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Organ distribution and antitumor activity of free and liposomal doxorubicin injected into the hepatic artery

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Summary. The plasma levels, organ distribution, and in vivo antitumor activity of free and liposomal doxorubicin injected into the hepatic artery of rats bearing W256 liver tumors were studied. The administration of liposomal doxorubicin resulted in liver-tumor and liver-parenchyma doxorubicin areas under the curve (AUCs) that were 4.7- and 3.8-fold, respectively, those obtained after the administration of free doxorubicin. Spleen and plasma AUCs were also increased by 2.8 and 2.5 times, respectively, following administration of the liposomal form. In contrast, liposomal doxorubicin did not affect heart AUCs; peak doxorubicin levels in heart tissue were three times lower in animals treated with liposomal doxorubicin. Following treatment with the liposomal form, the cumulative urinary excretion of doxorubicin at 8 h was 38 times lower. In good correlation with these findings, liposomal doxorubicin (2.35 mg/kg on day 7) was more effective than free doxorubicin against liver W256 tumors as measured by tumor-growth inhibition at 5 days after treatment (16% for liposomal doxorubicin versus –53.7% for free doxorubicin, $P < 0.05$) and increased life span (ILS; 108% for liposomal doxorubicin versus 27% for free doxorubicin, $P < 0.05$). These results demonstrate that as compared with free doxorubicin, the administration of liposomal doxorubicin into the hepatic artery results in higher drug levels in the liver tumor and enhanced antitumor activity while maintaining the cardioprotective effect of the liposome carrier as suggested by the decreased peak drug levels measured in the heart tissue.

Introduction

Liver tumors receive >90% of their blood supply from the hepatic artery, whereas the liver parenchyma is mainly supplied with blood (>70%) from the portal vein [4, 12, 19, 26]. On the basis of this anatomical characteristic, hepatic-artery administration of antitumor agents such as doxorubicin, fluorodeoxyuridine, and mitomycin C has been extensively explored in the presence and absence of concomitant embolization for the treatment of liver primary and metastatic tumors [2, 11, 14, 15, 18, 23, 24, 27]. Randomized clinical studies have shown that the tumor response rate is higher following hepatic-artery administration of antitumor agents than after i.v. administration, but hepatic-artery administration does not significantly prolong survival [16]. There is therefore a need for optimization of this form of antitumor therapy.

Although doxorubicin (DXR) is one of the drugs of choice for the treatment of hepatoma, it has limited antitumor activity against this disease [3, 6, 7]. In experimental systems, the administration of DXR into the hepatic artery results in a 3-fold increase in the tumor drug levels without significantly altering the drug levels in the heart [25]. In patients with malignant tumors, hepatic-artery administration of DXR reportedly reduces the plasma AUC by about 30% [6].

Liposomes have been extensively explored as carriers of different antitumor agents in an attempt to alter their pharmacokinetics and distribution and enhance their therapeutic index [10, 20]. Encapsulation of DXR in small liposomes has repeatedly been shown to result in lower heart drug levels and reduced cardiotoxicity in different animal species after i.v. administration [1, 9, 13] as well as in reduced vesicant toxicity after s.c. infiltration [1]. The i.v. administration of liposomal DXR has also been shown to enhance antitumor activity in different experimental mouse models of liver metastases [8, 21, 22]. However, in most of these studies, liposomal DXR was injected shortly after tumor inoculation, when microscopic liver metastases had yet to develop an independent vascularity.

The use of liposomal antitumor agents as a slow-release system for administration into the hepatic artery has received little attention thus far [17, 30]. Very few studies have investigated the effect of liposome encapsulation on the pharmacokinetics and tissue distribution of drugs given via the hepatic artery. It is conceivable that after adminis-

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tration into the hepatic artery, relatively large liposomes may remain there for some time in the tumor vasculature. If this is true, then higher and more prolonged drug levels should be achieved in liver tumors, thus enhancing therapeutic efficacy. To prove this hypothesis, we studied the tumor- and normal-tissue drug distribution and antitumor activity of free and liposomal DXR injected into the hepatic artery of rats bearing well-established, visible walker 256 liver tumors. We report herein the results of this study.

