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Preparation and pharmacokinetics of pirarubicin loaded dehydration–rehydration vesicles

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Abstract

Liposomes entrapped pirarubicin (THP, L-THP) were prepared by the modified dehydration–rehydration vesicle (DRV) method, and their pharmacokinetics and antitumor effects were evaluated in mice bearing M5076 liver metastasis tumor. After small unilamellar vesicles (SUVs) composed of egg lecithin, cholesterol (Ch), β-sitosterol β-D-glucoside (Sit-G) and oleic acid (OA) were freeze-dried with THP and sugars, rehydration of the lyophilized powders led to form the larger vesicles entrapping drugs, but the proper amounts of sugars and OA to lipids (sucrose/lipid = 8 (w/w)) maintained the small particle size (about 340 nm) with high entrapment (80.7%) of THP. After intravenous injection of L-THP, the accumulations of THP in the liver and heart were approximately 4-fold higher and half lower, respectively, than those of free THP (F-THP). L-THP had superior antitumor effect in 10 mg/kg intravenous administration without significant body weight loss. L-THP is a potential drug dosage form of liver cancer treatment since the liposomes carry THP to the liver.

Keywords: Liposome; Pirarubicin; Dehydration–rehydration vesicle; Pharmacokinetics; Liver metastasis tumor

1. Introduction

Pirarubicin (4′-*O*-tetrahydropyranyldoxorubicin; THP) is a doxorubicin (DXR) derivative with higher efficacy against several solid tumors, acute leukemia and malignant lymphoma (Kimura, 1986; Saito et al., 1986), and a lower incidence of side effects (e.g. cardiotoxicity, alopecia and gastrointestinal disorder) compared with DXR (Okuma et al., 1984; Saito et al., 1986). For these advantages, THP is utilized for intra-arterial or intraportal administration and for chemoembolization of lipiodol emulsion instead of DXR on liver metastasis and hepatocellular carcinoma (Izumi and Goto, 1990; Rougier et al., 1990; Ramirez et al., 1993). The higher cellular accumulation of THP was reported since THP shows higher lipophilicity than DXR (Sugiyama et al., 1999). However, the THP level in the liver was low after intravenous administration of THP solution compared with that of DXR (Iguchi et al., 1985). Liposomes are well-recognized drug delivery vehicles that have been shown to enhance the therapeutic activity of anticancer drugs in liver tumors. We previously reported that DXR entrapped in liposomes containing soybean-derived sterylglucoside (SG) accumulated in the liver, when administered to mice (Shimizu et al., 1998). Therefore, THP entrapped in liposomes containing SG could enhance the therapeutic efficacy of liver tumors. However,

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there are no studies about liposomal THP to our knowledge.

The high entrapment of drugs into small-sized liposomes by passive loading method is not high enough, and the pH gradients method that lead quantitative drug loading of vesicles through inward active drug diffusion are limited to drugs of small molecular weight, appropriate lipophilicity and certain structural characteristics (Maurer-Spurej et al., 1999). The dehydration–rehydration vesicle (DRV) method (Kirby and Gregoriadis, 1984) can entrap the drugs regardless of the molecular size and other characteristics such as carboxyfluorescein, albumin and DNA, but the size of liposomes was the relatively large, in some cases reaching micrometer size. Therefore, to prepare small-sized liposomes with high entrapment efficiency of THP, we used the DRV method as reported by Zadi and Gregoriadis (2000) with some modifications. In the modified DRV method, the dehydration of a mixture of empty small unilamellar vesicles (SUVs) and drug destined for entrapment is carried out in the presence of sugars on the external side of liposomes, i.e. sugars help more drug entry into the vesicles and prevents larger vesicle formation. Moreover, liposomes prepared by the DRV method can be stored in a lyophilized state, which improve shelf-life stability of liposomes used as drug carriers. This method was applied to DXR, but not THP and no in vivo studies were reported.

