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Reduction of Lethal Toxicity of Chloroethylnitrosoureas by Sugar Alcohols Without Loss of Antitumor Activity

Tazuko Tashiro, Makoto Inaba, and Yoshio Sakurai

Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 1-37-1, Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan

Summary. *Sugar alcohols, such as mannitol, sorbitol, galactitol, and inositol, selectively reduced the acute lethal toxicity of 1-(2-chloroethyl)-3-(methyl α-D-glucopyranos-6-yl)-1-nitrosourea (MCNU) without reducing its antitumor activity. Fifty mg MCNU/kg killed all $CD2F_1$ mice within about 10 days, while the administration of 3,000 mg sugar alcohols/kg immediately prior to MCNU protected mice from the lethal toxicity and all survived. The amelioration of MCNU toxicity by sugar alcohols was dose-dependent. Pretreatment with mannitol 1 day before MCNU administration was effective. In addition, a series of five daily treatments with lower doses of mannitol was also effective. This protection was accompanied by the reduction of both body weight loss and myelosuppression. The antitumor effects of MCNU on P388 leukemia and Lewis lung carcinoma were not significantly altered by mannitol treatment. These phenomena were not limited to MCNU, the lethal toxicity of GANU, ACNU, Me-CCNU, and mitomycin C also being reduced by mannitol treatment.*

Introduction

Various efforts have been made to enhance the selective toxicity of antitumor agents against tumor cells. One method is to use antitumor agents in combination with agents that do not themselves show antitumor activity. Recently, Yuhas et al. succeeded in reducing the host toxicity of nitrogen mustard, cyclophosphamide and *cis*-dichlorodiammine platinum(II) (PDD) with S-2(3-aminopropylamino)ethyl-phosphorothioic acid (WR-2721) without any alteration of their antitumor activity [5, 6]. Pera et al. also reported that IV infusion of an osmotic diuretic, mannitol, was effective in ameliorating not only the nephrotoxicity, but also the lethal toxicity, of PDD, thereby improving the therapeutic index of the drug [3, 4]. PDD is known to bind the bases of nucleic acid and exert its cytotoxic action in a manner similar to alkylating agents. Thus, we were highly interested to find whether mannitol and analogous sugar alcohols would reduce the host toxicity of alkylating agents as well as that of PDD.

In this paper, we describe the effects of several sugar alcohols, including mannitol, on the lethal toxicity of antitumor agents other than PDD in relation to their antitumor activity. In particular, with a new antitumor nitrosourea, 1-(2-chloroethyl)-3-(methyl α-D-glucopyranos-6-yl)-1-nitrosourea (MCNU), the dose- and schedule-dependency of sugar alcohols and the effects of mannitol on the myelosuppression induced by MCNU were studied in detail.

Materials and Methods

Mice. Female BALB/c × DBA/2 ($CD2F_1$) mice at about 10 weeks of age were used throughout the toxicity experiment. Female $CD2F_1$ and $B6D2F_1$ (C57BL/6 × DBA/2) mice were used for the experiment on antitumor activity. They were purchased from Charles River Japan, Inc. (Atsugi, Japan) and fed with CLEA CE-2 (Clea Japan, Inc., Tokyo, Japan) and water ad libitum.

Tumors. P388 leukemia and Lewis lung carcinoma were provided from the National Cancer Institute, NIH, USA and maintained by serial transplantation into DBA/2 and C57BL/6 mice, respectively.

Antitumor Agents. MCNU was kindly supplied by Tokyo Tanabe Co., Tokyo, Japan. Other antitumor agents used in the present study were 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea (GANU), 3-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride (ACNU); 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea

Reprint requests should be addressed to T. Tashiro

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(Me-CCNU), mitomycin C, and cyclophosphamide. GANU was provided by Meiji Seika Kaisha, Tokyo, Japan, ACNU by Sankyo Co., Tokyo, Japan, Me-CCNU and mitomycin C by Kyowa Hakko Kogyo Co., Tokyo, Japan, and cyclophosphamide by Shionogi & Co., Osaka, Japan. These drugs were dissolved in 0.9% NaCl solution and injected IP in a volume equal to 0.2 ml/20 g body weight. To dissolve Me-CCNU, one drop of Tween 80 was added.

