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Antitumor activity of vincristine encapsulated in glucuronide-modified long-circulating liposomes in mice bearing Meth A sarcoma

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Abstract

Liposomes modified with the uronic acid derivative palmityl-D-glucuronide (PGlcUA) have a long-circulation time and tend to accumulate in the tumors of tumor-bearing mice. Taking advantage of this character, we investigated the therapeutic effect of vincristine (VCR) encapsulated in liposomes containing PGlcUA (dipalmitoylphosphatidylcholine/cholesterol/PGlcUA = 4:4:1 as a molar ratio) on tumor-bearing mice. VCR was loaded into liposomes by a remote loading method, and then free or liposomal VCR was injected intravenously into BALB/c mice bearing Meth A sarcoma implanted subcutaneously 5 days before hand. Single-dose administration of VCR (3.0 mg/kg) in PGlcUA-liposomes significantly suppressed tumor growth, and prolonged the survival time (T/C = 1.37). Furthermore, two-dose administration of the liposomes cured one third of the animals. The therapeutic effect of PGlcUA-liposomes was greater than that of control liposomes containing dipalmitoylphosphatidylglycerol instead of PGlcUA. PGlcUA- liposomes might thus be a useful tool for delivering antitumor agents to tumor tissues.

Keywords: Liposome; Long-circulating liposome; Vincristine; Cancer therapy

1. Introduction

Liposomes can be used as ideal drug carriers in the field of DDS, and many previous studies have demonstrated the enhanced efficacy of encapsulated drugs and the reduction of the side effects of drugs so entrapped [1–4]. They have been effectively used especially as carriers of antitumor drugs, since liposomes can encapsulate and deliver the large amount of drugs to the tumor tissues, and can reduce the side effect of the drugs which is usually severe problem especially for antitumor agents. Conventional liposomes which are not specifically modified for obtaining long-circulating character, however, have a limitation, since they tend to be trapped by reticuloendothelial system (RES). Many attempts have been made to avoid the RES-trapping of liposomes and to get the longer half-lives of liposomes in the bloodstream, by the modification of liposomal surface. Successful results were obtained by the modification of liposomes with monosialoganglioside GM1 [5,6] poly(ethylene glycol) (PEG) [7–10] and so on, although GM1 modification was revealed to be effective only in mice and not in rats [11] nor rabbits [12]. We previously reported that the modification of liposomes with a glucuronic acid derivative, palmityl-D-glucuronide (PGlcUA), resulted in liposomes with a longer circulation time in the bloodstream of rats and mice [13,14]. Furthermore, a remarkable accumulation of PGlcUA-liposomes in tumor tissues in tumor-bearing mice, maybe due to passive targeting, was observed [14,15]. In the present study, we investigated the therapeutic effect of VCR encapsulated in PGlcUA-liposomes by use of Meth A sarcoma-bearing mice.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were the products of Nippon Fine Chemical (Takasago, Hyogo, Japan). Choles-

Abbreviations: PGlcUA, palmityl-D-glucuronide; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; Chol, cholesterol; VCR, vincristine.

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terol (Chol), vincristine (VCR) and octylglucoside were obtained from Sigma (St. Louis, MO). [G-^{3}H]Vincristine sulphate was purchased from Amersham (Buckinghamshire, UK). Palmityl-D-glucuronide (β-hexadecyl-D-glucuronide, PGlcUA) was synthesized as described previously [13].

