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Comparison of free and liposome encapsulated doxorubicin tumor drug uptake and antitumor efficacy in the SC115 murine mammary tumor

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Summary

Tumor drug uptake and antitumor efficacy of free and liposomal doxorubicin (DOX) were determined in the SC115 Shionogi mouse mammary tumor. Liposomal DOX systems were prepared by pH gradient-driven drug encapsulation in 170 nm egg phosphatidylcholine/cholesterol (55:45, mol ratio) vesicles. Intravenous injection of free DOX at 6.5 mg/kg, the maximum tolerated dose for free drug in the multiple dose therapy regimen, resulted in tumor-associated drug levels of 2.0 μg/g tissue at 1 h which remained constant over 24 h. Liposomal DOX injected at 6.5 mg/kg led to an accumulation of drug in the tumor from 2.6 μg/g tissue to 5.5 μg/g tissue between 1 h and 24 h, respectively. Increasing the dose of liposomal DOX to 13.0 mg/kg increased tumor drug uptake levels to 5.7 μg/g and 10.2 μg/g tissue at 1 h and 24 h, respectively. Administration of free or liposome encapsulated DOX every 7 days for 3 weeks resulted in a dose-dependent decrease in tumor growth rate. However, liposomal DOX injected at 6.5 mg/kg exhibited enhanced tumor growth inhibition compared to an equivalent dose of free drug. Further, the ability to administer increased doses of the less toxic liposomal DOX not only resulted in a greater inhibition of tumor growth but also significantly reduced tumor weight. Tumors weighing as much as 5 g were diminished to less than 0.5 g upon treatment with liposomal DOX at a dose of 13 mg/kg. In addition, groups receiving the highest liposomal DOX dose exhibited 25% complete tumor regression which persisted over the 50-day study period. These results demonstrate the ability of appropriately designed liposomal DOX systems to significantly enhance the delivery and retention of drug at solid tumor sites, resulting in increased therapeutic activity.

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Abbreviations: EPC, egg phosphatidylcholine; Chol, cholesterol; DOX, doxorubicin; MTD, maximum tolerated dose; EDTA, ethylenediaminetetraacetic acid.

Keywords: liposomal doxorubicin; tumor drug uptake; antitumor efficacy.

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Introduction

Numerous studies have addressed the use of liposome encapsulated DOX in the treatment of various tumor types. Results from several laboratories indicate that liposome encapsulation of DOX does not compromise drug potency while buffering toxic side effects [1,6,13,14,18,19,22], most notably cardiotoxicity [1,5,6,13,18,22]. This ability of liposomes to enhance the therapeutic potential of DOX has been demonstrated in a variety of metastatic [6—8,15], ascitic leukemia [13,14,18—20] and solid tumor models [5,19,22].

Although the above studies clearly establish the potential utility of liposome encapsulation in improving the therapeutic activity of DOX, little is known about the mechanism whereby this effect is achieved. For example, recent reports have demonstrated that manipulation of liposome characteristics can result in enhanced vesicle accumulation in solid tumors [9]. However, it is unclear whether this capability to improve delivery of entrapped drugs to tumor sites necessarily increases antitumor efficacy. Investigations by Gabizon et al. [7] employing a metastatic liver tumor model implied that such a relationship may be important in determining antitumor activity. The relevance of this implication to tumors residing at sites that do not inherently accumulate liposomes is still unresolved.

In order to better understand the comparative antitumor activities of free and liposome encapsulated DOX, we have investigated tumor drug uptake characteristics and efficacy behaviour of these systems in the transplantable androgen-responsive Shionogi mouse mammary carcinoma (SC115). This tumor arose spontaneously in a female mouse of the DD/S strain. After 19 passages in male mice, an androgen-responsive variant was isolated [16]. Although this tumor grows in males, selection of this tumor model was based on the observations that this mouse mammary tumor is similar to many human breast cancers in its sensitivity to different classes of steroid hormones, including androgens [2,10], estrogens [17] and glucocorticoids [23]. In addition, we have previously shown that free DOX administered for three weeks at 7 day intervals is effective in causing growth delay of the SC115 tumor [4]. It is demonstrated here that appropriately designed liposomal DOX systems permit the use of higher DOX doses and increases delivery of drug to the tumor, resulting in improved antitumour activity over free DOX.

Materials and methods

DOX was obtained from Adria Laboratories (Mississauga, Ont.). Egg PC was obtained from Avanti Polar Lipids (Birmingham, Al). Cholesterol salts were obtained from Sigma Chemical Co. (St. Louis, MO).

