Targeting of Soybean-Derived Sterylglucoside Liposomes to Liver Tumors in Rat and Mouse Models

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The blood clearance, tissue uptake and antitumor efficacy against liver metastasis of M5076 reticulosarcoma in mice and against primary liver cancer in rats of doxorubicin (DOX) encapsulated in two types of liposomes, with and without a soybean-derived sterlyglucoside mixture (SG), were examined. Liposomes entrapping DOX were composed of dipalmitoylphosphatidylcholine (DPPC), SG and cholesterol (Ch) at a molar ratio of 6 : 1 : 3, (SG-liposomes) and 6 : 0 : 4 (non-SG-liposomes). Pharmacokinetic analysis of drug disposition was based on the area under the curve (AUC) for liposomes up to 24 h following i.v. injection. SG-liposomes showed lower DOX concentrations in blood and higher concentrations in liver compared with non-SG-liposomes. The highest AUC of SG-liposomes in tissue was in liver, 2.4 times higher than that of the free drug. The antitumor efficacy of SG-liposomes was compared with that of free DOX and non-SG-liposomes at a dose of 5 mg DOX/kg. SG-liposomes displayed stronger antitumor activity than the free drug and non-SG-liposomes in murine reticulosarcoma M5076 tumor models and primary liver cancer models reflecting accumulation in hepatocytes. The antitumor activity of SG-liposomes in rats with primary liver cancer was significantly higher compared with free DOX and non-SG-liposomes (ILS: 92.7%).

Key words liposome; sterlyglucoside; M5076; primary liver cancer; doxorubicin

The chemotherapy of liver cancer is very difficult because many drugs do not reach therapeutic concentrations in liver tumors. Liposomes are versatile drug delivery vehicles that have proven useful in reducing toxicity and enhancing the activity of a variety of pharmacologically active agents, including antineoplastic drugs.

When liposomes are given intravenously, almost all are taken up by the reticuloendothelial system. Liposomes are being successfully used for passive targeting of anticancer agents entrapped in long-circulating liposomes and can lead to significant improvement in the therapeutic efficacy of the entrapped drug. The asialoglycoprotein receptor has been used as a ligand of targeted delivery to the liver. However, lipids with galactose residues are expensive to synthesize.

We have already demonstrated that dipalmitoylphosphatidylcholine (DPPC) liposomes containing a soybean-derived sterlyglucoside mixture (SG) entrapping calcein accumulate in hepatocytes. SG is a natural product being a mixture of steryl β-D-glucosides (Fig. 1) and is cheaper than its counterpart modified with galactose residues because of the waste occurring during the preparation of soybean-oil.

Doxorubicin (DOX) is one of the most commonly used antitumor drugs. If liposomes with SG entrapped DOX (SG-liposomes) accumulate metastatic cancer of the liver, they will be a practical means of treating liver tumors.

In this study, we examined the blood clearance, tissue uptake and antitumor effects of DOX entrapped in SG-liposomes compared with liposomes without SG (non-SG-liposomes) against liver metastases of M5076 reticulosarcoma in mice and against primary liver cancer in rats.

MATERIALS AND METHODS

Materials DPPC was purchased from NOF (Tokyo, Japan). Cholesterol (Ch) was purchased from Sigma Chemical Co. (MO, U.S.A.). SG was provided by Ryukakusan Co. (Tokyo, Japan). Diethylaminoethylamine (DEEA) and DOX were purchased from Wako Pure Chemical Industries (Tokyo, Japan).

![Chemical Structure of SG](https://example.com/structure.png)

Fig. 1. Chemical Structure of SG

The numbers in parentheses represent the mixture ratio in SG.

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Preparation of Liposomes Entrapped DOX Liposomes were prepared from DPPC and Ch (DPPC : Ch = 6:4, molar ratio) and SG (DPPC : SG : Ch = 6:1:3, molar ratio) by the reverse-phase evaporation method as reported previously. Liposomes were successively extruded through a polycarbonate membrane (Nuclepore, U.S.A.) with a pore size of 200 nm at 60°C. The size distribution of liposomes estimated was homogeneous and the mean diameter was about 200 nm, measured by an ELS-800 instrument (Otsuka Electronics, Osaka, Japan).