Sugar Alcohols. Mannitol was purchased from Koso Chemical Co., Tokyo, Japan, sorbitol and galactitol from Tokyo Kasei Kogyo Co., Tokyo, Japan, and inositol from Wako Pure Chemicals Ind., Osaka, Japan. All of them were dissolved in distilled water and administered in a volume equal to 0.5 ml/mouse.

Acute Lethal Toxicity Assay. A lethal dose (50 mg/kg) of MCNU was given IP to groups of six mice. Just (1 min or less) before the MCNU administration, sugar alcohols (mannitol, sorbitol, galactitol, and inositol) were given IP or PO. The control group received no sugar alcohol treatment. All mice were weighed daily for 2 weeks and the mortality rate of the mice was observed for 30 days. To study the schedule-dependency of the effects of sugar alcohols, mannitol or sorbitol was administered once IP the day before or within 5 days after the MCNU treatment. In some experiments, lower doses of sugar alcohols were administered daily for 5 days, starting immediately before the MCNU treatment.

Myelosuppression Assay. $CD2F_1$ mice were given MCNU (6.25–50 mg/kg) IP alone or in combination with 3,000 mg mannitol/kg 1 min or less before MCNU administration. At 2–6 days after MCNU treatment, five mice from each group were sacrificed by decapitation. One femur was removed from each mouse and the marrow cells were washed out into a test tube with 2 ml Hanks' balanced saline. A portion of the cell suspension was stained by Turk's solution and all nucleated cells were counted by means of a hemocytometer. The degree of myelosuppression was expressed as the percentage of all nucleated cells in a femur from an MCNU-treated mouse compared with those from a control animal.

In vitro Culture of P388 Cells. The culture medium used in this experiment was RPMI 1640 (Grand Island Biological Co., Grand Island, NY, USA), supplemented with 5% heat-inactivated fetal bovine serum (Microbiological Associates Inc., Bethesda, Md, USA), 5 μM 2-hydroxyethyldisulfide (Aldrich Chemical Co., Inc., Milwaukee, Wis., USA) and kanamycin (100 μg/ml) (Meiji Seika Kaisha, Ltd, Tokyo, Japan). Fifty thousand P388 ascites cells were suspended in 1 ml culture medium and incubated with varying concentrations of MCNU in the presence or absence of mannitol in a CO_2 incubator at 37° C for 48 h. The number of P388 cells was then counted by means of a Coulter counter (model ZBI) and the percentage of cells in MCNU-treated compared with that in control tubes was calculated.

In vivo Antitumor Activity. Groups of six $CD2F_1$ mice were inoculated with 10^6 P388 cells IP on day 0. On day 1, several doses of MCNU were administered IP. Mannitol was given IP once (3,000 mg/kg) on day 1 or five times on days 1–5 (500 mg/kg/day). The percentage increase in the lifespan of each treated group against the control group was calculated. With Lewis lung carcinoma, 5×10^5 viable cells were transplanted SC in an axillary region to groups of six $B6D2F_1$ mice. Seven days after the transplantation, MCNU was injected IP with or without 3,000 mg mannitol/kg immediately before. Long (a) and short (b) diameters were measured twice a week thereafter, and the size of each tumor was expressed as $1/2a \times b^2$.

Table 1. Effects of sugar alcohols on lethal toxicity of MCNU[a]

Sugar alcohol[b]	Dose (mg/kg)	30-Day survivors	
		IP	PO
Mannitol	3,000	6/6	2/6
Sorbitol	3,000	6/6	2/6
Galactitol	3,000	6/6	2/6
Inositol	3,000	6/6	3/6
–	–	0/6	0/6

[a] Female $CD2F_1$ mice received 50 mg MCNU/kg IP
[b] Sugar alcohol was administered IP or PO 1 min or less before MCNU

Table 2. Dose response of protective effects of sugar alcohols on lethal toxicity of MCNU[a]

Dose[b] (mg/kg)	30-Day survivors	
	Mannitol	Sorbitol
3,000	6/6	6/6
1,000	5/6	3/6
500	4/6	2/6
300	3/6	2/6
0	0/6	0/6

[a] Female $CD2F_1$ mice received 50 mg MCNU/kg IP
[b] Sugar alcohol was administered IP 1 min or less before MCNU

Results

Effects of Sugar Alcohols on Acute Lethal Toxicity of MCNU

Female $CD2F_1$ mice given 50 mg MCNU/kg IP died from toxicity 7–10 days after treatment. Table 1 shows the effects of sugar alcohols (3,000 mg/kg) administered 1 min or less before MCNU on its lethal toxicity. No mouse given 3,000 mg sugar alcohols/kg IP in combination with MCNU had died more than 30 days afterwards, and all of them were healthy. Oral administration of sugar alcohols also reduced the lethal toxicity of MCNU, but only 33%–50% of the mice survived for over 30 days. Mannitol alone, of course, exerted no apparent change on the mice.

