Hyperthermia-enhanced tumor accumulation and antitumor efficacy of a doxorubicin-conjugate with a novel macromolecular carrier system in mice with non-small cell lung cancer

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Abstract. A novel drug delivery system (DDS) compound was formed by binding doxorubicin hydrochloride (DXR) to the macromolecular carrier carboxymethyldextran polyalcohol (CM-Dex-PA) via the peptidyl spacer (GGFG: Gly-Gly-Phe-Gly). Its use in a murine tumor model confirmed that the DDS (CM-Dex-PA-GGFG-DXR) was retained in the blood and distributed in tumor tissue. The combined use of hyperthermia (HT: 41-42˚C for 40 min) and DXR-conjugate (5, 10 or 20 mg/kg i.v.) on tumor accumulation and efficacy was investigated in a murine model of non-small cell lung cancer. Tumor size was measured and the tumor inhibition rate (IR) was calculated. The mean tumor concentration of conjugated DXR in the DXR-conjugate group was 9.40 μg/g compared with 19.04 μg/g in the DXR-conjugate + HT group (p=0.0008). The antitumor efficacy of the DXR-conjugate was significantly enhanced in the groups receiving the combination therapy (p=0.0039, p=0.0250). Significant differences were found between the groups given DXR and those given DXR-conjugate (p=0.0492, p=0.0104). The results demonstrate that the antitumor efficacy of DXR-conjugate is significantly superior to that of DXR alone and the combined use of DXR-conjugate and HT increases the drug's concentration in the tumor, with significant enhancement of antitumor efficacy.

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Introduction

Conventional anticancer drugs have poor selective cytotoxicity, and severe side effects are a dose-limiting factor (1). Therefore, selective targeting of tumors by anticancer drugs is needed. Solid tumors generally possess some pathophysiological characteristics that lead to what is known as the 'EPR' effect (enhances permeability and retention) (2-6): a) hypervascularity; b) incomplete vascular architecture; c) secretion of vascular permeability factors that stimulate extravasation within the cancer; and d) little drainage of macromolecules and particles, which results in their long-term retention in the tumor.

The doxorubicin hydrochloride (DXR)-conjugate [macromolecular carrier peptidyl spacer (GGFG)-DXR, carboxymethyldextran polyalcohol (CM-Dex-PA)-GGFG-DXR] synthesized in the present study is retained in high concentration in the blood for a long time because of the nature of the CM-Dex-PA carrier, which enables passive tumor targeting based on the EPR effect (7-9). CM-Dex-PA has high water-solubility, thus enabling its conjugate to also be water-soluble. The main chain, dextran polyalcohol (Dex-PA), has structural flexibility and a similarity to polyethylene glycol (PEG) so it is not recognized as a foreign body by the reticuloendothelial system. Because its mean molecular weight is approximately 300 kilodaltons (kDa), it does not pass through the glomerular filter. Accumulation of DXR-conjugate in tumor tissue is proportional to the tumor blood flow and vascular permeability. The macromolecule is incorporated into tumor cells by endocytosis and DXR is released from the peptidyl spacer (GGFG: Gly-Gly-Phe-Gly) by lysosomal enzymes (i.e., cathepsins). After the DXR is released, CM-Dex-PA is slowly depolymerized in the lysosomal acidic environment and excreted (10,11) (Fig. 1).

We reviewed the literature for a new drug delivery system (DDS) compound and focused our attention on hyperthermia (HT), which is a recognized modality in interdisciplinary oncotherapy. HT alters the local tumor environment (12,13) and has a lethal effect on cancer cells, even when it is used