2.2. Liposomalization of VCR

Liposomes were prepared according to the method described previously [14]. In brief, DPPC and Chol with PGlcUA or DPPG (4:4:1 as a molar ratio) were dissolved in chloroform, dried under reduced pressure and stored in vacuo for at least 1 h. Resulting thin lipid film was hydrated with 0.3 M sodium citrate, pH 4.0. Then liposomes were subjected to freeze-thawing for three cycles using liquid nitrogen, and extruded through a polycarbonate membrane filter with a 100-nm pore size (Nucleopore, Costar, Cambridge, MA). The size distribution of liposomes was determined by dynamic light scattering with a Submicron Particle Analyzer (Nicomp Model 370) after preparing liposomes with the same procedures. Volume-weighted particle sizes of PGlcUA- and DPPG-liposomes were 123 and 117 nm with standard deviations of 45 and 36 nm, respectively, and number-weighted particle sizes of those were 69 and 77 nm with standard deviations of 21 and 28 nm, respectively. VCR-loading into liposomes were performed by a modification of the remote loading method developed by Mayer et al. [16]. Briefly, the pH outside of the liposomes was adjusted to 7.5 by the addition of sodium bicarbonate, and the liposomal solution was incubated with VCR (VCR/liposomal lipids = 0.2:1 as a weight ratio) for 1 h at 60°C. Encapsulation efficiency was more than 90% as determined by the absorbance at 297 nm in the presence of 0.7% octylglucoside after separation of free and liposomal VCR by centrifugation of the liposomal suspension ($100\,000 \times g$ for 5 min with Hitachi, CS120EX). Untrapped VCR was removed by centrifugation at $100\,000 \times g$ for 5 min. Liposomal pellet was resuspended in saline appropriately.

2.3. Assay of liposomal stability

VCR-encapsulated liposomes composed of DPPC, Chol, and PGlcUA or DPPG (4:4:1 as a molar ratio) were incubated in phosphate-buffered saline, pH 7.2, or in 90% fetal bovine serum (Hezleton Biologics, Lenexa, KS) at 37°C. At selected times, 400 μl of sample was removed and centrifuged at $100\,000 \times g$ for 5 min to pellet the liposomes. An aliquot of the supernatant was collected and its VCR content was determined photometrically.

2.4. Study on biodistribution of liposomal VCR

Meth A sarcoma grown in the ascites of BALB/c mice under an appropriate schedule was diluted with saline to obtain $5 \cdot 10^6$ cells/ml suspension. Then 0.2 ml of the suspension was carefully injected s.c. into the posterior flank of 5-week-old BALB/c male mice ($n = 5$). Liposomes encapsulating cold and a trace of ^{3}H-labeled VCR were prepared just prior to administration. Liposomal and free VCR (1.5 mg/kg as VCR, 74 kBq/mouse) were i.v. injected into Meth A sarcoma bearing-mice. Mice were killed with ether anesthesia for collection of blood 6 h after injection, and the radioactivity in the plasma was measured after centrifugation of the blood. The lungs, heart, liver, spleen, kidneys and tumor were then removed after bleeding, washed with saline and weighed. About 100 mg of each tissue, or the whole tissue when it weighed less than 100 mg, were minced and solubilized in 1 ml of Solvable (NEN Research Products, Boston, MA) overnight at 40°C. After treatment with hydrogen peroxide, a Hionic-Fluor (Packard Japan, Tokyo, Japan) scintillation cocktail was added and the radioactivity in each sample was determined in a liquid scintillation counter (Aloka, LSC-3500). The total blood volume (7.3% of body weight) and correction factors for the blood content of various tissues were determined as described previously by use of ^{51}Cr-labeled erythrocytes [17].

2.5. Therapeutic experiment and statistical analysis

Meth A sarcoma ($1 \cdot 10^6$ cells/0.2 ml) was carefully injected s.c. into the posterior flank of five-week-old BALB/c male mice ($n = 10$–12). Liposomes encapsulating VCR were prepared just prior to administration. Liposomes and free drugs were i.v. injected with single- or two-dose administration into Meth A sarcoma bearing-mice. Mouse weight and tumor volume were monitored every day after administration of drugs. Tumor volume was determined by measuring two bisecting diameters of each tumor with slide calipers, and calculated by the formula $0.4(a \times b^2)$ where 'a' is the largest and 'b' the smallest diameter. Tumor volume obtained by this measurement is known to correlate well with actual tumor weight ($r = 0.980$). Variance in a group was evaluated by the F-test, and differences in mean tumor volume were evaluated by Student's t-test. Differences were considered significant when the P value of comparison was less than 0.05.

