0/10
Lipo-BCNU	20	27, 27, 27, 28, 28, 28	>40.5±15.9	>412.0	4/10
s-Lipo BCNU	20	24, 25, 25, 28, 29, 29, 29, 29	>33.8±13.2	>327.3	2/10
BCNU	30	21, 25, 26, 27, 28, 29	>39.6±16.7	>400.6	4/10
Lipo-BCNU	30	35	>57.7±7.9	>626.9	9/10
s-Lipo BCNU	30	35	>57.5±7.9	>626.9	9/10

($**$) ($*$)

CDF_1 mice were inoculated intraperitoneally with 10^5 L1210 cells on day 0 and were subsequently treated i.v. on days 2, 9, and 16. The %ILS (percent increase lifespan) was determined as the median survival time of treated animals/the median survival time of control animals. Average diameters of s-lipo BCNU and lipo-BCNU were 50 nm and 200 nm, respectively.
Significant difference ($*$ $P<0.05$, $**$ $P<0.01$) by Student's t test. All BCNU-treated groups had a significantly better survival than controls.

implantation was attempted to increase the efficacy while reducing the toxicity (Table III). Treatment with BCNU alone significantly prolonged the lifespan by 36.5% at a dose of 10 mg/kg, by 97.2% at 20 mg/kg, and by 400.6% at 30 mg/kg, indicating that the intermittent treatment was for superior to the consecutive treatment in experiment 1. In the high-dose group, 4 out of 10 mice survived longer than 60 days after tumor implantation and were regarded as cured.

Treatment with lipo-BCNU resulted in a significantly longer lifespan than the corresponding dose of BCNU alone. Treatment with lipo-BCNU at 10 mg/kg prolonged the lifespan by 65.6% as compared with the LM controls, while 20 mg/kg prolonged it by 412% and at 30 mg/kg prolonged it by 626.9%. In both the medium- and high-dose lipo-BCNU groups, 4/10 and 9/10 mice respectively survived for longer than 60 days. It is apparent from the survival rates in both lipo-BCNU groups that the lipo-BCNU had a significantly better antitumor activity against L1210 leukemia.

Treatment with s-lipo BCNU also resulted in a significantly longer lifespan than that of the corresponding BCNU-treated groups. Administration of s-lipo BCNU at 10 mg/kg prolonged the lifespan by 61.8%, while at 20 mg/kg it was 327.3% and at 30 mg/kg it was 626.9% longer. Two and 9 mice out of the 10 animals each in the medium- and high-dose groups receiving s-lipo BCNU survived longer than 60 days.

The 60-day survivors were killed, and the body and spleen weights were measured along with determination of the white blood cell (WBC) count and hemoglobin (Hb) concentration. In the BCNU-treated group, weight

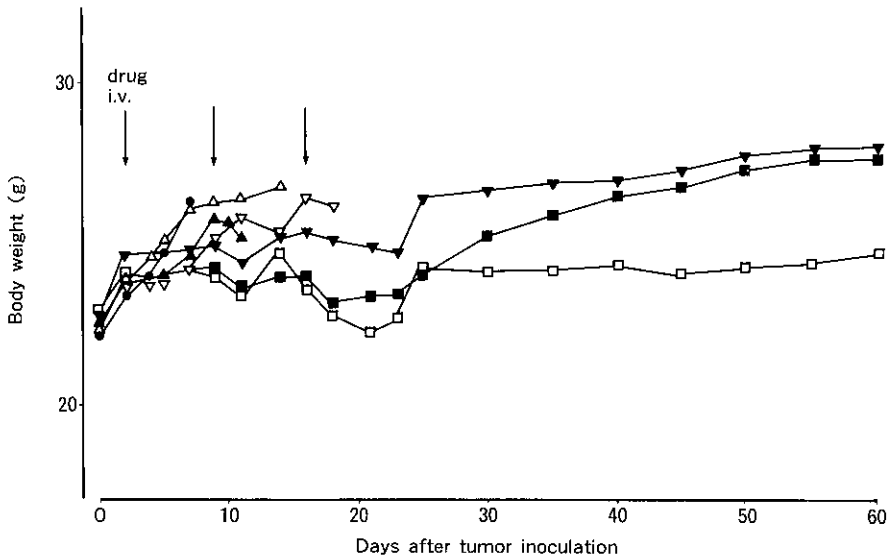


Fig. 2. Body weight of L1210 tumor-bearing mice. Values are the mean weight in grams of surviving mice. Control (●); BCNU (10 mg/kg △, 20 mg/kg ▽, 30 mg/kg □); lipo-BCNU (10 mg/kg ▲, 20 mg/kg ▼, 30 mg/kg ■).