In this study, we prepared THP-entrapping liposomes containing β-sitosterol β-D-glucoside (Sit-G), a major component of SG, and evaluated their pharmacokinetics and antitumor effects against the liver metastasis of M5076 in mice.

2. Materials and methods

2.1. Materials

THP hydrochloride for injection was supplied by Nippon Kayaku Co. Ltd. (Tokyo, Japan). Purified yolk lecithin (EPC) was supplied by Q.P. Co. (Tokyo, Japan). Cholesterol (Ch) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sit-G was purchased from Essential Sterolin Products (Midrand, South Africa). Oleic acid (OA) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Sucrose was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Chemicals for HPLC were of HPLC grade and, all other chemicals were of analytical grade.

2.2. Animals and tumor cells

Specific pathogen-free female C57BL/6j mice (17–19 g) were purchased from Tokyo Animal Experiment Center Co. Ltd. (Tokyo, Japan). Murine histiocytoma M5076 cells were supplied by the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research (Tokyo, Japan). After four transplant generations, the tumor cells were used in this study. The cells were kept as a solid tumor in C57BL/6j mice and transplanted every 3 weeks.

2.3. Preparation of L-THP

Liposomal THP (L-THP) was prepared from EPC, Ch, Sit-G and OA (EPC:Ch:Sit-G:OA = 7:3:2:0–3, in molar ratio). All lipids were dissolved in a chloroform–methanol mixture (1:1) and the solvent was evaporated. The dried lipid mixture was hydrated with distilled water to form multilamellar vesicles (MLV), that were changed to SUVs (average particle size, about 80–100 nm) by sonication using an ultrasonic disruptor (TOMY UD-200, Tomy Seiko Co. Ltd., Tokyo, Japan). The pH value of SUV suspensions was adjusted at 7.4 by 0.1 M NaOH solution to prevent the aggregation of SUV after mixing with THP. THP and sugars (glucose, lactose or sucrose) were mixed with the SUV suspensions at each indicated weight ratio. The resulting mixtures were then freeze-dried overnight. The powders obtained after freeze-drying were rehydrated with water in a manner similar to that applied in the DRV technology (Kirby and Gregoriadis, 1984; Zadi and Gregoriadis, 2000). Finally, the concentration of THP in liposome suspensions was adjusted to 0.5 mg/ml.

2.4. Measurement of size, entrapment efficiency and micrographs of L-THP

The average particle size of liposomes was determined using a dynamic laser light scattering instrument (ELS-800, Ostuka Electronics Co. Ltd., Osaka, Japan). Entrapment efficiency of THP was calculated

from the THP concentration of supernatant (C_f) after separation of 1/20 diluted L-THP suspension by ultracentrifugation (100,000 × g, 60 min, 4 °C) determining by fluorescent spectrometer (Hitachi F-4010, Tokyo, Japan) at 482 nm for excitation and 550 nm for emission $(1 - C_f/C_{total}) \times 100$ (%), where C_{total} is the THP concentration of total liposome suspension. The shape of liposomes before and after rehydration was observed using the scanning electron microscope (JSM-5600LV, JEOL Co. Ltd., Tokyo, Japan).