3. Results

3.1. Stability of VCR-encapsulated liposomes

At first, we examined the stability of PGlcUA- and DPPG-liposomes containing VCR in the presence and absence of serum (Fig. 1). Both liposomes were quite stable in phosphate-buffered saline and in serum. These in

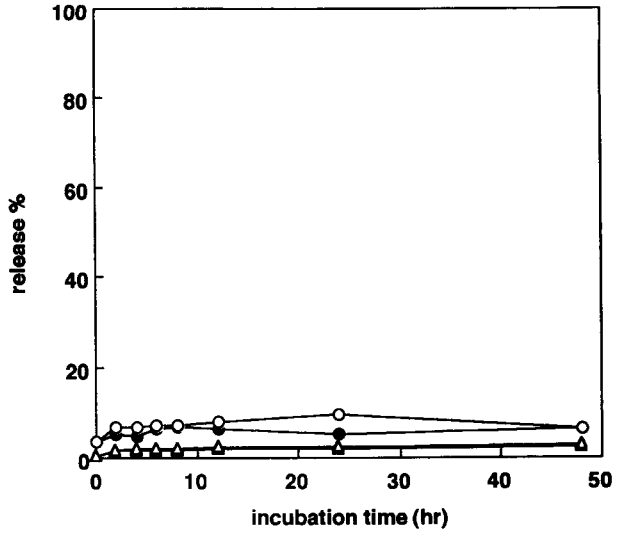

Fig. 1. VCR release from long-circulating liposomes in serum. VCR-encapsulated liposomes composed of DPPC/Chol/PGlcUA (4:4:1, closed symbols) or DPPC/Chol/DPPG (4:4:1, open symbols) were incubated in 90% serum (circles) or in phosphate-buffered saline (triangles) at 37°C for the indicated times. The released VCR was determined as described in Section 2.

vitro data suggest that VCR will not leak out during circulation.

3.2. Therapeutic effect of liposomal VCR after single-dose administration

Therapeutic effect was determined by the suppression of tumor growth and the increase in life time of tumor-bearing mice after treatment with free or liposomal VCR. The treatment was performed at day 5 after tumor implantation. The dose of 1.5 mg/kg was used for free VCR and 3 mg/kg dose (15 mg/kg as liposomal lipids) was for liposomal VCR, since 3 mg/kg free VCR was toxic to the animal whereas liposomal formulation could reduce the acute toxicity (data not shown). As shown in Fig. 2, administration of VCR-encapsulated PGlcUA-liposomes was most efficient for suppressing tumor growth. Survival time and T/C are presented in Table 1. PGlcUA-liposomes with encapsulated VCR were the most efficient for prolonging survival time of tumor bearing mice.

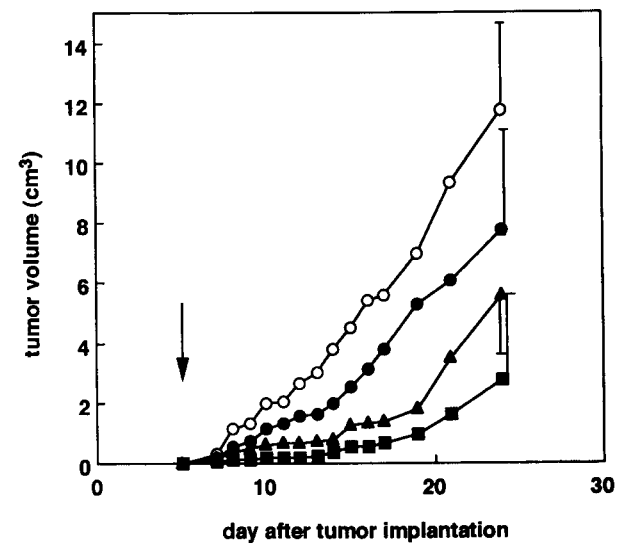

Fig. 2. Suppression of tumor growth by treatment with liposomal VCR. Meth A sarcoma ($1 \cdot 10^6$ cells/0.2 ml) was carefully implanted subcutaneously into the posterior flank of 5-week-old BALB/c male mice. These mice (ten per group) were injected i.v. with saline (○), 1.5 mg/kg free VCR (●), 3.0 mg/kg VCR encapsulated in DPPG-liposomes (▲), or 3.0 mg/kg VCR in PGlcUA-liposomes (■), at day 5. The tumor volume was determined at the indicated days as described in Section 2. S.D. bars are shown only for the last points for the sake of graphic clarity.