Liposomes were prepared by hydrating a thin film of EPC/chol (55:45 mol ratio) in 300 mM citric acid buffer (pH 4.0) at a concentration of 100 mg lipid/ml buffer with vortex mixing. After freezing and thawing the multilamellar vesicles 5 times, the liposomes were extruded 10 times through 2 stacked 0.2 μm pore size polycarbonate filters [12]. The resulting vesicles had a mean diameter of 170 nm as determined by quasielastic light scattering. The vesicles were then titrated to pH 7.8 with 0.5 M Na_2CO_3. These vesicles were then heated at 60 °C for 5 min and added to solid DOX (Adriamycin® from Adria Labs) to achieve a drug-to-lipid ratio of 0.27:1 (w/w). The mixture was subsequently heated at 60 °C for 10 min. Under these conditions, greater than 98% of the drug was sequestered into the liposomes as determined by column chromatography [11]. Liposomal DOX solutions were diluted with sterile saline to achieve drug concentrations appropriate for administering proper drug doses to mice in 0.2 ml.

The Class I SC115 subline was used in these experiments [3]. It was maintained by serial transplantation in male mice of the DD/S strain as previously described [4]. Tumors weighing approximately 2 g were dissociated in 0.05% trypsin (1:250) and 0.025% EDTA

(Sigma Chemical Co., St. Louis, MO) in Ca^{2+}- and Mg^{2+}-free saline A (pH 7.3) and the cell suspension centrifuged at 80 × g for 4 min to enrich the epithelial cell population. The pellet was resuspended in Dulbecco's modified Eagle's medium (DME; Terry Fox Laboratory, Vancouver, B.C.) and passed through a 150 μm Nitex filter (Tetko, Inc., Elmsford, NY) to collect single cells and small cell aggregates. Viable cells, determined by Trypan blue exclusion, were counted on a hemacytometer. Suspensions of 3×10^6 cells in 0.1 ml of DME were injected s.c. into the interscapular region of male mice 2—4 months old. Mice were randomly distributed to the different treatment groups ($n = 9$) immediately following tumor cell injection.

Tumor uptake of DOX was determined for free and liposomal DOX at the maximum tolerated dose (MTD) of free drug (6.5 mg/kg, 3 × weekly regimen) as well as for the MTD of liposomal drug (13.0 mg/kg, 3 × weekly regimen). Mice bearing the SC115 subcutaneous tumor (0.5—1.0 g) were injected i.v. with the indicated dose of free DOX or liposomal DOX containing [^{3}H]cholesterol hexadecylether as a lipid marker (200 dpm/μg lipid). At the indicated times mice, (4 per group) were anesthetized with ether and plasma was recovered from blood collected via heart puncture into 'Microtainer' tubes (Becton-Dickenson, Richmond, B.C.) containing EDTA beads. Tumors were removed and stored frozen at −70°C. Tumor liposomal lipid levels were determined by preparing a 10% tissue homogenate and determining the radioactivty in an aliquot of solubilized (employing Protosol, NEN Nuclear, Mississauga, Ont.) tumor by scintillation counting. Plasma and tumor DOX levels were determined by monitoring the fluorescence of extracted samples [2] at 550 nm. Tumor drug and liposomal lipid levels were corrected for endogenous plasma volume contributions in individual mice on the basis of ^{14}C-containing 100 nm distearoylphosphatidylcholine/cholesterol liposome levels in the tumor 5 min after their injection i.v. at a dose of 100 mg lipid/kg. Under the conditions employed, >95% of these liposomes were recovered in the plasma and tissue blood volume corrections can consequently be made for each tumor drug level determination. The validity of such corrections were confirmed by determining tumor blood volumes for representative mice injected with ^{51}Cr-labelled red blood cells.

For efficacy experiments, tumor-bearing mice were palpated 3 times/week until tumors were measurable, after which caliper measurements were made. Tumor weights were calculated according to the formula [21]:

$$\frac{\text{length (cm)} \times [\text{width (cm)}]^2 = \text{g}}{2}$$

Comparisons of calculated and actual (measured) tumor weights indicated that calculated tumor weights were accurate within ± 10%. Upon growth of the tumor to 0.3—2.5 g mice were administered saline, empty liposomes (administered at a dose equivalent to that given for a liposomal DOX dose of 13 mg/kg), free DOX and liposomal DOX i.v. at the indicated doses at 7 day intervals (3 injections of the indicated dose). Mice whose tumors did not reach a size of 0.5 g by day 25 post tumor transplant were characterized as 'no takes' and were removed from the study. Treatment doses were based on the initial animal weights prior to tumor inoculation. Tumor growth was monitored 50 days post first treatment. Statistical significance of differences in the group means of tumor weights were determined employing Student's *t*-test (2 sided).