2.5. Biodistribution of L-THP

The solid tumors of M5076 were dispersed with sterile saline by gentle homogenization on the mesh and this suspension was injected on Day 0 via the tail vein (1×10^5 cells/animal). L-THP (EPC:Ch:Sit-G:OA = 7:3:2:1, molar ratio) with sucrose to total lipid weight ratio at 8, which was almost isotonic solution, was used. Free THP (F-THP) was THP solution of 5% glucose (0.5 mg/ml). At 10 days after M5076 cells inoculation, the mice were injected F-THP or L-THP at a dose of 5 mg/kg intravenously. Four mice were used at each predetermined time after i.v. injection (0.5, 1, 2, 6, 24 h) and blood and their tissues (heart, lung, liver, kidney, and spleen) were collected. The concentration of THP in serum and tissues was measured by the HPLC assay method (Matsushita et al., 1983; Morikawa et al., 1998). The HPLC system was composed of an LC-10AS pump (Shimadzu Co., Japan), a SIL-10A autoinjector (Shimadzu Co., Japan), a RF-10AXL fluorescence detector (Ex, 482 nm, Em, 550 nm, Shimadzu Co., Japan) and a YMC-Pack ODS-A, 150 mm × 4.6 mm I.D. column (YMC Co. Ltd., Japan). The mobile phase was 0.1 M ammonium formate (pH 4.0):acetonitrile = 7:3 (v/v) and the flow rate was 1.0 ml/min. The concentration of THP in each sample was determined using a calibration curve, using daunomycin as the internal standard. Pharmacokinetic parameters for THP in serum were estimated by the nonlinear least squares program (MULTI) (Yamaoka et al., 1981). The areas under the biodistribution curves (from 0 to 24 h) (AUC_{0-24}) of THP in tissues were calculated using the trapezoid method. Statistical comparison was performed using the Bonferroni/Dunn post-hoc test with $P < 0.05$ indicative of significance.

2.6. Antitumor experiment

M5076 cells were injected on Day 0 via the tail vein (1×10^5 cells/animal). The treatment of F-THP or L-THP started on the Day 3, was injected intravenously at a dose of 10 mg/kg. The control group was injected with the same volume of sterile 5% glucose solution. Antitumor effects were determined by comparing the mean survival time of treated groups (T) with that of control group (C) and expressed as an increase in life span (ILS), ILS = $(T/C - 1) \times 100$ (%). For the survival test, Kaplan–Meier curves were constructed and the survival ratios were compared by the log-rank test. Differences were considered significant when the P-value was less than 0.05.

3. Results and discussion

3.1. Preparation of L-THP by DRV method

To deliver drugs to the liver using liposomes, it is important to control the particle size under about 400 nm to evade uptake by the reticuloendothelial system and also to maintain a high entrapment efficiency of the drugs. Usually, it is very difficult to entrap drugs highly in small-sized liposomes. The active entrapment method using a transmembrane pH gradient failed to entrap THP probably because of its low solubility in the buffer solution. Therefore, we selected the modified DRV method to prepare liposomal THP. In this method, the particle size of liposomes and the entrapment efficiency of drugs are influenced by the kinds of sugars and the ratio of the drugs, sugars and OA to lipids in liposomes. We fixed the THP/EPC ratio at 0.05 (w/w), because at the higher ratio of THP (THP/EPC = 0.1 (w/w)), the rehydrated liposomes consisting of EPC:Ch:Sit-G:OA = 7:3:2:1 became 1254.9 ± 223.5 nm while those at THP/EPC = 0.05 was 341.0 ± 41.1 nm (data not shown).

3.2. The effect of sugars and OA on the particle size and entrapment efficiency of L-THP

First of all, we confirmed the effect of sugars on the average particle size and the THP entrapment of L-THP with or without OA as shown in Table 1. Three kinds of sugars were tested, i.e. glucose, sucrose and

Table 1
The effect of sugars on the average particle size and entrapment efficiency of L-THP at sugar:lipid = 1:1 (w/w)

Molar ratio (EPC:Ch:Sit-G:OA)	Sugar	Average particle size (nm)	Entrapment efficiency (%)
L-THP (7:3:2:0)	Without sugar	1113.7 ± 146.3	31.7 ± 1.3
	Glucose	187.3 ± 6.1	31.4 ± 4.2
	Lactose	389.5 ± 18.1	33.8 ± 2.9
	Sucrose	147.2 ± 0.9	35.0 ± 0.1
L-THP (7:3:2:1)	Without sugar	1510.3 ± 52.3	84.8 ± 8.0
	Glucose	675.5 ± 72.8	87.0 ± 0.4
	Lactose	1245.4 ± 236.7	77.7 ± 1.1
	Sucrose	768.1 ± 102.7	83.9 ± 8.7

THP/EPC = 0.05 (w/w). Each value represents the mean ± S.D. ($n = 3$).