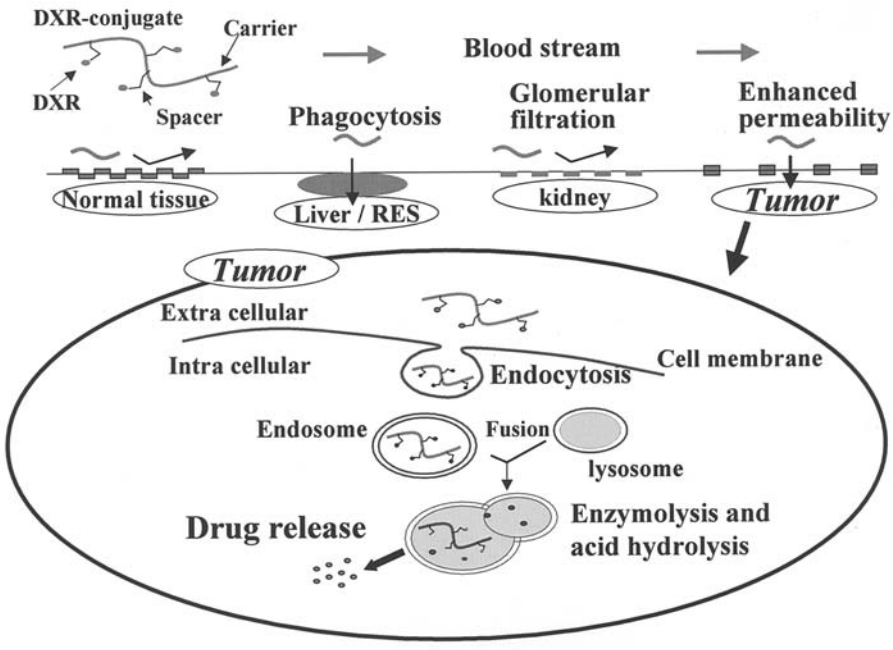

Figure 1. Schematic of passive tumor-targeting based on the EPR (enhanced permeability and retention) effect and drug-release mechanism of the doxorubicin (DXR)-conjugate.

alone (14). Tumor blood flow (15,16) and the permeability of tumor vessels (17-20) are increased at temperatures of 41-43˚C. Efficacy is enhanced by combined use with irradiation or anticancer drugs, so the present study was designed to investigate both the usefulness of DXR-conjugate in comparison with DXR alone and the usefulness of combining HT with DXR-conjugate in mice with non-small cell lung cancer (NSCLC).

Materials and methods

Animals and tumor model. Male 5-week-old BALB c-nu/nu nude mice (CLEA Japan Inc.) were used and the tumor model was created by subcutaneously injecting NSCLC strain LU99 cells (large cell carcinoma; $1x10^5$ cells). The long (L) and short (W) diameters of the tumor were measured with calipers and the weight was estimated as: V = (L*W*W)/2 (mg). The experiment was started when the estimated weight of the implanted tumors reached 200-400 mg (10-16 days after implantation).

DXR-conjugate. The DXR-conjugate (CM-Dex-PA-GGFG-DXR; Daiichi Pharmaceutical Co.) had 0.4% carboxymethylation, whereas the DXR content was 6.8% (Fig. 2).

Hyperthermia. Hyperthermia was achieved with a Thermotoron RF IV, a radiofrequency-type warmer apparatus used for animals and now widely used in the clinical setting (frequency, 8 MHz; maximum output, 200 W; Yamamoto Vinita Co., Ltd.). Radiofrequency waves at frequencies <100 MHz will warm deep tissue. The mice were anesthetized with pentobarbital (40 mg/kg i.p.) for immobilization during the 40-min HT period. The subcutaneous tumor only was warmed and when the temperature reached 40˚C, it was treated at 41-42˚C for 40 min. Rectal temperature was monitored and controlled so that it did not exceed 38˚C during HT.

Tumor drug concentrations. Two groups were established: group A in which 20 mg/kg of DXR-conjugate was administered intravenously (n=6), and group B which received 20 mg/kg of DXR-conjugate and HT (n=6). The dose of DXR-conjugate was determined as DXR equivalents. HT was completed in all animals within 1 h of the DXR-conjugate being administered and the mice were sacrificed 4 h (n=6 in each group) or 24 h (n=3 in each group) after the injection. The tumor was excised, weighed, and homogenized, 50% MeCN/0.5 N HCl was added to the homogenate, and then the mixture was centrifuged. The supernatant was collected and the concentrations of conjugated DXR and free DXR in the tumor were determined by high-performance liquid chromatography. The results were analyzed by Mann-Whitney U test, and statistically significant differences were identified.