Table 1
Survival time of Meth A sarcoma-bearing mice treated with VCR-encapsulated long-circulating liposomes

Treatment	Mean survival (days ± S.D.)	T/C
Control	31.8 ± 2.4	1.00
Free VCR (1.5 mg/kg)	35.3 ± 6.1	1.11
DPPG-liposomes (3.0 mg/kg)	38.6 ± 1.1	1.21
PGlcUA-liposomes (3.0 mg/kg)	43.6 ± 4.3 *	1.37

* Significantly different from others.

3.3. Biodistribution of liposomal VCR in Meth-A-sarcoma-bearing mice

To examine the long-circulating activity of PGlcUA liposomes, the biodistribution of free and liposomal VCR 6 h after injection was determined by use of radioactive VCR. As shown in Fig. 3, the amounts of VCR remained in plasma and accumulated in tumor were extremely higher

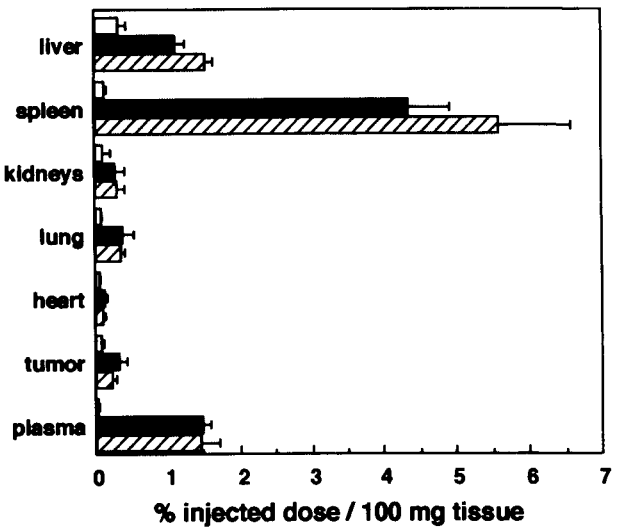

Fig. 3. Biodistribution of liposomal VCR in tumor-bearing mice 6 h after i.v. administration. Free [^{3}H]VCR (open bars), or liposomes encapsulating [^{3}H]VCR (PGlcUA-liposomes, closed bars: DPPG-liposomes, hatched bars) were injected into a tail vein in Meth-A-sarcoma-bearing mice (5 per group). The animals were killed under ether anesthesia 6 h after administration and the biodistribution of [^{3}H]VCR was determined as described in Section 2.

when both liposomal formulations were injected than those after the administration of free VCR. Furthermore, the higher radioactivity was detected in tumor tissues by use of PGlcUA-liposomes than by use of DPPG-liposomes, consistent with the previous observation for liposomal biodistribution [14].

3.4. Therapeutic effect of liposomal VCR after two-dose administration

Next we examined the therapeutic effect of liposomal VCR after two-dose administration at days 5 and 12 after tumor implantation. As shown in Fig. 4, two-dose administration was more efficient than single-dose administration especially by use of liposomal formulation. The result indicates that liposomal formulation is efficient for suppressing grown tumor whereas free drug is inefficient for suppressing grown one. Especially, VCR-encapsulated PGlcUA-liposomes which are long-circulating liposomes were efficient due to the high accumulation in grown tumor.

We also determined the side effect of VCR in liposomal formulation. Fig. 5 shows the change in body weight of mice, which is one of criteria of side effect, after administration of drugs. Obvious weight loss was observed in mice injected with 1.5 mg/kg free VCR, which is slightly lower than the LD_{50}, and with 3.0 mg/kg liposomal VCR. The toxicity of VCR was, however, reduced by the liposomal formulation, since none of the mice died after treatment with 3.0 mg/kg VCR by the liposomal formulation.