Materials and methods

Liposome preparation

L-α-Dipalmitoyl phosphatidylcholine (DPPC; mol. wt., 735 kDa; purity, >98%; Nippon Oil & Fats Co., Ltd., Tokyo, Japan) was dissolved in a diisopropylether/chloroform (1 : 1, v/v) solution. DXR (Adriamycin Injection; Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) was dissolved in TRIS-HCl buffer (pH 6.01). Liposomes composed of DPPC were prepared by the reverse-evaporation vesicle method [28]. The encapsulated drug was separated from the nonencapsulated drug by centrifugation at 75,000 *g* at 4° C for 20 min, and the pelleted vesicles were resuspended in TRIS-HCl buffer. This step was repeated three times. The final lipid-to-drug weight ratio was 4 : 1. The concentration of total DXR was adjusted to 7.08 mg/ml. The concentration of nonencapsulated drug was <0.05% of the total concentration in the final preparation. The average diameter and size distribution of the liposomes was determined by a Laser Particle Analyzer (LPA-3000/3100; Otsuka Electronics Co., Ltd., Osaka, Japan) using the dynamic light-scattering (DLS) method.

The encapsulation efficiency (%E) was calculated using the following formula:

$$\%E = \frac{\text{Liposome-encapsulated DXR}}{\text{Total initial DXR}} \times 100.$$

The liposome-encapsulated drug was separated from the free drug by gel filtration through a Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, N. J., USA) column (10 × 200 mm) in TRIS-HCl buffer (pH 6.01). The amount of DXR was determined by high-performance liquid chromatography (HPLC) [31]. Briefly, the HPLC-analysis conditions were: Waters 6000 HPLC; Nova-Pak, 4 μm; 3.9 × 150 mm C18 column; mobile phase, 0.067 M KH_2PO_4: CH_3CN = 75 : 25 (v/v); flow rate, 0.8 ml/min; fluorescent detector, 585 nm emission and 470 nm excitation; sample solvent, 0.1 M KH_2PO_4 : CH_3OH = 1 : 1 (v/v); sample injection volume, 100 μl [28]. The lipid was quantitated by measuring the phosphorus content by the Bartlett assay [2].

Liver-tumor model

Walker 256 carcinosarcoma (W256; Shionogi & Co., Ltd., Osaka, Japan) cells (5×10^6 cells/rat) were surgically implanted into the left liver lobe of SLC Wistar/ST male rats (8 weeks old, 224–240 g; Japan SLC Co., Ltd., Hamamatsu, Shizuoka, Japan). Animals bearing W256 liver tumors were used for the biodistribution and antitumor-activity studies described below.

Biodistribution studies

At 9 days after W256 tumor implantation in the liver, all animals underwent a laparotomy. In animals showing visible liver tumor (90%), the prehepatic artery was cannulated via the gastric artery. Animals were then divided into 2 groups of 30 rats each. Each group received 2.35 mg/kg of either liposomal or free DXR injected into the hepatic artery through the cannulae. This dose was selected because it had been found to be the optimal dose of liposomal DXR against this tumor model in preliminary studies. The injection time was 1 min in all cases. The position of the cannulae was checked by a soft X-ray system (Softex-CSM-2; Softex Co., Ltd., Tokyo, Japan). The cannulation methods have been described by us in detail elsewhere [30]. At 5 min and 2, 8, 24, and 120 h after drug administration, six animals in each group were killed by exsanguination. The tumor, liver, heart, kidney, and spleen were resected and a plasma sample was obtained. Urine was also collected on an hourly basis through a bladder catheter. A volume of Kuthoff buffer equivalent to 5 times the tissue-sample weight was added, the tissues were homogenized, and the DXR was extracted with a 1 : 1 (v/v) butanol : toluene solution (5 ml/ml homogenized tissue). The samples were centrifuged at 3,000 *g* for 10 min. The supernatants were collected, dried, and dissolved in a 1 : 1 (v/v) phosphate-buffered saline (PBS) : methanol solution (1 ml/ml homogenized tissue). DXR concentrations in these samples were determined by the HPLC method described above. The area under the concentration-time curve (AUC) was calculated as the sum of the areas between every two time points.