DOX was entrapped in liposomes, with and without SG, by the pH gradient loading method (SG-liposomes and non-SG-liposomes, respectively). Briefly, the pH of the liposome suspension (initially pH 4.0) was raised to pH 7.8 with 1 N NaOH and then heated to 60°C for 5 min. The liposome suspension was then mixed with a preheated (60°C) solution of DOX in Hepes buffer (pH 7.8). The mixture was incubated with periodic mixing for 15 min at 60°C.

Animals and Tumor Models Specific-pathogen-free male C57BL/6 mice (19–20 g, 7 weeks old) and male Wistar rats (190–200 g) were purchased from Tokyo Laboratory Animal Science Company Co., Ltd. and Saitama Experimental Animal Supply, respectively. Murine reticulosarcoma (MS076) was supplied by Dr. T. Tashiro of the Cancer Chemotherapy Center, Japan Foundation for Cancer Research (Tokyo, Japan) and kept in vivo as an ascitic tumor in C57BL/6 mice. MS076 cells were maintained by transplantation every two weeks into the peritoneal cavity. To obtain a suspension of tumor cells for transplantation, the ascitic fluid containing MS076 cells was diluted with sterile saline, and the diluted suspension was injected intraperitoneally, about $5 \times 10^4$ cells per mouse.

The model of primary liver cancer was established in rats by giving 25 mg DENA per kg weight 13 times at 7-d intervals. DENA was administered orally as a saline solution. As DENA is sensitive to light, a fresh solution was prepared for each administration and kept in dark bottles for short periods.

Tissue Distribution Study Liposomal DOX was injected via the tail vein at a dose of 5 mg DOX/kg to groups of three to seven MS076-bearing C57BL/6 mice. At indicated times after injection the mice were killed by cervical dislocation, blood was collected via the cervical vein and carotid arteries, and the liver, heart, lung, kidney and spleen were immediately removed. Tissues were lightly blotted to remove any excess blood and weighed. These tissues, as well as 0.2 ml serum, were homogenized and extracted with chloroform/methanol (4:1, v/v); the extracts were then subjected to high performance liquid chromatography (HPLC) assay.

The HPLC system consisted of a Shimadzu LC-10AS high-pressure pump, a Shimadzu SIL-10A autoinjector, a Shimadzu RF-10AXL fluorescence detector (EX, 470 nm; EM, 585 nm) and a YMC-Pack C18 column. The mobile phase consisted of methanol/water/acetic acid (50:45:5, v/v/v) and a flow rate of 1.0 ml/min. Measurements were made using the ratio of the peak area to that of an internal standard (daunomycin).

Evaluation of Antitumor Activity In the therapeutic experiments of liver metastatic cancer, groups of 10 C57BL/6 mice were inoculated i.v. on day 0 with $5 \times 10^4$ MS076 cells. Treatment was initiated 9 d after implantation of tumor cells.

In the therapeutic experiments on primary liver cancer, groups of 9 or 10 rats, 100 d after administration of DENA, were used.

The animals (both mice and rats) were given as a single i.v. dose, free or liposomal DOX, 5 mg/kg. The control group was given sterile saline. Mice and rats were weighed every other day after DOX administration. The survival time was recorded in days following DOX administration. Antitumor activity was evaluated by comparing the mean survival time of the treated animals (T) with that of the controls (C), i.e., by calculation of the increase in life-span (ILS), $(T/C−1)\times100$ (%). The ILS value indicates the ratio of the median survival time of liposomal-treated mice (L) vs. mice treated with an equivalent dose of 5.0 mg free DOX (F).

In the primary liver cancer model, photos of the liver were taken at day 0, 50, 98 and 105 after DENA administration and then imaged by Adobe® Photoshop®.

Statistical Analysis The statistical significance of the results was evaluated by the nonparametric test of Kruskal-Wallis for the survival experiment and Student's t-test for the tissue distribution experiment.

RESULTS

Tissue Distribution of Liposomes in MS076-Bearing Mice Figure 2 shows SG-liposomes, non-SG-liposomes and free DOX remaining in serum and the distribution in heart, liver, spleen, kidney and lung in MS076-bearing mice. Table 1 shows the calculated pharmacokinetic parameters. The amount of SG-liposomes remaining in serum 24 h after injection was almost the same as that of non-SG-liposomes (Fig. 2A). The areas under the curve (AUC) of SG-liposomes (283.73 ± 46.11 µg/ml) in serum was significantly higher than that of free DOX (17.93 ± 5.14 µg/ml) ($p<0.001$) and a little lower than that of non-SG-liposomes (356.99 ± 53.27 µg/ml) (Table 1).