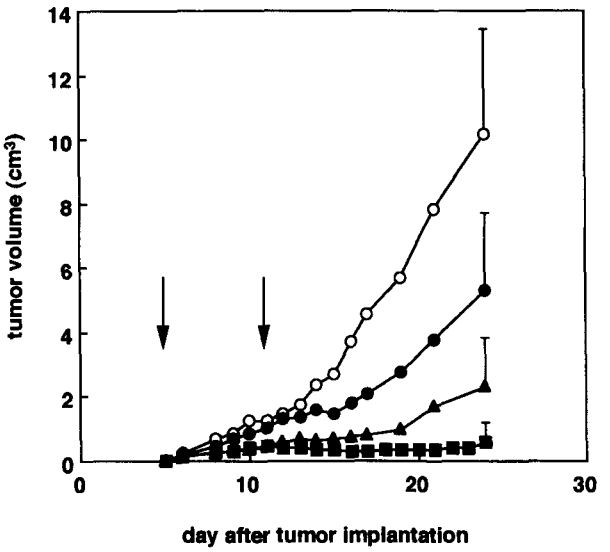

Fig. 4. Suppression of tumor growth by two-dose treatment with liposomal VCR. Meth A sarcoma implantation and tumor volume determination was performed as described in the legend of Fig. 2. These mice (12 per group except for control liposomes in which 10 mice were used) were injected i.v. with saline (●), 1.5 mg/kg free VCR (▲), 3.0 mg/kg VCR encapsulated in DPPG-liposomes (■), or 3.0 mg/kg VCR in PGlcUA-liposomes (■), at days 5 and 12 indicated by arrows. S.D. bars are shown only for the last points for the sake of graphic clarity.

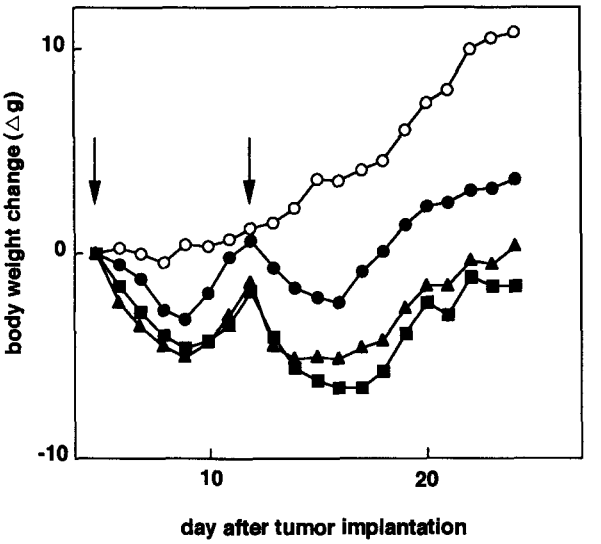

Fig. 5. Effect of liposomal VCR on the body weight of tumor-bearing mice. Meth A sarcoma-bearing mice were treated with saline (○), 1.5 mg/kg free VCR (●), 3.0 mg/kg VCR encapsulated in DPPG-liposomes (▲), or 3.0 mg/kg VCR in PGlcUA-liposomes (■), at days 5 and 12 as described in the legend of Fig. 4.

Fig. 6 shows the survival time of treated animals. PGlcUA-liposomes encapsulating VCR were the most efficient for prolonging survival time of tumor bearing mice, and 33% of the animals were cured. These mice were apparently tumor free and remained alive throughout the experimental period (more than 180 days). Mean survival of the other 67% of the VCR-PGlcUA-liposome-treated animals was 50.9 ± 17.0 days, which was 1.5-fold longer than the survival of the saline treatment group (33.7 ± 3.2 days).