Results

The liposomal DOX preparation employed here demonstrated reduced toxicity compared to the free drug. The MTD for free and liposomal DOX was 6.5 mg/kg per injection and 13.0 mg/kg per injection, respectively, for the day 1, 8, 15 i.v. dose regimen utilized in therapy evaluations. At the 13.0 mg/kg liposomal DOX dose, no deaths could be directly related to drug toxicity. In contrast, administration of

free DOX at a dose of 6.5 mg/kg resulted in one toxic related death and a dose of 13.0 mg/kg per injection caused a 70% mortality rate (data not shown). This 2-fold increase in MTD compares well with our previous studies employing similar liposomal DOX systems in other mouse strains [13]. Plasma and tumor drug uptake levels were therefore determined over 24 h post injection for free DOX at 6.5 mg/kg and liposomal DOX at 6.5 mg/kg and 13.0 mg/kg (Table 1). Low plasma DOX levels were observed between 1 h and 24 h after administration of free DOX. The 1-h value of 0.8 μg/ml corresponded to 0.5% of the injected dose being present in the plasma compartment. This value decreased to 0.03 μg/ml plasma at 24 h. Free DOX accumulation in tumor tissue occurred within the first hour after drug injection, yielding approximately 2 μg/g tissue which was maintained over the 24 h time course.

Liposomal DOX administered at a dose equal to free DOX displayed significantly different pharmacological properties. Plasma drug levels at 1 h and 4 h were 20—25-fold higher than observed for free DOX and 2.3-fold higher at 24 h. More importantly, whereas free DOX tumor levels remained constant over 24 h, tumor drug levels increased from 2.6 μg/g to 5.5 μg/g between 1 h and 24 h for liposome encapsulated DOX. It should be noted that the drug to liposomal lipid ratio present in tumor tissue decreased with increasing time. This is due to the fact that the liposomes employed in this study release entrapped DOX while in the circulation (see plasma drug and lipid levels, Table 1). Hence, liposome accumulation in the tumor at later time points resulted in relatively smaller increases in tumor drug levels. Increasing the dose of liposomal DOX to the MTD (13.0 mg/kg) increased both plasma and tumor DOX levels compared to the 6.5 mg/kg dose. The magnitude of the increase in tumor DOX uptake was comparable to the 2-fold increase in liposomal DOX dose.

Figure 1 shows the rate of tumor growth in individual mice treated with saline, free DOX

Table 1. Plasma and tumor doxorubicin levels after i.v. injection of free and liposomal doxorubicin to Shionogi mice bearing SC115 tumors[a].

Sample	Drug dose (mg/kg)	Time (h)	Plasma		Tumor[b]	
			Dox (μg/ml)	Lipid (μg/ml)	Dox (μg/g tissue)	Lipid (μg/g tissue)
Free Dox	6.5	1	0.8 ± 0.13	—	2.0 ± 0.7	—
		4	0.4 ± 0.06	—	2.1 ± 0.8	—
		24	0.03 ± 0.01	—	1.9 ± 0.8	—
Lipodox	6.5	1	19.8 ± 1.2	290 ± 35	2.6 ± 0.4	4.6 ± 1.3
		4	8.2 ± 1.1	143 ± 27	4.2 ± 0.5	23.3 ± 3.1
		24	0.07 ± 0.01	14.7 ± 1.4	5.5 ± 1.1	47.1 ± 16.3
Lipodox	13.0	1	47.5 ± 7.4	421 ± 31	5.7 ± 1.1	19.3 ± 4.5
		4	37.0 ± 5.9	369 ± 41	12.1 ± 2.7	65.0 ± 12.0
		24	1.4 ± 0.4	44.5 ± 19.2	10.2 ± 3.6	102.6 ± 26.3

[a]Mice (4 per group) were injected with free or liposomal doxorubicin via a lateral tail vein. Drug levels represent the mean and standard deviation.

[b]Tumor drug and liposomal lipid levels were corrected for tumor plasma volume contributions as described in Materials and methods. For liposomal doxorubicin groups, drug and liposomal lipid levels were determined by monitoring fluorescence at 550 nm and radioactivity, respectively as described in Materials and methods.