lactose when the mass ratio of sugar to total lipid was 1. In both liposomes prepared without sugar, the average particle size was significantly larger (greater than 1000 nm), whereas with sugar it was small. The particle sizes of L-THP (7:3:2:1) with glucose and sucrose were 675.5 and 768.1 nm, respectively. The effect of sucrose and glucose was almost equal and greater than that of lactose. Since it has been reported that sucrose, i.e. disaccharide is effective to prevent fusion than glucose, i.e. monosaccharide (Crowe and Crowe, 1993; Zadi and Gregoriadis, 2000), sucrose was selected as sugars.

The entrapment efficiency of L-THP without OA was 31–35% regardless of with or without sugar, but that with OA was 78–87%. Incorporation of negatively charged lipid, i.e. OA, into liposomes increased the retention of positively charged THP in liposomes and, therefore, the particle size was increased.

To determine the optimal ratio of sucrose to lipids, the ratio of sucrose to lipid was examined. When increasing the amount of sucrose, the increasing average particle size after the dehydration–rehydration steps was moderately inhibited without reduction of entrapment efficiency (Table 2). At the 8:1 ratio of sucrose to lipid, we obtained the smallest vesicle with a high entrapment efficiency.

The effect of OA on the particle size of liposomes and the entrapment efficiency is shown in Fig. 1. The entrapment efficiency was increased with increase of OA ratio in liposomes (EPC:Ch:Sit-G:OA = 7:3:2:0–3 in molar ratio, THP/EPC = 0.05 in weight and sucrose:total lipid = 8:1 in weight), but the particle size was also increased, perhaps because of interaction of THP with OA. From these results, L-THP (EPC:Ch:Sit-G:OA = 7:3:2:1 in molar ratio) prepared with THP/EPC = 0.05 in weight had less than 400 nm particle size (size about 340 nm) with higher entrapment efficiency (about 80%), therefore, this L-THP (7:3:2:1) with a sucrose to lipid ratio of 8:1 was used for the animal experiments.

3.3. The scanning electron micrograph of L-THP

To confirm the liposome structure in each preparation stage, the scanning electron micrographs of L-THP (7:3:2:1) were taken (Fig. 2). Under the lyophilized state with sucrose, liposomes appeared to be buried in a glassy matrix of sucrose (Fig. 2(a)).

Table 2
The effect of the sucrose to lipid ratio on the average particle size and entrapment efficiency of L-THP

Molar ratio (EPC:Ch:Sit-G:OA)	Sucrose/lipid (w/w)	Average particle size (nm)	Entrapment efficiency (%)
L-THP (7:3:2:1)	0	1510.3 ± 52.3	84.8 ± 8.0
	1	768.1 ± 129.6	83.9 ± 8.7
	2	423.7 ± 35.8	82.4 ± 10.7
	5	412.4 ± 1.5	82.6 ± 4.9
	8	341.0 ± 41.1	80.7 ± 11.3

THP/EPC = 0.05 (w/w). Each value represents the mean ± S.D. ($n = 3$).

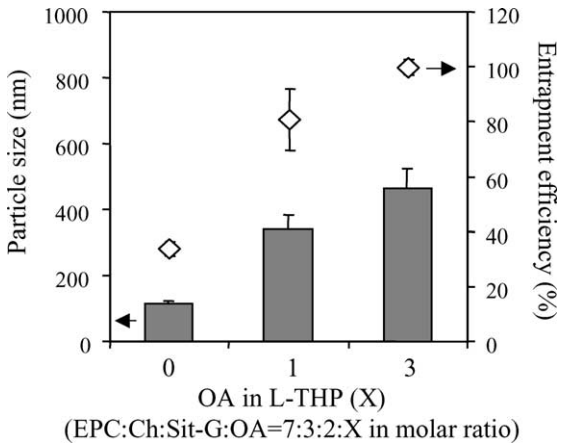

Fig. 1. The effect of OA ratio in L-THP on the particle size of liposomes (bar) and the entrapment efficiency (◇). L-THP was prepared from EPC:Ch:Sit-G:OA = 7:3:2:*X* (molar ratio) at sucrose:total lipid = 8:1 (w/w) and THP/EPC = 0.05. Each value represents the mean ± S.D. ($n = 3$).