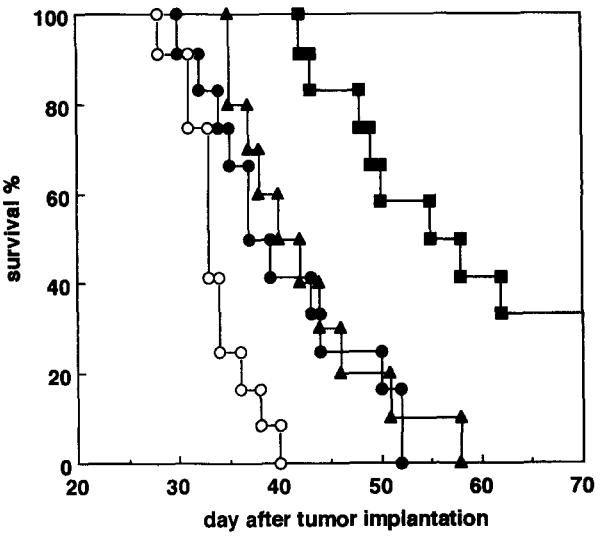

Fig. 6. The survival of Meth A-bearing mice by treatment with VCR encapsulated in long-circulating liposomes. Mice implanted s.c. with Meth A sarcoma ($1 \cdot 10^6$ cells) were injected i.v. on days 5 and 12 as described in the legend of Fig. 4 with saline (○), 1.5 mg/kg free VCR (●), 3.0 mg/kg VCR encapsulated in DPPG-liposomes (▲), or 3.0 mg/kg VCR in PGlcUA-liposomes (■). The mean survival time (days) was 33.7 ± 3.2, 40.4 ± 7.7, 42.6 ± 7.4, and 50.9 ± 7.0, respectively.

4. Discussion

Liposomalization of antitumor drugs has been shown to reduce the side effect of the drugs and to enhance their therapeutic efficacy [1–4]. Since conventional liposomes tend to be trapped in the reticuloendothelial system (RES), avoidance of the RES-trapping in order to prolong the circulation time of liposomes has been attempted. For this purpose, we synthesized a glucuronate derivative, palmityl-D-glucuronide (PGlcUA), that is rather easily prepared and readily incorporated into the liposomal bilayer [13]. These long-circulating liposomes showed further advantage for tumor therapy; they accumulate passively in tumor tissues, since the vasculature in the tumor tissues is leaky enough to extravasate small-sized liposomes.

This passive-targeting character of long-circulating liposomes to tumor tissues might be useful for tumor imaging [15] and for cancer therapy, since there is a positive correlation between the circulation time of liposomes and their accumulation into tumor tissues. In fact, it was demonstrated that anthracyclines, such as doxorubicin, encapsulated in long-circulating liposomes were selectively delivered to the tumor tissues than free drugs. Therefore, a significant enhancement of the antitumor activity was achieved by such long-circulating liposomes [18–20] including PGlcUA-liposomes [17]. Furthermore, PEG-coated long-circulating liposomes containing doxorubicin were effective in suppressing spontaneous metastasis [21,22].

VCR was also encapsulated into PEG-coated long-circulating liposomes which showed a marked therapeutic efficacy on mammary carcinoma-bearing mice [23]. Similarly, GM1-modified liposomes were revealed to be effective against P388 tumors [24]. In the present study shows the obvious therapeutic effect of VCR encapsulated in PGlcUA-liposomes on tumor-bearing mice. Especially when liposomal VCR was administered twice at days 5 and 12, a significant therapeutic effect of VCR was obviously observed with the long-circulating liposomes than in the case of free-VCR treatment or treatment with VCR encapsulated in conventional liposomes. These findings suggest that the efficacy of VCR in long-circulating liposomes comes from the tumor accumulation of the liposomes. Furthermore, the reduction of toxic effect of VCR in terms of liposomal formulation allowed us to use higher doses of the drugs to be given. This also may cause significant therapeutic efficacy by liposomal formulation.

In conclusion, VCR encapsulated in PGlcUA-liposomes showed enhanced therapeutic efficacy in tumor-bearing mice in comparison with the free VCR or VCR encapsulated in conventional liposomes. These effect might be caused by the intense accumulation of long-circulating PGlcUA-liposomes in tumor tissues. Therefore, the long-circulating liposomes presented here may be practically useful for delivering anticancer drugs.

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