Antitumor efficacy. Eight groups (n=6) were established: group I, no treatment; group II, DXR 10 mg/kg i.v.; group III, DXR-conjugate 10 mg/kg i.v.; group IV, DXR-conjugate 10 mg/kg i.v. + HT; group V, DXR 5 mg/kg i.v.; group VI, DXR-conjugate 5 mg/kg i.v.; group VII, DXR-conjugate 5 mg/kg i.v. + HT; and group VIII, HT. The experiment was started when the estimated weight of the tumors reached 200-400 mg. Tumor size was measured daily for 14 days after the treatments began. Mild HT at 41-42˚C was induced for 40 min in groups A and B under the same conditions used for the experimental determination of the drug concentrations in the tumors. Because tumors implanted in nude mice do not usually exhibit sustained growth, antitumor efficacy was evaluated on the basis of relative growth. Estimated tumor volume at the start of the experiment was designated as V0, and tumor volume on the day (n) of each determination (Vn) was divided by V0 (Vn/V0). The mean Vn/V0 was calculated for each treatment group (VT) and in the control group (VC). The tumor inhibition rate (IR) was calculated as: IR = (1 - VT/VC) 100% (21,22).

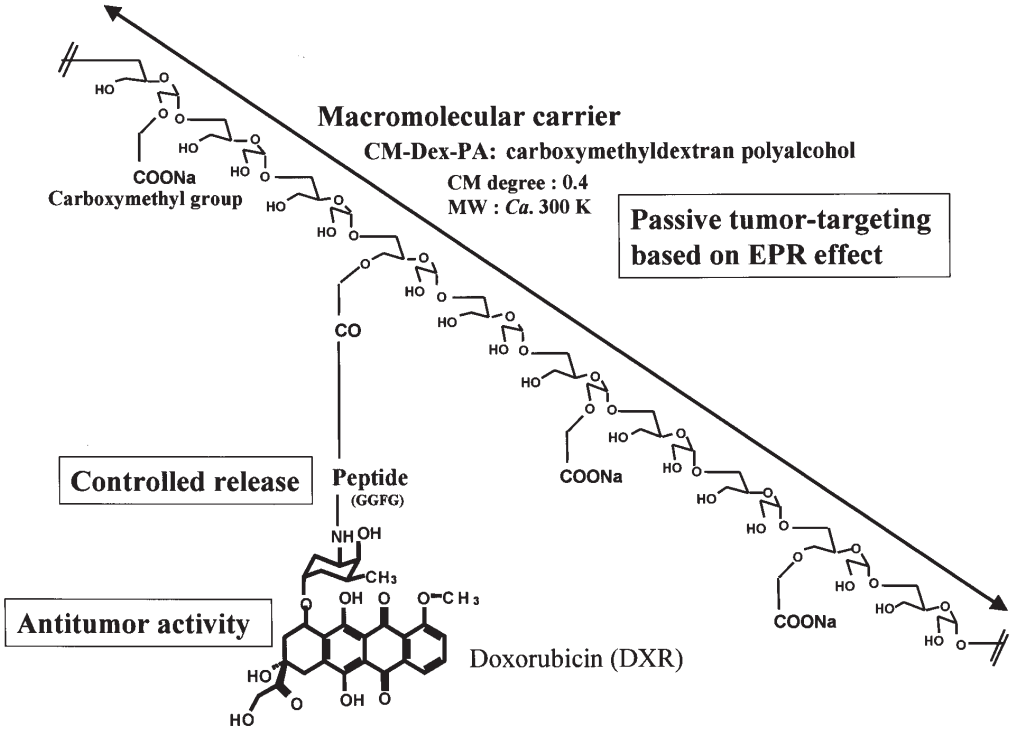

Figure 2. Partial structure of the doxorubicin (DXR)-conjugate (CM-Dex-PA-peptide-DXR).