The AUC of SG-liposomes (462.16 ± 109.4 µg/ml, $p<0.001$) and 219.44 ± 34.2 µg/ml, $p<0.05$) and non-SG-liposomes (389.65 ± 82.5 µg/ml, $p<0.01$ and 147.27 ± 20.5 µg/ml, $p<0.05$) in liver and spleen tumors was significantly greater than that of free DOX (190.66 and 87.46 µg/ml, respectively) (Fig. 2B, Table 1). The AUC of SG-liposomes (32.73 ± 4.3 µg/ml) was lower than that of free DOX (44.36 ± 5.1 µg/ml) (Table 1). The liposome uptake by other tissues (spleen, lung, and kidney) was lower than that by liver for SG-liposomes (Fig. 2D, E, F) and the AUC values were similar (Table 1).

Antitumor Activity in MS076-Bearing Mice The antitumor efficacy of liposomal and free DOX, as measured in the liver metastatic cancer model using MS076, is shown in Table 2 and Fig. 3. The mean survival time of SG-liposomes (18.2 d) and non-SG-liposomes (15.5 d) indicated a greater antitumor activity than saline (11.4 d) ($p<0.01$) and free DOX (13.1 d). The ILS values of SG-liposomes (27.3%) and non-SG-liposomes (27.3%) were greater than free DOX (9.1%). The ILS and L/F values of SG-liposomes (27.3% and 1.16, respectively) were equal to those of non-SG-liposomes. 2 of the 10 mice following administration of the SG-liposomes survived for over 30 days, but mice given the non-SG-liposomes did not.

Antitumor Activity in Primary Liver Cancer Figure 4
Fig. 2. Drug Levels in Various Tissues after i.v. Injection of SG-Liposomes (—□—), Non-SG-Liposomes (—△—) and Free DOX (—■—) in M5676-Bearing Mice at a Dose of 5 mg/kg

Each value represents the mean±S.D. (n=3—7).

Table 1. Tissue AUC Values after i.v. Injection of SG-Liposomes and Non-SG-Liposomes in M5676-Bearing Mice at a Dose of 5 mg/kg

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Serum (h·µg/g)</th>
<th>Liver (Tumor) (h·µg/g)</th>
<th>Spleen (Tumor) (h·µg/g)</th>
<th>Heart (h·µg/g)</th>
<th>Lung (h·µg/g)</th>
<th>Kidney (h·µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free DOX</td>
<td>17.93 (1.05)</td>
<td>190.66 (23.82)</td>
<td>87.46 (26.35)</td>
<td>44.36 (4.20)</td>
<td>20.17 (3.30)</td>
<td>28.61 (4.57)</td>
</tr>
<tr>
<td>SG-liposomes</td>
<td>283.73 (19.90)**</td>
<td>462.16 (67.81)**</td>
<td>219.44 (84.38)*</td>
<td>32.73 (7.02)</td>
<td>17.31 (3.60)</td>
<td>36.48 (3.40)</td>
</tr>
<tr>
<td>Non-SG-liposomes</td>
<td>356.99 (116.44)</td>
<td>389.65 (34.37)**</td>
<td>147.27 (10.40)*</td>
<td>33.56 (2.40)</td>
<td>16.84 (1.81)</td>
<td>37.36 (3.12)</td>
</tr>
</tbody>
</table>

a) AUC values were calculated for 0—24h (n=3—7). b) Serum AUC is given as h·µg/ml (n=3—5). The numbers in parentheses represent S.D. *: p<0.05, **: p<0.01, ***: p<0.001, significantly different from free DOX.