The dose response of the protective effects of sugar alcohols on the lethal toxicity of MCNU is shown in Table 2. Even 300 mg mannitol/kg exhibited the protective effect, though 30-day survival was recorded in only three of the six mice. With 1,000 mg sorbitol/kg only 50% of the mice survived 30 days. At lower doses mannitol was more effective than sorbitol.

The schedule-dependency of the protective effect of mannitol on the lethal toxicity of MCNU is shown

Table 3. Schedule dependency of protective effects of sugar alcohols on lethal toxicity of MCNU[a]

Sugar alcohol[b]		30-Day survivors	
Dose (mg/kg)	Schedule	Mannitol	Sorbitol
3,000	Day − 1	4/6	
	Day 0	6/6	
	Day + 1	0/6	
	Day + 2	0/6	
	Day + 3	0/6	
	Day + 4	1/6	
	Day + 5	0/6	
1,000	Days 0–4	5/6	4/6
500	Days 0–4	6/6	3/6
250	Days 0–4	6/6	
100	Days 0–4	2/6	
0	Days 0–4	0/6	0/6

[a] Female $CD2F_1$ mice received 50 mg MCNU/kg IP on day 0
[b] Sugar alcohols were administration IP on one or five occasions, as indicated

in Table 3. When administered 1 day before MCNU treatment, mannitol had a similar effect to when it was given simultaneously, and four of six mice survived. However, no effect was observed as a result of treatment with mannitol once between 1 and 5 days after MCNU administration. In contrast, five daily treatments with lower doses of mannitol, starting immediately before and ending 4 days after MCNU administration, protected mice from the lethal toxicity and, even with 250 mg/kg/day, all mice survived 30 days. Sorbitol showed less effect.

Effects of MCNU Alone or With Mannitol on Body Weight of Mice

As shown in Fig. 1, mice receiving 50 mg MCNU/kg alone markedly lost weight until death. Although mice administered 3,000 mg mannitol/kg immediately before MCNU also lost weight for 5 days, recovery was observed thereafter, although the rate was relatively slow. Groups receiving mannitol once 1 day before (3,000 mg/kg) or daily (500 mg/kg/day) on days 0–4 with MCNU showed a similar pattern of weight loss and recovery. Mannitol administered on the day after MCNU had no effect on body weight loss.

Effects of Mannitol on Myelosuppression Induced by MCNU

MCNU alone induced intensive myelosuppression on female $CD2F_1$ mice. By day 3 bone marrow nucleated

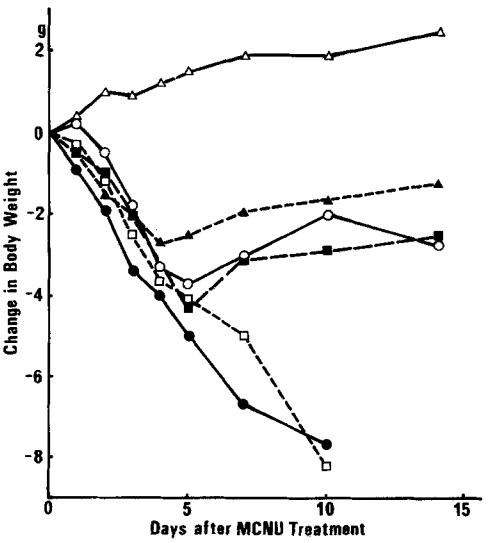

Fig. 1. Effects of MCNU alone or with mannitol on change in body weight of $CD2F_1$ mice. MCNU (50 mg/kg) was given IP on day O. △——△, untreated control; (●——●, ○——○, ■– – –■, □– – –□) MCNU alone; MCNU with mannitol (3,000 mg/kg) on day 0, day −1, or day 1, respectively; ▲- - - -▲, MCNU with mannitol (500 mg/kg × 5) on days 0–4

cells in the femur were reduced to less than 10% of the control level with 50, 35, or 25 mg MCNU/kg, as illustrated in Fig. 2a–c. The mice given mannitol 1 min prior to MCNU exhibited a similar reduction in femur nucleated cells by day 2 to the mice given MCNU alone, while on day 3 a statistically significant difference was observed between the two groups, the mannitol-treated groups showing somewhat higher nadir values and a faster recovery from myelosuppression. On day 6 groups receiving 35 and 25 mg MCNU/kg recovered to the control level. At lower doses of MCNU, milder myelosuppression (Fig. 2d and e) and earlier recovery were observed in the mannitol-treated groups than in those given MCNU alone.