Table I. Tumor concentrations of doxorubicin (DXR)-conjugate and free DXR at 4 h after administration with or without hyperthermia.

Group	Conjugated DXR (μg/g)	Free DXR (μg/g)	Total (μg/g)	Free/Total (%)
A (DXR-conjugate 20 mg/kg i.v.)	9.17±1.18	0.56±0.05	9.73±1.16	6.23±0.97
B (DXR-conjugate 20 mg/kg i.v. + HT)	19.04±2.51[a]	0.96±0.17	20.00±2.61[b]	4.98±0.78

[a]Statistically significant compared with conjugated DXR of group A, p=0.008. [b]Statistically significant compared with total of group A, p=0.007. Data are expressed as mean ± SE.

Toxicity was determined by the decrease in body weight. The results were analyzed by Mann-Whitney U test, and statistically significant differences were identified.

Results

Tumor accumulation of DXR-conjugate with or without HT. Table I shows the concentrations of conjugated DXR and free DXR in the excised tumors from group A (DXR-conjugate 20 mg/kg i.v.) and group B (DXR-conjugate 20 mg/kg i.v. + HT). The weight of the excised tumors was 541.07±236.99 mg in group A and 550.72±292.21 mg in group B, and the difference between the groups was not significant (p=0.379). The mean concentration of conjugated DXR was 9.17±1.18 μg/g in group A and 19.04±2.51 μg/g in group B. Thus, the concentration of conjugated DXR was approximately doubled by combined use of HT, and the difference was significant (p=0.008). The mean concentration of free DXR was 0.56±0.05 μg/g in group A and 0.96±0.17 μg/g in group B, and the difference was not significant (p=0.066). The mean total concentration of conjugated DXR and free DXR was 9.73±1.16 μg/g in group A and 20.00±2.61 μg/g in group B, and thus the drug concentration in the tumor was approximately doubled by the combined use of HT, and the difference was significant (p=0.007). The mean rate of DXR release calculated as the ratio of the concentration of free DXR to the total concentration (free DXR/total DXR) was 6.23±0.97% in group A and 4.98±0.78% in group B, and the difference between the groups was not significant (p=0.262).

Antitumor efficacy of DXR-conjugate with or without HT. Because tumor blood flow volume is generally large when the growth rate is high, and decreases when the growth rate is low (23), the experiment was started at an appropriate time for assessing antitumor efficacy (i.e., when the estimated tumor weight reached ~300 mg). There were no significant

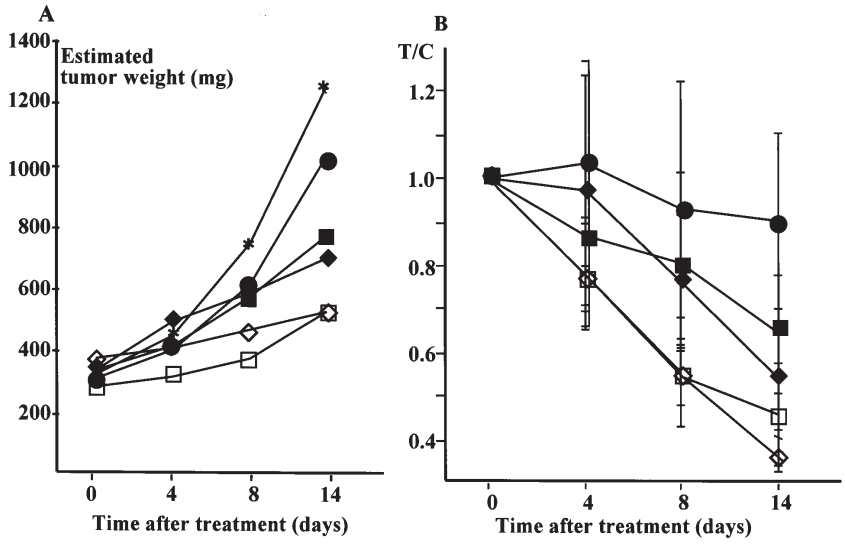

Figure 3. Individual tumor growth curves (A) and antitumor efficacy of doxorubicin (DXR)-conjugate with or without hyperthermia (HT) (B). *, no treatment; ■, DXR-conjugate 5 mg/kg i.v.; ◆, 10 mg/kg i.v.; □, 5 mg/kg i.v. + HT; ◇, 10 mg/kg i.v. + HT; ●, HT.