Table 2. Antitumor Efficacy of SG-Liposomes and Non-SG-Liposomes Injected at Day 9 via the Tail Vein Following Implantation of Reticulum Cell Sarcoma (M5076) at Day 0 via the Tail Vein

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Dose (mg/kg)</th>
<th>Survival time (d)</th>
<th>%ILS[a]</th>
<th>L/E[b]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30 d Mean (S.D.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>5.0</td>
<td>0/10</td>
<td>11.4 (1.6)</td>
<td>11.5</td>
</tr>
<tr>
<td>Free DOX</td>
<td>5.0</td>
<td>0/10</td>
<td>13.1 (3.1)</td>
<td>12.5</td>
</tr>
<tr>
<td>SG-liposomes</td>
<td>5.0</td>
<td>0/10</td>
<td>18.2 (10.2)*</td>
<td>14.5</td>
</tr>
<tr>
<td>Non-SG-liposomes</td>
<td>5.0</td>
<td>0/10</td>
<td>15.5 (3.9)*</td>
<td>14.5</td>
</tr>
</tbody>
</table>

a) Percentage increase in life span, [(T/C−1)×100 (%)], where T and C represent the median survival time (days) of the treated and control animals, respectively. b) Ratio of median survival time of liposomal-treated mice (T) vs. mice treated with an equivalent dose of 5.0 mg of free DOX (C). *: p<0.01, significantly different from saline.
shows photographs of the liver cancer induced by oral administration of DENA. No effect of DENA on tumor development was observed till 50 d had elapsed (Fig. 4A and B). The liver at 98 d seemed to be still normal (Fig. 4C). At 105 d, the liver exhibited severe cirrhosis and haemorrhaging.

The antitumor efficacy of liposomal and free DOX, as measured in the primary liver cancer model, is shown in Table 3 and Fig. 5. The non-SG-liposomes did not display antitumor activity because the survival time of non-SG-liposomes (25.6 d) was almost as the same as that of saline (25.8 d). However, the mean survival time of SG-liposomes (48.2 d) was significantly higher than that of saline (25.8 d) ($p<0.05$) and free DOX (19.7 d). The mean survival time of SG-liposomes was significantly higher than that of the free DOX and non-SG-liposomes (25.6 d) ($p<0.01$, $p<0.05$, respectively). The ILS value of the SG-liposomes (92.7%) was greater than that of the non-SG-liposomes (−5.5%) and free DOX (−49.1%). The $L/F$ value of the SG-liposomes (3.9) was about 4 times greater than that of saline and about 2 times greater than that of non-SG-liposomes (1.9).

**DISCUSSION**

DOX is a useful antitumor drug and DOX entrapped in liposomes has been reported to reduce the in vivo toxicity and maintain or increase the antitumor activity. Mayhew et al. and Gabizon et al. demonstrated that liposomes entrapping DOX were more active than free DOX in the treatment of established liver metastatic tumors. Qi et al. reported that the antitumor efficacy of DOX entrapped in liposomes at a dose of 5 mg/kg against hepatoma 22 and sarcoma 180 was more effective than free DOX at a dose of 10 mg/kg. Yachi et al. also reported that the antitumor efficacy of DOX entrapped in liposomes at a dose of 7.5 mg/kg against M5076 was more effective than free DOX at a dose of 12.0 mg/kg. These reports suggest that DOX entrapped in liposomes at a dose of 5 mg/kg is enough to evaluate the antitumor efficacy of liposomes in this study.

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**Fig. 3.** Effects of Free DOX and Liposomal DOX on Survival of Mice Injected at Day 9 via the Tail Vein Following Implantation of Reticulum Cell Sarcoma (M5076) at Day 0 via the Tail Vein (n=10).

Key: (○), SG-liposomes; (■), non-SG-liposomes; (△), free DOX; (△), saline.

**Fig. 4.** Photographs of the Liver at 0 (A), 50 (B), 98 (C) and 105 d (D) after Oral Administration of DENA

**Table 3. Antitumor Efficacy of SG-Liposomes and Non-SG-Liposomes in a Primary Model of Liver Cancer Induced by Diethylnitrosamine in Vivo**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Dose (mg/kg)</th>
<th>Survival time (d)</th>
<th>%ILS$^{a}$</th>
<th>$L/F^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (S.D.)</td>
<td>Median</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>5.0</td>
<td>25.8 (17.1)</td>
<td>27.5</td>
<td>−49.1</td>
</tr>
<tr>
<td>Free DOX</td>
<td>5.0</td>
<td>19.7 (14.1)</td>
<td>14.0</td>
<td></td>
</tr>
<tr>
<td>SG-liposomes</td>
<td>5.0</td>
<td>48.2 (23.3)</td>
<td>53.0</td>
<td>92.7</td>
</tr>
<tr>
<td>Non-SG-liposomes</td>
<td>5.0</td>
<td>25.6 (15.5)</td>
<td>26.0</td>
<td>−5.5</td>
</tr>
</tbody>
</table>