Lyophilization without sucrose led liposomes aggregating completely (data not shown), and resulted in larger vesicles (greater than 1000 nm) after rehydration. After rehydration of lyophilized L-THP (7:3:2:1) with sucrose, small vesicles were reconstructed (Fig. 2(b)).

3.4. Pharmacokinetics and antitumor effects of L-THP

The pharmacokinetics and antitumor effects of L-THP (EPC:Ch:Sit-G:OA = 7:3:2:1 in the molar ratio and sucrose:total lipid = 8:1 in weight) are evaluated in mice bearing liver metastasis of M5076 cells. Murine histiocytoma M5076, which arises spontaneously in mice ovaries (Talmadge et al., 1981), is highly invasive and metastasizes to several organs including the liver and spleen (Hart et al., 1981). At 14 days after tumor inoculation, metastatic nodules were seen mainly in the liver and spleen (data not shown).

The serum and tissue $AUC_{0–24}$ values calculated from the biodistribution curves of F-THP and L-THP are shown in Table 3. F-THP accumulated highly in lung and spleen, which correspond well with Iguchi et al. (1985) and Fujita et al. (1986). L-THP changed the biodistribution of THP, i.e. increasing the $AUC_{0–24}$ value of liver and decreasing those of heart, lung, kidney and spleen. The $AUC_{0–24}$ value of L-THP in the liver (24.4 μg h/g), where the tumor represented, was approximately 4-fold that of F-THP (6.6 μg h/g). The drug accumulation in the heart correlated to the cardiotoxicity. THP is less cardiotoxic than DXR, because the accumulation of THP in the heart was lower than that of DXR (Iguchi et al., 1985; Shimizu et al., 1998). L-THP significantly decreased the AUC value in the heart (3.7 μg h/g) compared with that of F-THP (7.7 μg h/g), suggesting that L-THP is safer than F-THP.

After administration at a dose of 10 mg/kg, the mice treated both F-THP and L-THP prolonged the median survival time than control and the ILS values of F-THP and L-THP were 28.6 and 42.9%, respectively (Table 4). L-THP did not decrease largely the body weight of mice unlike F-THP that decreased to

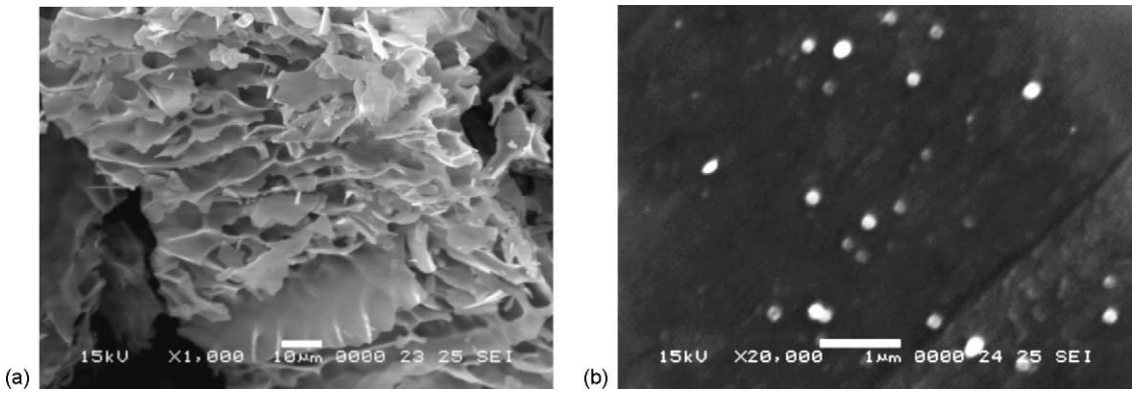

Fig. 2. Scanning electron micrographs of L-THP before (a) and after (b) rehydration. L-THP was prepared from EPC:Ch:Sit-G:OA = 7:3:2:1 (molar ratio) at sucrose:lipid = 8:1 (w/w).