Effects of Mannitol Treatment on Antitumor Activity of MCNU

To ascertain whether the amelioration of MCNU toxicity with mannitol was due to the inactivation of MCNU or not, the following in vitro and in vivo experiments were carried out. Figure 3 shows the growth inhibition curves of MCNU against primary-cultured P388 cells in the presence or absence of 10 mg mannitol/ml. In the combination with mannitol, no change was observed in the growth inhibition curve of MCNU on P388, indicating that mannitol might not inactivate MCNU. In addition, the therapeutic efficacy of MCNU on P388-bearing mice was

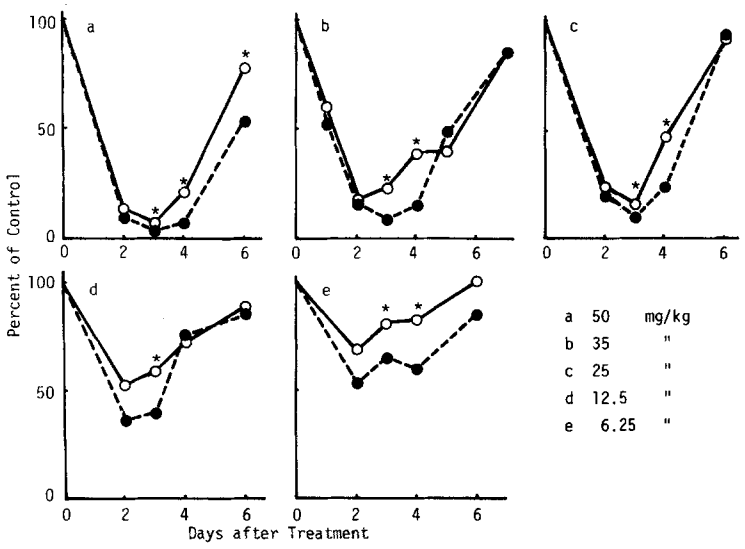

Fig. 2a–e. Effects of mannitol on myelosuppression induced by MCNU. Female $CD2F_1$ mice were given MCNU IP at the doses indicated. Mannitol (3,000 mg/kg) was administered IP 1 min or less before MCNU. (●-----●), MCNU alone; (○———○) MCNU with mannitol.*, significant at $P < 0.05$ compared with the group given MCNU alone

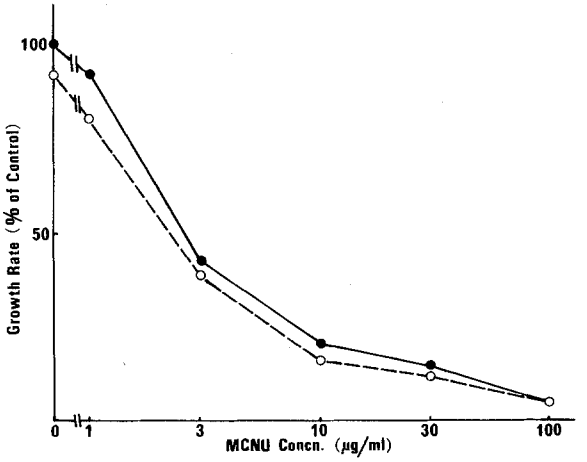

Fig. 3. Growth-inhibitory effects of MCNU on cultured P388 cells in the absence or presence of mannitol. P388 cells (5×10^4 cells/ml) were incubated with varying concentrations of MCNU in the presence (●———●) or absence (○-----○) of mannitol (10 mg/ml) at 37° C for 48 h