Table II. Antitumor efficacy of doxorubicin (DXR)-conjugate with or without hyperthermia.

Group	Treatment	Estimated tumor weight (mg), mean ± SE	Relative tumor volume	Inhibition rate (%)
I	No treatment	1254.67±163.3	1	0
II	DXR 10 mg/kg i.v.	989.3±251.2	0.738	26.2[a]
III	DXR-conjugate 10 mg/kg i.v.	701.9±105.3	0.539	46.1[a,b]
IV	DXR-conjugate 10 mg/kg i.v. + HT	527.4±52.2	0.375	63.5[b]
V	DXR 5 mg/kg i.v.	1175.6±195.9	0.919	8.0[c]
VI	DXR-conjugate 5 mg/kg i.v.	915.5±189.1	0.638	36.2[c,d]
VII	DXR-conjugate 5 mg/kg i.v. + HT	528.2±106.5	0.479	52.0[d]
VIII	HT	1032.8±143.7	0.864	13.6

Estimated tumor volume (mg) = 0.5 x (length x width^2). Relative tumor volume = (average tumor volume of each group)/(average tumor volume of group I). Inhibition rate (%) = (1-relative tumor volume) x 100. [a]p=0.0492, [b]p=0.0039, [c]p=0.0104, [d]p=0.0250.

differences between any of the groups in estimated tumor weight at the start of the experiment. The growth rate of the implanted tumor increased after the estimated weight exceeded 300 mg, and the growth rate increased ~1.5-fold after the weight exceeded 400 mg (Fig. 3A). Tumor size in the control group increased ~4-fold within 14 days (Fig. 3A). In group II (DXR 10 mg/kg i.v.) and group V (DXR 5 mg/kg i.v.). The inhibition rate (IR), which reflects antitumor efficacy, was 26.2 and 8.0%, respectively, as opposed to 46.1 and 36.2% in group III (DXR-conjugate 10 mg/kg i.v.) and group IV (DXR-conjugate 5 mg/kg i.v.), respectively. Comparison of DXR-conjugate and DXR at the same doses revealed significant enhancement of drug activity in groups III and VI (p=0.0492, p=0.0104), demonstrating the usefulness of the DXR-conjugate as a DDS compound (Table II). IR was significantly increased by HT at every dose (Table II) and was cytocidal in group VIII.

Kumazawa *et al* have shown that a DDS using the CM-Dex-PA carrier reduced toxicities (10) and Shiose *et al* reported that the DXR-conjugate has lower toxicities than DXR alone (personal communication). In the present study the side effects of DXR-conjugate with HT were evaluated on the basis of body weight loss (Fig. 4, Table III). Comparison of the weight loss in groups III and IV and groups VI and VII showed that the differences were not significant (p=0.1282, p=0.1093), but the weight loss in the groups with HT (IV and VII) tended to be less than in the groups without HT (III and VI). Group VIII (HT) lost the least body weight.

Discussion

There is a long history of macromolecular drugs and a number of studies have been conducted since 1975 when the basic model was proposed by Ringsdorf (24). The DXR-conjugate

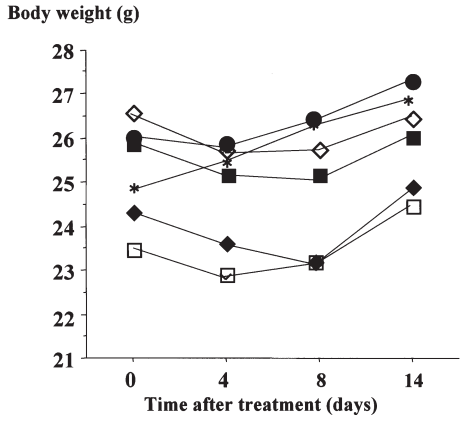

Figure 4. Change in body weight. *, no treatment; ■, DXR-conjugate 5 mg/kg i.v.; ◆, 10 mg/kg i.v.; □, 5 mg/kg i.v. + HT; ◇, 10 mg/kg i.v. + HT; ●, HT.