Table 3
Tissue AUC_{0-24} values after injection of F-THP and L-THP in mice bearing M5076 at a dose of 5 mg/kg at 10 days after tumor inoculation ($n = 4$)

	Tissue AUC_{0-24} (μg h/g)					
	Serum[a]	Liver	Heart	Lung	Kidney	Spleen
F-THP	1.13	6.6 (0.8)	7.7 (1.0)	485.1 (179.5)	90.2 (50.5)	130.7 (24.4)
L-THP	1.45	24.4* (3.2)	3.7* (0.9)	93.0* (4.3)	24.0* (1.8)	101.3 (20.9)

[a] Serum AUC is given as μg h/ml. The numbers in parentheses represent S.D.
* $P < 0.05$, compared with F-THP.

Table 4
Antitumor effects of F-THP and L-THP in mice bearing M5076 liver metastasis tumors treated at 10 mg/kg at 3 days following tumor inoculation ($n = 7$)

	Median survival time (days)	ILS[a] (%)
Control	14.0	
F-THP	18.0	28.6*
L-THP[b]	20.0	42.9*

[a] Percentage increase in life span $(T/C - 1) \times 100$ (%), where T and C represent the median survival time of the treated and control animals, respectively.
[b] L-THP consisted of EPC:Ch:Sit-G:OA = 7:3:2:1 (molar ratio).
* $P < 0.05$, compared with control.

about 10% of the initial body weight (data not shown), reflecting an adverse effect. No lethal toxicity was observed in C57BL/6j mice treated with THP up to 15 mg/kg (data not shown), while it was reported that the LD_{50} value is 14.1 mg/kg in female ddY mice (Tone et al., 1986). This finding suggested that L-THP kept equal or superior antitumor effect to F-THP on M5076 liver metastasis with less adverse effects. Systemic injection of L-THP is a potential treatment for liver cancer, because the liposomes can safely carry THP to the liver.

4. Conclusions

Using the modified DRV method, we obtained small-sized liposomes highly entrapping THP by the addition of OA into a formulation (EPC:Ch:Sit-G:OA = 7:3:2:1 in molar ratio), and by the addition of sucrose in preparations. L-THP increased the accumulation of THP in the liver, and had potential antitumor effects than F-THP in M5076 tumor bearing mice. A novel potential application of THP in liver tumors was found by the liposome dosage form.

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References

Crowe, J. H., Crowe, L. M., 1993. Preservation of liposomes by freeze-drying. In: G. Gregoriadis (Ed.), Liposome Technology, vol. I, 2nd ed. CRC Press, Boca Raton, FL, pp. 229–252.

Fujita, H., Ogawa, K., Tone, H., Iguchi, H., Shomura, T., Murata, S., 1986. Pharmacokinetics of doxorubicin, (2′′*R*)-4′-*O*-tetrahydropyranyladriamycin and aclarubicin. Jpn. J. Antibiot. 39, 1321–1336.

Hart, I.R., Talmadge, J.E., Fidler, I.J., 1981. Metastatic behavior of a murine reticulum cell sarcoma exhibiting organ-specific growth. Cancer Res. 41, 1281–1287.

Iguchi, H., Tone, H., Ishikura, T., Takeuchi, T., Umezawa, H., 1985. Pharmacokinetics and disposition of 4′-*O*-tetrahydropyranyladriamycin in mice by HPLC analysis. Cancer Chemother. Pharmacol. 15, 132–140.

Izumi, N., Goto, Y., 1990. A clinical trial of transarterial chemoembolization for hepatocellular carcinoma using 4′-*O*-tetrahydropyranyladriamycin. Jpn. J. Cancer Chemother. 17, 1303–1307.