Antitumor-activity studies

Tumor-growth inhibition. On day 7, 6-week-old rats bearing single W256 liver tumors underwent a laparotomy. Animals with visible liver tumor were divided into three groups of six animals each. They were injected with an equivalent volume of normal saline plus either free or liposomal DXR (2.35 mg/kg) into the hepatic artery using the cannulation techniques described above. The longest and shortest diameters (a and b, respectively) of the tumors were measured with calipers [5, 29] (error, <10%) before drug administration and 5 days later during a second laparotomy. The tumor volume was calculated as $a \times b^2/2$ [5, 29]. The tumor-size range on the day of drug administration was: $0.6 < a < 0.9$ cm and $0.4 < b < 0.6$ cm. The tumor-growth inhibition (%TGI) was calculated using the following equation:

$$\%TGI = \left(1 - \frac{\text{Tumor volume on day 12}}{\text{Tumor volume on day 7}}\right) \times 100.$$

Increased life span. The percentage of increase in life span (%ILS) was determined in a separate experiment. The design was identical to that of the TGI experiment, except for the addition of a control group (no treatment) of 15 animals. Before drug administration, the presence of liver tumors was surgically confirmed. The survival of each animal was recorded, and the %ILS was calculated by the following equation:

$$\%ILS = \left(\frac{\text{Median survival of treated group}}{\text{Median survival of control group}} - 1\right) \times 100.$$

Statistical analysis

Differences in biodistribution and TGI were analyzed for statistical significance using Student's *t*-test, and differences in median survival between the animal groups were analyzed by log-rank test.

Results

Characterization of liposomal DXR

As assessed by laser DLS, the liposomal DXR preparation was a homogeneous liposome suspension with a mean particle size of 850 ± 50 nm in diameter, the size of all particles (100%) ranging from 772 to 903 nm. The encapsulation efficiency of DXR in the liposomes was 38.14% ± 1.64% as determined by gel-filtration analysis of at least five different samples.