Table III. Toxicity of treatment with doxorubicin (DXR)-conjugate with or without hyperthermia.

	BWLmax % (day)
No treatment	0
DXR-conjugate (10) i.v.	4.9 (8)
DXR-conjugate (10) + HT	3.4 (4)
DXR-conjugate (5) i.v.	3.9 (8)
DXR-conjugate (5) + HT	3.2 (4)
HT	0.5 (4)

BWLmax, maximum rate of body weight loss.

Table IV. Tumor concentrations of doxorubicin (DXR)-conjugate and free-DXR 24 h after administration with or without hyperthermia.

	Conjugate DXR (μg/g)	Free DXR (μg/g)	Total (μg/g)	Free/Total (%)
DXR-conjugate 20 mg/kg i.v.	4.41±1.55	4.02±0.85	8.427±2.38	50.20±5.53
DXR-conjugate 20 mg/kg i.v. + HT	9.50±2.06	6.28±0.90	15.78±2.97	40.57±2.09

Data are expressed as mean ± SE.

was developed from the concept that long-term high retention of the conjugate in the blood leads to an efficient EPR effect, and release of DXR in the tumor, but not in the blood (Shiose *et al,* personal communication). The DXR-conjugate also showed both biocompatibility (i.e., not recognized as a foreign body in the reticuloendothelial system and no antigenicity) and appropriate excretion, because of its hydrophilia and the charge of the CM-Dex-PA [polyalcoholization (PA) and carboxymethylation (CM) of Dex] carrier. Because of its high mean molecular weight (approximately 300 kaD), the DXR-conjugate does not pass through the glomerular filter, and high concentrations are maintained in the blood. The anti-cancer effect of DXR is dose-dependent, and its rate of release is an important factor in its efficacy. The rate of release is controlled by the peptidyl spacer and varies with the combination of amino acids. Because a peptidyl spacer with a high release rate is considered appropriate for use with dose-dependent anticancer drugs such as DXR, we used GGFG (Gly-Gly-Phe-Gly) for the DXR-conjugate. The biotransformation of DXR-conjugate and DXR in MethA-cancer-bearing mice was shown by Shiose *et al* (personal communication). The area under the blood concentration-time curve of DXR-conjugate in the tumor during the period from 2 to 48 h ($AUC_{2\text{-}48\ h}$) was approximately twice as high as that of DXR, and the $AUC_{2\text{-}48\ h}$ of DXR-conjugate in the liver was approximately 50% higher than that of DXR. The tumor selectivity of the DXR-conjugate (tumor $AUC_{2\text{-}48\ h}$/liver $AUC_{2\text{-}48\ h}$) was approximately 4-fold higher than that of DXR.

Tumor accumulation of DXR-conjugate with or without HT. Cancer therapy based on the EPR effect of macromolecular and liposome preparations has attracted interest in the last decade (25-30), and research on treatment methods that combine administration of such preparations with HT is also progressing. Some studies have shown that tumor accumulation of macromolecular and liposome preparations is increased by HT-induced microenvironmental changes in tumor tissue (29,31-34). In general, mild HT at 39-42˚C induces an increase in tumor tissue blood flow volume (15,16), tumor vessel hyper-permeability and extravasation (35-39). However, HT at 43˚C or higher temperatures induces vascular injury, haemorrhage, and collapse, and a decrease in extravasation (38,40-43). Thus, the effect of HT on tumor tissue and tumor drug accumulation varies with the thermal dose. In the present study HT was conducted at 41-42˚C for 40 min to increase tumor accumulation of DXR-conjugate. The drug's concentration in the tumors was determined 4 h after administration (i.e., when the change in tumor tissue induced by HT was most prominent). After the combination treatment, the concentration of conjugated DXR and the total drug concentration (conjugated DXR + free DXR) in the tumor was approximately doubled. At 4 h after administration there was no significant difference in the rate of DXR release (free DXR/total dose) between administration with and without HT, suggesting that the rate of drug release is unaffected by HT. The drug's concentration in the tumors was determined 24 h after administration to investigate differences in drug concentrations in the tumor. Both the