Table IV. Body and Spleen Weight, WBC Count, and Hb Concentration in L1210-bearing Mice on Day 0 and Day 60

Group	Dose (mg/kg)	n	Body weight (g)	Spleen weight (mg)	WBC count ($\times 10^2/\mu l$)	Hb concentration (g/dl)
Day 0						
L1210-bearing mice	—	123	22.6±0.47	—	69.4±9.12	16.5±1.21
Survivors on day 60						
BCNU	30	4	24.8±0.34	124.2±55	227.5±187.2	14.4±2.3
lipo-BCNU	20	4	28.1±1.22	74.2±12.6	82.8±17.6	16.4±0.1
	30	9	27.8±1.15	78.3±10.2	71.4±11.8	16.4±0.69
s-lipo BCNU	20	2	28.9	76.0	68.5	16.6
	30	9	28.1±1.0	81.1±6.7	68.6±4.2	16.9±0.51
14-week-old normal mice	—	5	28.4±0.23	71.2±3.96	57±17.5	17.1±0.74

Values are the mean±SD.

gain was retarded and the minimun was reached on day 20 (Fig. 2). The mean body weight of survivors on day 60 was 24.8 g (Table IV), while that of survivors treated with lipo-BCNU or s-lipo BCNU was 27.8 and 28.1 g, respectively. The ratio of spleen weight to body weight and the WBC count of BCNU-treated survivors were higher than those of animals treated with lipo-BCNU or s-lipo BCNU, while the Hb concentration was lower. In addition, the mean body weight of normal 14-week-old mice was 28.4±0.23 g, while the spleen weight was 71.2 ±3.96 mg, the WBC count was 57±17.5/μl, and the Hb concentration was 17.1±0.74 g/dl (n=5, mean±SD). These values were similar to those of lipo- or s-lipo BCNU-treated survivors.

Toxicity In order to assess the toxicity of lipo-BCNU, intermittent intravenous injection was performed in normal mice according to the same schedule as in experiment 3 (Table V). Three doses of 10 or 30 mg/kg of lipo-BCNU had little effect over 60 days, despite transient weight loss after drug treatment. However, mice given 60 mg/kg all died within 14 days after showing marked weight loss. After treatment at 90 mg/kg, 7 mice died within 7 days, and the last one died on day 8.

As compared to lipo-BCNU, BCNU appeared to be somewhat less toxic. With treatment at 60 mg/kg, 4 out of 8 mice died within 14 days, and the others died on days 15, 16 and 17. After treatment at 90 mg/kg, 4 mice died by day 7, but the others remained alive until days 9 and

Table V. Toxicity of BCNU and Lipo-BCNU in Normal Mice

Preparation	Drug dose (mg/kg)	Total dose (mg/kg)	Survival time (day)		BWD (%)				
			60 days	Mean±SD	Day 3	7	14	26	60
Saline	0	–	8/8	60>	+1.7	+6.5	+9.5	+15.2	+29.0
BCNU	10	30	8/8	60>	−0.9	+4.0	+8.5	+13.7	+27.0
	30	90	8/8	60>	−4.3	−2.6	−6.0	−5.6	+15.8
	60	*a*)	0/8	14.6±1.32	−7.2	−21.1	−40.8	–	–
	90	*b*)	0/8	7.63±2.0	−8.1	−27.3	–	–	–
Lipo-BCNU	10	30	8/8	60>	+0.9	+5.1	+7.3	+13.4	+24.7
	30	90	8/8	60>	−3.1	+2.2	−3.5	−3.5	+19.6
	60	*c*)	0/8	13.1±1.27	−11.4	−26.5	−27.7	–	–
	90	*d*)	0/8	6.6±0.99	−13.4	−26.8	–	–	–

Normal CDF_1 mice were treated intravenously with drugs three times (days 0,7, and 14). BWD (body weight differences) are expressed as %.
a) 180 mg/kg (n=4), others 120 mg/kg. *b*) 120 mg/kg. *c*) 90 mg/kg (n=4), others 180 mg/kg. *d*) 90 mg/kg (n=7), other 180 mg/kg.