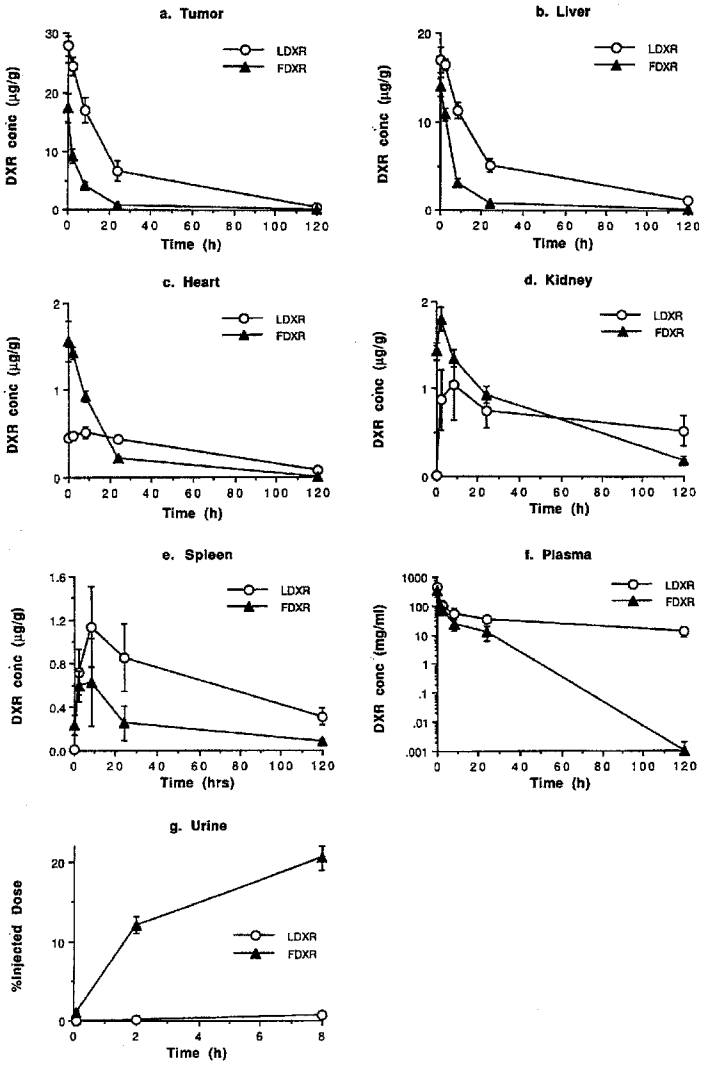

Fig. 1 a–g. Biodistribution of DXR after the administration of free and liposomal drug into the hepatic artery. Wistar rats bearing W256 liver tumors (30 rats/group) received a single dose (2.35 mg/kg) of free (*FDXR*) or liposomal DXR (*LDXR*) via the hepatic artery. DXR levels in **a** the liver tumor, **b** the liver parenchyma, **c** the heart, **d** the kidney, **e** the spleen, **f** the plasma, and **g** the collected urine are shown. Sampling points were 5 min and 2, 8, 24, and 120 h after drug administration. Tissue and plasma DXR levels are expressed as μg/g wet tissue and μg/ml plasma, respectively. Cumulative urinary excretion is expressed as a percentage of the DXR dose delivered. The lines with *round symbols* (○) represent the DXR levels determined after liposomal drug administration, and those with *dark triangular symbols* (▲) represent the levels measured after free drug injection. The *error bars* represent standard deviations

Organ distribution

Tumor DXR levels were increased by 1.6- to 7.9-fold at different time points in animals treated with liposomal DXR as compared with those receiving free DXR (Fig. 1 a). Liver parenchyma DXR levels were also increased by 1.2- to 8.3-fold in the rats that received liposomal DXR compared with those that received free drug (Fig. 1 b). Peak DXR levels measured in the liver tumor and liver parenchyma after the administration of liposomal DXR were 27.784 ± 1.688 and 17.062 ± 1.395 μg/g tissue, respectively, and values of 17.364 ± 2.371 and 14.006 ±

Table 1. AUCs, peak levels, and tumor/tissue ratios after administration of free and liposomal DXR into the hepatic artery[a]

Tissue and fluid	AUC (μg h g^{-1} or ml^{-1})		Peak level (μg/g or ml)		Tumor tissue ratios			
					AUC		Peak level	
	Free	Liposomal	Free	Liposomal	Free	Liposomal	Free	Liposomal
Tumor	155.4	725.2*	17.36 ± 2.37	27.78 ± 1.69*	1.0	1.0	1.0	1.0
Liver	142.6	553.5*	14.01 ± 1.08	17.06 ± 1.40**	1.1	1.3	1.2	1.6
Heart	29.5	36.4	1.56 ± 0.24	0.52 ± 0.06*	5.3	19.9	11.1	53.4
Kidney	84.2	81.8	1.80 ± 0.13	1.04 ± 0.40*	1.8	8.9	9.6	26.7
Spleen	27.9	78.4**	0.62 ± 0.40	1.14 ± 0.37**	5.6	9.2	28.0	24.4
Plasma	1.6	4.0**	0.33 ± 0.04	0.45 ± 0.09**	97.1	181	52.6	61.7
Urine	110.5	2.9*	20.51 ± 1.43	0.75 ± 0.13*	–	–	–	–

[a] The mean AUC, peak level, and AUC ratios or peak level ratios of tissue or plasma/tumor were calculated on the basis of the data in Fig. 1 (5 time points, 6 animals per point)

* $P < 0.01$, ** $P < 0.05$ (Student's t-test comparing two groups given free or liposomal DXR)