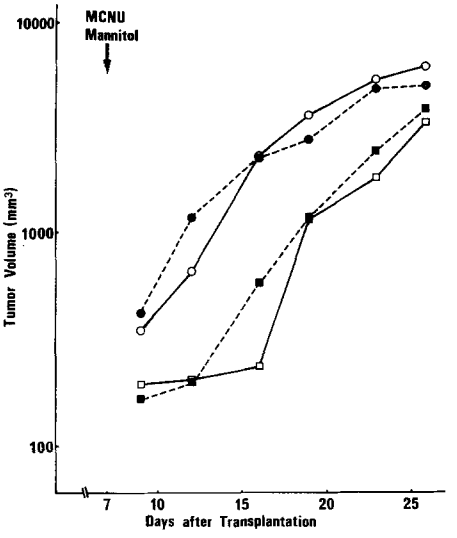

Fig. 4. Effects of mannitol on antitumor activity of MCNU against Lewis lung carcinoma. Lewis lung Ca cells (5×10^5 viable cells) were implanted SC on day 0. MCNU (20 mg/kg) and mannitol (3,000 mg/kg) were given IP on day 7. (○———○) control; (●-----●) mannitol; (□———□) MCNU; (■-----■) MCNU with mannitol

Table 4. Effects of mannitol on antitumor activity of MCNU against P388 leukemia[a]

MCNU[b] (mg/kg)	% ILS[c]		
	Alone	Mannitol[d] (mg/kg)	
		3,000	500 × 5
1	44	55	39
2.5	68	62	68
5	118	125	88

[a] $CD2F_1$ mice (six mice/group) were inoculated with 10^6 P388 cells IP on day 0

[b] MCNU was injected IP on day 1

[c] The percent increase in lifespan was calculated by the formula: (1−T/C) × 100, where T and C are the median survival in days of the treated and the control groups, respectively

[d] Mannitol was given IP once on day 1 (3,000 mg/kg) or five times, on days 1–5 (500 mg/kg/day)

not affected by the mannitol treatment either. As shown in Table 4, the increase in lifespan with 1 mg MCNU/kg, which was the minimum effective dose, was 44%, while when a single dose of 3,000 mg and five daily doses of 500 mg mannitol/kg were co-administered with MCNU the corresponding values were 55% and 39%, respectively.

Figure 4 demonstrates the growth delay of SC-transplanted Lewis lung carcinoma as a result of MCNU alone or in combination with mannitol. At 20 mg MCNU/kg tumor growth delay was observed. Mannitol treatment had no significant effect on the growth inhibition of this tumor with MCNU.

Table 5. Effects of mannitol on lethal toxicity of various antitumor agents

Antitumor agent[a]	Dose (mg/kg)	30-Day survivors	
		Alone	Mannitol[b]
MCNU	50	0/6	6/6[c]
GANU	50	0/6	6/6[c]
ACNU	60	0/6	4/6[d]
Me-CCNU	80	0/6	6/6[c]
Mitomycin C	8	1/6	4/6[d]
Cyclophosphamide	500	1/6	2/6

[a] Antitumor agents were administered IP into $CD2F_1$ mice (six mice/group)

[b] 3,000 mg mannitol/kg was administered IP 1 min or less before antitumor agents

[c,d] Significant at $P < 0.01$ (c) or $P < 0.05$ (d) by the Fisher exact test, compared with the group without mannitol

Effects of Mannitol on Lethal Toxicity of Other Antitumor Agents

To examine whether the reduction of host toxicity by mannitol was limited to MCNU, the lethal toxicity of several antitumor agents against $CD2F_1$ mice was tested with or without mannitol treatment. Table 5 shows that mannitol also protected mice from the lethal toxicity of several antitumor nitrosoureas, such as GANU, ACNU, and Me-CCNU, and of mitomycin C.

Discussion

The data presented above indicate that sugar alcohols, such as mannitol, sorbitol, galactitol, and inositol, possess activity to protect mice from acute lethal toxicity of antitumor agents. With a higher dose of sugar alcohols (3,000 mg/kg), a single IP administration 1 min or less before MCNU resulted in complete protection of the mice. The effects of sugar alcohols were dose-dependent and the protection with lower doses was incomplete.

Pera et al. [3, 4] succeeded in reducing the nephrotoxicity and lethal toxicity of PDD by mannitol with a 30-min IV infusion, but we found that an IP bolus injection or even oral administration of mannitol also had similar effects. Furthermore, it was demonstrated that divided treatment with lower doses of sugar alcohol over 5 days was as effective in ameliorating MCNU toxicity as a single higher dose.