10. After a single dose of BCNU, mice given 90 mg/kg died on days 5, 6, 7, 10, and 14 (7.83±3.24 days, mean ±SD). The LD_{50} values of lipo-BCNU and BCNU were calculated by Behrens' method, and were 71 mg/kg and 73.5 mg/kg, respectively.

DISCUSSION

The present study has shown that lipo-BCNU not only has enhanced activity against L1210 leukemia in mice but also has reduced toxicity, and its potency was much greater than that of the equivalent dose of BCNU alone.

Optimal therapy was achieved by intermittent treatment with lipo-BCNU. Besides increasing the lifespan, the number of 60-day survivors was significantly greater in the lipo-BCNU-treated groups of mice. Also, the body weight of the survivors recovered faster in the lipo-BCNU-treated groups than in the BCNU-treated groups, which showed splenomegaly, an increased WBC count, and a decreased Hb concentration. Splenomegaly might have reflected the recurrence of leukemia in the survivors, since this often induces splenomegaly. In contrast, the weight gain and spleen/body weight ratio were within the normal ranges in lipo-BCNU-treated survivors. All of these findings indicate that intermittent administration of lipo-BCNU was superior for the treatment of L1210 leukemia in mice.

This improvement may be explained partly by the modification of BCNU distribution in the body, which was induced by the LM drug carrier. Previously, we have shown that LM accumulate mainly in inflammed tissues and vascular lesions *in vivo*.[16-18] Tumors always have a high energy supply, and would actively take up LM particles.[19-21] The fusion of LM to lipoprotein particles may take place *in vivo*. Also, we have already demonstrated that LM can be easily taken up by tumor cells *in vitro*.[22] It is well known that an oil such as lipiodol is retained selectively in tumor tissues.[23] Accordingly, this improved therapeutic efficacy would be obtained by an alteration of the distribution and longer retention of BCNU in tumor sites.

The toxicity of lipo-BCNU appeared to be the same as that of BCNU in normal mice. This may also be because LM-encapsulated BCNU is likely to be retained and have a prolonged clearance. In tumor-bearing mice, LM-encapsulated BCNU may be retained efficiently in tumor compartment.

The tissue distribution of liposomes is considered to be influenced by vesicle size and lipid composition.[6, 15] The BCNU-containing LN with an average diameter of 50 nm tended to show higher antitumor activity *in vivo*. However, there was no significant improvement. In our preliminary experiment, it was observed that reducing the size of LM decreased *in vitro* incorporation by tumor cells when compared to that of standard LM, although accumulation in tumor sites *in vivo* was satisfactory. These findings would account for the present results.

A number of reports have shown that the lipid components can also influence the antitumor activity or can exhibit such activity themselves.[24-29] Plant phosphatidylinositol (PI) is one such component.[29] However, the antitumor activity of lipo-BCNU prepared using plant PI instead of egg lecithin was almost the same as that of standard lipo-BCNU (data not shown). Thus, plant PI might only be active against certain types of tumors. However, LM prepared from other glyceryl ether phospholipids or eicosapentadecanoic acid, which has been reported[28] to have antitumor activity, may be worth investigating for their targeting ability and/or antitumor activity.

In this study, we were able to get stable lipo-BCNU or s-lipo BCNU, because of its lipophilicity. BCNU has been reported to decompose at relatively rapid rates under physiological conditions without enzymatic assistance.[30] Actually, BCNU solution in which BCNU was no longer detectable by HPLC exhibited no antitumor activity. Our present study has demonstrated that LM containing BCNU are stable at 4°C. This may be partly because lecithin surrounds and protects the oil phase containing BCNU, since it was confirmed that BCNU remained intact in soybean oil at more than 37°C.

Indeed, lipo-BCNU seemed to leak out from LM, and be destroyed in the aqueous phase under physiological conditions. We have already shown[31] that the amount of a drug (PGE_1) retained in the LM after incubation with human serum was 0.5%. In spite of that, lipo-PGE_1 is well known to show dramatic potency, particularly in clinical use.[32] After intravenous administration, it would not require a long time for BCNU in the LM to be transferred to the tumor sites.

In summary, our present study has shown that lipo-BCNU has greater efficacy than BCNU alone. Liposome-encapsulated BCNU has already been reported to exhibit higher antitumor activity against L1210 leukemia and Ehrlich ascites.[33] As compared to liposomes, LM are superior in terms of stability and safety. Therefore, LM (including LN) could well serve as useful carriers for other lipophilic agents besides BCNU.

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