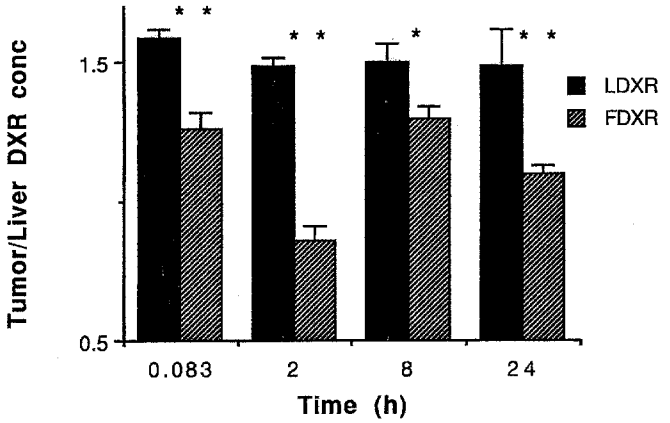

Fig. 2. Tumor/liver DXR ratios observed in the experiment described in Fig. 1. At each sampling time, six rats in each group were killed and DXR levels in the liver tumor and liver parenchyma were determined. The *dark bars* represent the ratios obtained in rats injected with liposomal drug (*LDXR*), and the *striped bars* represent those obtained in rats injected with free drug (*FDXR*). The *error bars* indicate the standard deviations. * P <0.001, ** P <0.001 (Student's t-test comparing two groups at different time points)

1.077 μg/g tissue, respectively, were obtained after the administration of free drug, thus suggesting a slightly more selective distribution of the drug into the tumor following administration of the liposomal form (P <0.01).

Heart DXR levels are shown in Fig. 1c. The peak level measured in animals treated with liposomal drug was 3 times lower than that determined in animals treated with free drug (0.520±0.062 vs 1.558±0.235 μg/g). In addition, the peak heart level occurred at 8 h after the injection of liposomal drug and at 5 min after treatment with free drug. After 24 h, no significant difference in heart drug levels was observed between the two groups of animals. In the kidney, the drug level was 2 times higher in animals receiving free DXR than in those treated with liposomal DXR until 24 h (Fig. 1d). In the spleen, DXR levels found in animals treated with the liposomal drug were about 3-fold those measured in rats treated with free drug (Fig. 1e). Figure 1f shows the plasma clearance of free and liposomal DXR. Plasma levels attained following the administration of liposomal DXR were 1.35- to 2.69-fold those reached after the injection of free drug. The peak plasma drug levels were 0.451±0.087 and 0.333± 0.043 μg/ml after the administration of liposomal and free drug, respectively. Urinary excretion of free DXR at 8 h was 20.514% of the injected dose, while the cumulative urinary excretion of liposomal DXR was only 0.746% (Fig. 1g).

Table 1 shows the DXR AUCs and peak levels obtained in the different tissues, plasma, and urine of rats and the AUC and peak level tumor/tissue ratios. The AUCs calculated after liposomal drug administration were several times greater than those determined after free drug injection in the tumor (4.7-fold, P <0.01), liver parenchyma (3.8-fold, P <0.01), spleen (2.8-fold, P <0.05), and plasma (2.5-fold, P <0.05). In the heart, no significant difference was observed. The cumulative urinary excretion of DXR was 37.7 times higher (P <0.001) in animals treated with free drug than in those receiving liposomal drug. Except in the spleen, all AUC or peak level ratios were higher in the liposomal DXR groups than in the free DXR group.

Table 2. Antitumor activity of free or liposomal DXR injected into the hepatic artery against W256 liver tumor[a]

Treatment	%TGI[b]	%ILS[b]
Control	–	0 (15)
Normal saline	–381.2±69.3 (6)	–5 (6)
Free DXR	–53.7±23.3 (6)	+27 (6)
Liposomal DXR	+16.1± 5.6* (6)	+108* (6)

[a] Results were obtained from two separate experiments: tumor-growth inhibition (TGI) and increased life span (ILS) of rats bearing liver tumors. A single injection (2.35 mg/kg) of free or liposomal DXR was given via the hepatic artery at 7 days after the implantation of 5×10^6 viable W256 cells into the left liver lobes of the animals

[b] The number of animals used in experiment are shown in parentheses

* P <0.05 as compared with the group given free DXR. In both experiments, the results obtained in DXR-treated groups differed significantly from those obtained in the normal saline or control groups