These phenomena were accompanied by reduced body weight loss and myelosuppression, as shown in Figs. 1 and 2. During the first 5 days after MCNU administration all treated groups lost body weight, but thereafter only the protected groups regained weight, the rest continuing to lose weight until death.

As for myelosuppression, reductions in bone marrow nucleated cells were observed at all doses of MCNU tested and the degree of this phenomenon was dose-dependent. The nadir of cell numbers was observed 2 or 3 days after MCNU administration. Mannitol treatment (3,000 mg/kg) alleviated the suppression to some degree and therefore resulted in earlier recovery. At 35 mg MCNU/kg alone, which was almost the maximum tolerated dose in $CD2F_1$ mice, the cells began to increase 5 days after treatment and within a week they attained the control level. Even at a lethal dose of 50 mg/kg, recovery to 50% of the control value was observed. Thus, myelosuppression alone seems not to be the direct cause of death, but some damage subsequently induced by myelosuppression might be. Intestinal and/or renal toxicity of MCNU might also be alleviated with mannitol treatment.

From the standpoint of chemotherapy, it is very important that the protection of the host from the toxicity of MCNU does not decrease the antitumor activity. Howell et al. [1] reported that although sodium thiosulfate protected mice from the lethal damage induced by PDD, partial reduction of its antitumor activity was also observed. We examined in vitro and in vivo whether mannitol treatment in combination with MCNU would affect the antitumor activity or not. With P388 mouse leukemia, neither the growth inhibition curve of MCNU nor the percentage increase in the lifespan of tumor-bearing mice at the minimum effective dose of MCNU was altered by concomitant use of mannitol at all, as demonstrated in Fig. 3 and Table 4, respectively. These results indicate that mannitol does not inactivate the cytotoxic activity of MCNU.

In the experiment with the IP–IP system of P388, tumor cells were directly exposed to the drug. There is the other possibility that mannitol reduces tissue levels of MCNU by its diuretic action. If this is the case, mannitol treatment might result in the decrease of antitumor activity of MCNU when both drugs are given IP to mice bearing some solid tumors. Therefore the growth-inhibitory effect of the combined use of MCNU and mannitol on SC-implanted Lewis lung carcinoma was compared with that of MCNU alone. The results shown in Fig. 4 clearly demonstrate that mannitol does not also influence the antitumor activity of MCNU under such experimental conditions.

Furthermore, it is noteworthy that the protection of the host toxicity by sugar alcohols was not limited to MCNU, mannitol being effective in reducing the

host toxicity of several of the nitrosoureas described above and mitomycin C.

The mechanism by which sugar alcohols can selectively protect mice from the lethal toxicity of antitumor agents remains unknown. Pera et al. [4] reported that, with PDD, mannitol did not increase the excretion of PDD in urine, accelerate plasma clearance of platinum, or decrease renal levels of platinum. Thus, the protection observed with sugar alcohols might not be due to the rapid excretion of the excessive drug by their osmotic diuretic action. Panasci et al. [2] postulated that a nitrosourea, chlorozotocin, containing a glucose moiety in its structure might share the same cellular uptake mechanism into bone marrow cells as glucose, but they failed to show the amelioration of neutropenia and thrombocytopenia with combined chlorozotocin and glucose therapy. MCNU and GANU contain glucose moiety in their structure. In fact, in our experiment the reduction of lethal toxicity of other types of antitumor alkylating agents was also shown by mannitol, so it would be difficult to explain the mechanism by competitive inhibition of drug uptake on the common carrier protein.

It should be particularly noted that mannitol cannot prevent the initial toxic manifestation of MCNU, but plays an important role in recovery of the host damage induced by MCNU, as suggested by the results concerning changes in body weight and myelosuppression (Figs. 1 and 2).

In the present study, we demonstrated that sugar alcohols were able to protect mice from the lethal toxicity of antitumor agents, such as MCNU, while they did not reduce the antitumor activity against P388 leukemia and Lewis lung carcinoma at all. In the case of such alkylating agents, severe side-effects limit the clinical dosage. Even if the available dose could be slightly increased, enhancement of the therapeutic effects would surely be expected. Thus, concomitant use of sugar alcohols, such as mannitol, might be helpful in improving the therapeutic effectiveness of alkylating agents, including nitrosoureas, in clinical cancer chemotherapy.

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