concentration of conjugated DXR and total dose of drugs were less than at 4 h after administration in both the DXR-conjugate group and DXR-conjugate + HT group. However, the total dose of drugs was still approximately 2-fold higher than baseline at 24 h after administration, and the drug concentration in the tumor was still higher when HT was used (Table IV). These results support the suggestion that HT has an effect on the pharmacokinetics of DXR-conjugate, and the results were similar to those of conventional studies in which HT has been combined with liposomes (31,44). The widening and opening of gaps between endothelial cells may account for the increased tumor drug delivery (31,36,38,39,45). Functional and structural studies have shown that large pores exist in tumor vessels (46). Hyperthermic conditions lead to a rapid reduction and rearrangement of endothelial cell F-actin stress fibres (47,48), which would allow larger pores to be formed between cells (39). If the HT does not exceed 43˚C, the change in the endothelium appears to be reversible within 24 h (47). One study in a model of ovarian carcinoma (SKOV-3) showed that 6 h after HT (41˚C for 1 h) the degree of extravasation of nanoparticles had returned to baseline (38). Thus the tumor model used, the size of the drug, and the thermal dose affect the results.

Antitumor efficacy of DXR-conjugate with or without HT. From the findings of the dose-response relationship after administering 1.25-30 mg/kg of DXR-conjugate (data not shown), the maximum tolerated dose (MTD) of DXR-conjugate in nude mice was estimated to be approximately 40 mg/kg, and one-quarter and one-eighth of the MTD were used in the assessment of antitumor efficacy. The IR increased from 26.2 to 46.1% in the DXR 10 mg/kg group and DXR-conjugate 10 mg/kg group and from 8.0 to 36.2% ($p<0.05$) in the DXR 5 mg/kg group and the DXR-conjugate 5 mg/kg group respectively. Tashiro reported that the MTD of DXR in nude mice is 12 mg/ kg, with an IR of LU99 cells of 28% (22). In the present study the IR of DXR 10 mg/kg was nearly equal to that of DXR at the MTD. Another study has shown that the sensitivity of LU99 to DXR is probably low because of the distribution of DXR in the tumor tissue, which depends on the pH (49,50) and is higher in the range of 6.2-7.6 (50). The results of the present study show that antitumor efficacy was enhanced by using a DDS with a macromolecular carrier (i.e., accumulation of DXR in tumor tissue).

Comparison of the IR in the DXR-conjugate 10 mg/kg group and the DXR-conjugate 10 mg/kg i.v. + HT group showed that it increased from 46.1 to 63.5%, and from 36.2 to 52.0% ($p<0.05$) between the DXR-conjugate 5 mg/kg and DXR-conjugate 5 mg/kg + HT groups. Thus, antitumor efficacy was enhanced to almost the same extent, 16-17 points, in the groups in which HT was used. Because the IR was 13.6% in the group treated with HT alone, we consider that enhancement of the antitumor efficacy of the DXR-conjugate by HT is an additive effect to its own cytocidal effect of HT (Tables III and IV). The evaluation of the toxicity of DXR-conjugate using weight loss as an indicator showed that toxicity tended to be reduced by the combined use of HT. The specific mechanism remains unknown, but one possible reason is that the drug's concentration in the tumor was enhanced and thus the tumor selectivity of the DXR-conjugate was increased by the combined use of HT.

A newly synthesized compound, DXR-conjugate, exerted antitumor efficacy that was significantly superior to that of DXR alone in a nude mouse model. In addition, combined use of DXR-conjugate and HT resulted in an increase in the DXR-conjugate concentration in the tumor, significantly enhancing the drug's antitumor efficacy. Using weight loss as an indicator, toxicity was not increased by the combined use of HT.

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