$^{a}$ Percentage increase in life span, $[(T-C) \times 100 / C]$, where T and C represent the median survival time (days) of the treated and control animals, respectively. $^{b}$ Ratio of median survival time of liposomal-treated mice (T) vs. mice treated with an equivalent dose of 5.0 mg free DOX (C). $^{*}$ $p<0.05$, $^{**}$ $p<0.01$ significantly different from saline and SG-liposomes.
The M5076 tumor is a reticulum cell sarcoma of histiocytic origin that arises spontaneously in mouse ovary and is highly invasive and metastasizes to several organs including the liver and spleen. This model has been used to evaluate liposomal formulations of anthracyclins and cisplatin derivatives. The SG-liposomes in M5076-bearing mice accumulated more in the liver tumor than saline (Fig. 2B) and the survival time of the SG-liposomes (18.2 d) was significantly (p<0.01) greater than that of saline (11.4 d) (Fig. 3 and Table 2). The AUC of the SG-liposomes in heart (32.73 h·μg/g) was smaller than that of free DOX (44.36 h·μg/g) and non-SG-liposomes (33.56 h·μg/g) (Table 1). These data suggest that SG-liposomes reduce heart toxicity and are effective for liver targeting in liver metastatic cancer.

We have reported that liposomes with and without SG (corresponding to SG-liposomes and non-SG-liposomes not entrapping DOX) entrapping calcine accumulated in the liver to about 80 and 15% of the dose 1 h after i.v. injection, respectively, and the ratio of calcine in blood to liver (blood/liver value) of liposomes with and without SG entrapped calcine was 0.14 and 5.09, respectively. In this study, SG-liposomes and non-SG-liposomes accumulated to about 20 and 14 μg DOX/g liver 1 h after i.v. injection, respectively, and blood/liver value of liposomes and non-SG-liposomes was 2.15 and 3.36, respectively. Blood/liver values of non-SG-liposomes (5.09) and liposomes without SG entrapped calcine (3.36) were similar. However, the blood/liver value of SG-liposomes (2.15) was larger than that of liposomes with SG entrapped calcine (0.14). These results suggest that SG-liposomes are distributed in blood to a greater extent since DOX may affect SG-liposomes or the method of loading DOX (pH gradient loading method) may favor SG-liposomes rather than non-SG-liposomes.

We have reported that liposomes with SG entrapped calcine, given via the tail vein in normal mice accumulated in hepatocytes. The M5076 tumor is a reticulum cell sarcoma so that the tumor in hepatocytes is more suitable for estimating liver targeting (hepatocytes). We used a model of primary liver cancer in rats given DENA. Steinhoff reported that DENA given by oral administration in the rat makes it simple to induce primary liver cancer a suitable model for testing new treatments. In this study, we confirmed that DENA induces primary liver cancer at 105 d (Fig. 4D).

The survival time of SG-liposomes (48.2 d) in primary liver cancer was significantly higher than that of saline (25.8 d) (p<0.05) (Table 3). The %ILS of SG-liposomes (92.7%) was greater than that of free DOX (−49.1%) and non-SG-liposomes (−5.5%). This result indicates that SG-liposomes are an effective dosage form in the primary liver cancer model because SG-liposomes selectively accumulate in hepatocytes. The %ILS of free DOX (−49.1%) and non-SG-liposomes (−5.5%) indicate that free DOX and non-SG-liposomes do not reduce the toxicity of DOX compared with control (saline).

We have reported the uptake mechanism of liposomes with SG entrapped calcine in hepatocytes using rat primary cultured hepatocytes and confocal laser scanning microscopy and shown that liposomes with SG may not be taken up into hepatocytes by galactose receptor-mediated endocytosis. This study suggests that in the case of liposomes with SG entrapped DOX, SG-liposomes accumulated and increased the antitumor efficacy in murine reticulosarcoma M5076 tumor and primary liver cancer models.

CONCLUSIONS

SG-liposomes displayed stronger antitumor activity than free DOX and non-SG-liposomes against murine reticulosarcoma M5076 tumor and primary liver cancer models reflecting accumulation into hepatocytes. The antitumor activity of SG-liposomes in primary liver cancer-bearing rats was significantly higher compared with free DOX and non-SG-liposomes.

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REFERENCES