Tumor/liver and tumor/heart DXR ratio

The tumor/liver drug ratio after liposomal drug administration ranged between 1.49 and 1.59 until 24 h (Fig. 2). Following free drug administration, the tumor/liver ratio ranged between 0.86 and 1.30. At each different time point, the tumor/liver ratio was significantly higher for liposomal DXR than for free DXR (P <0.01). Therefore, higher drug levels were observed in the tumor in relation to the liver parenchyma following treatment with the liposomal drug. The tumor/heart ratio decreased from 57.62±1.57 at 5 min to 16.62±2.88 at 24 h after administration of the liposomal drug and from 10.55±0.73 at 5 min to 4.13±0.95 at 24 h after the injection of free drug. Therefore, at all time points, the tumor/heart ratio was about 5 times higher (P <0.001) in the animals treated with liposomal drug than in those treated with free drug.

Antitumor activity

One single dose of liposomal DXR (2.35 mg/kg) significantly inhibited the growth of W256 liver tumors and extended the life span of rats bearing liver tumors (Table 2). The same dose of free DXR did not result in significant antitumor activity. In the TGI experiment, the size of tumors in animals treated with liposomal drug decreased by 16%±5.6% over 5 days, whereas the size of tumors in animals treated with free drug increased by 53.7%±23.3% (P <0.05). In the survival experiment, the ILS was significantly prolonged in animals treated with liposomal drug as compared with those treated with free drug (108% vs 27%, P <0.05).

Discussion

In this study, the encapsulation of DXR in liposomes resulted in a preferential distribution of the drug into an

experimental liver tumor when the hepatic artery was used as the route of administration. The tumor AUC and peak drug levels were increased by 4 and 1.5 times, respectively, in animals that received the liposomal form of the drug. In good correlation with these findings, the antitumor activity was also increased in the animals treated with liposomal DXR as shown by a >4-fold increase in the %TGI and %ILS. An additional pharmacological advantage associated with the use of liposomal DXR was a 3-fold decrease in heart peak levels, thus suggesting that the previously described cardioprotective effect of liposome encapsulation is preserved by the use of the intraarterial route of administration.

Liver parenchyma levels of DXR were similar after the administration of free and liposomal DXR. Peak liver parenchyma levels were observed at 5 min for both forms of the drug; therefore, they were probably the result of a first-passage effect in both cases. Because liposomal DXR is to a greater extent delivered to liver Kupffer's cells, liver DXR levels after the administration of liposomal DXR probably represent a combination of drug taken up by Kupffer's cells, drug taken up by hepatocytes, and liposomes arrested in the hepatic-artery system vasculature. By contrast, liver DXR levels after the administration of free DXR mainly represent drug taken up by hepatocytes and in the process of being excreted in the biliary tree.

The i.v. administration of liposomal DXR results in increased peak plasma drug levels, prolonged elimination half-life, and preferential drug distribution to the liver and spleen, whereas heart and kidney drug levels are decreased. The present plasma pharmacokinetics and tissue levels are consistent with these findings, which suggests that most drug reaching the systemic circulation through the hepatic vein was in the liposomol form in animals treated with liposomal DXR.

In the present study, peak plasma levels measured in animals treated with liposomal DXR were no lower than those found in animals treated with free DXR. Given the similar liver-parenchyma levels and the higher tumor drug levels resulting from treatment with liposomal DXR, lower or similar peak plasma levels were expected following the injection of the liposomal form. However, the confinement of liposome-encapsulated drug within the vesicles in the vascular compartment and the negligible urinary excretion of liposomal DXR as compared with free DXR are the most likely explanations for the observed difference in peak plasma levels.

In summary, this study demonstrates a potential advantage for the treatment of well-developed liver tumors via the hepatic artery using liposomes as carriers of DXR. Although free DXR may not be the most suitable drug for intraarterial administration due to its low extraction ratio, the advantage provided by the liposome carrier as suggested by the present study can certainly be applied and deserves to be explored using other chemotherapeutic agents that are commonly delivered via the hepatic artery.

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