Kimura, K., 1986. A phase II study of (2′′*R*)-4′-*O*-tetrahydropyranyladriamycin (THP) in patients with hematological malignancies. THP Study Group. Jpn. J. Cancer Chemother. 13, 368–375.

Kirby, C.J., Gregoriadis, G., 1984. Dehydration–rehydration vesicles (DRV): a new method for high yield drug entrapment in liposomes. Biotechnology 2, 979–984.

Matsushita, Y., Iguchi, H., Kiyosaki, T., Tone, H., Ishikura, T., Takeuchi, T., Umezawa, H., 1983. A high

performance liquid chromatographic method of analysis of 4′-*O*-tetrahydropyranyladriamycin and their metabolites in biological samples. J. Antibiot. 36, 880–886.

Maurer-Spurej, E., Wong, K.F., Maurer, N., Fenske, D.B., Cullis, P.R., 1999. Factors influencing uptake and retention of amino-containing drugs in large unilamellar vesicles exhibiting transmembrane pH gradients. Biochim. Biophys. Acta 1416, 1–10.

Morikawa, N., Mori, T., Takeyama, M., Hori, S., 1998. Pharmacokinetics of intra-arterially administered pirarubicin in plasma and cerebrospinal fluid of patients with glioma. Biol. Pharm. Bull. 21, 297–299.

Okuma, K., Furuta, I., Ota, K., 1984. Acute cardiotoxicity of anthracyclines: analysis by using Holter ECG. Jpn. J. Cancer Chemother. 11, 902–911.

Ramirez, L.H., Munck, J.N., Bognel, C., Zhao, Z., Ardouin, P., Poupon, M.F., Gouyette, A., Rougier, P., 1993. Pharmacology and antitumour effects of intraportal pirarubicin on experimental liver metastases. Br. J. Cancer 68, 277–281.

Rougier, P., Munck, J.N., Elias, D., Herait, P., Bognel, C., Gosse, C., Lasser, P., 1990. Intra-arterial hepatic chemotherapy with pirarubicin. Preclinical and clinical studies. Am. J. Clin. Oncol. 13 (Suppl. 1), S1–S4.

Saito, T., Kasai, Y., Wakui, A., Furue, H., Majima, H., Nitani, H., Niijima, T., Takeda, C., Abe, O., Koyama, Y., 1986. Phase II study of (2′′*R*)-4′-*O*-tetrahydropyranyladriamycin (THP) in patients with solid tumors. Multi-Institutional Cooperative Study. Jpn. J. Cancer Chemother. 13, 1060–1069.

Shimizu, K., Qi, X.R., Maitani, Y., Yoshii, M., Kawano, K., Takayama, K., Nagai, T., 1998. Targeting of soybean-derived sterylglucoside liposomes to liver tumors in rat and mouse models. Biol. Pharm. Bull. 21, 741–746.

Sugiyama, T., Sadzuka, Y., Nagasawa, K., Ohnishi, N., Yokoyama, T., Sonobe, T., 1999. Membrane transport and antitumor activity of pirarubicin, and comparison with those of doxorubicin. Jpn. J. Cancer Res. 90, 775–780.

Talmadge, J.E., Key, M.E., Hart, I.R., 1981. Characterization of a murine ovarian reticulum cell sarcoma of histiocytic origin. Cancer Res. 41, 1271–1280.

Tone, H., Shirai, M., Onoue, F., Kumagai, H., 1986. Toxicological studies on (2′′*R*)-4′-*O*-tetrahydropyranyladriamycin, a new antitumor antibiotic, acute toxicity study in mice. Jpn. J. Antibiot. 39, 250–258.

Yamaoka, K., Tanigawara, Y., Nakagawa, T., Uno, T., 1981. A pharmacokinetic analysis program (multi) for microcomputer. J. Pharmacobiodyn. 4, 879–885.

Zadi, B., Gregoriadis, G., 2000. A novel method for high-yield entrapment of solutes into small liposomes. J. Liposome Res. 10, 73–80.