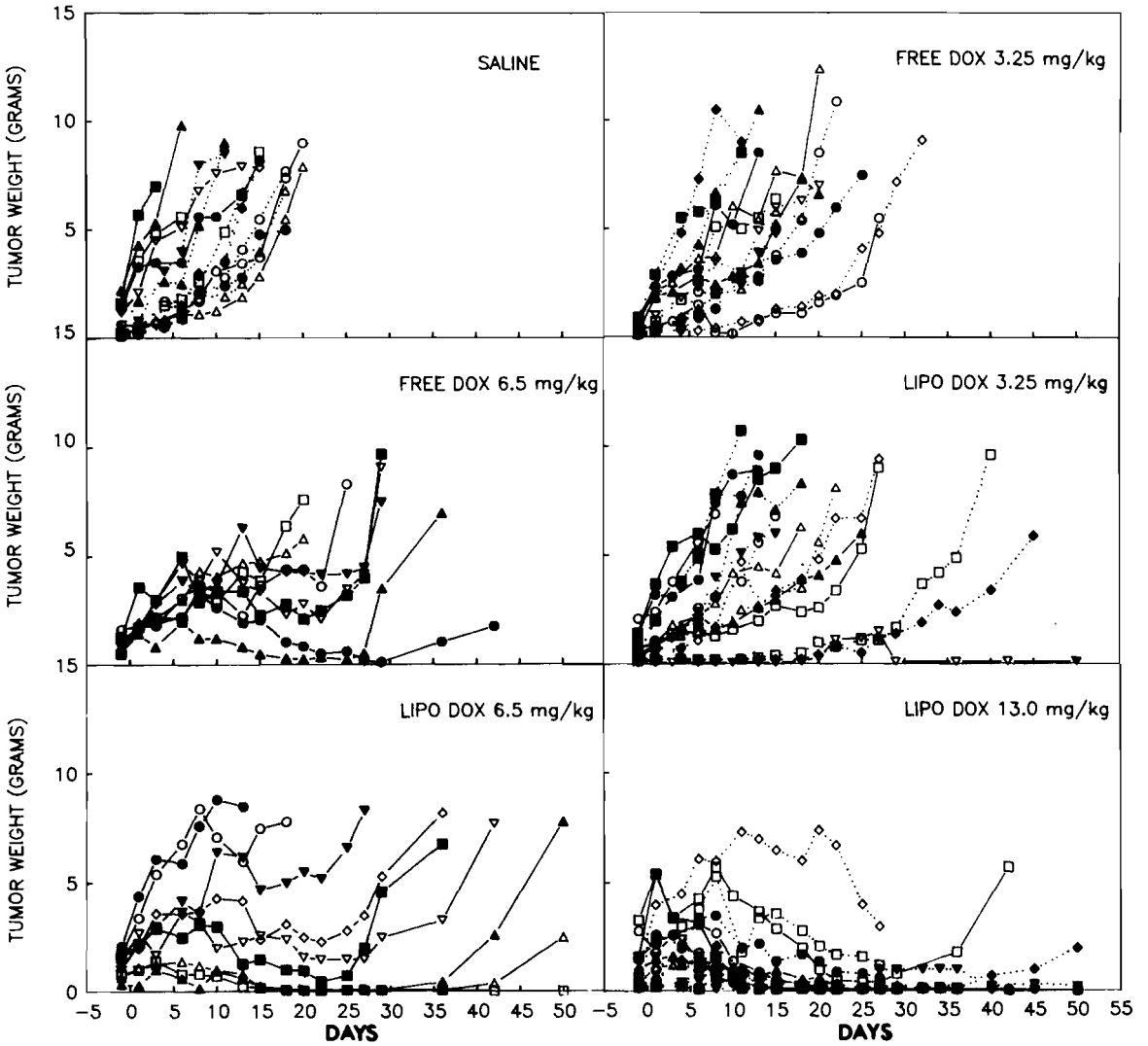

Fig. 1. Tumor growth curves of individual mice receiving the indicated treatments of saline and DOX in free and liposomal form. Experimental conditions are as described in Materials and methods. The indicated drug doses were administered on days 1, 8 and 15. The solid and dotted lines represent separate experiments.

at 3.25 mg/kg per injection and 6.5 mg/kg per injection and liposomal DOX at 3.25 mg/kg per injection, 6.5 mg/kg per injection and 13.0 mg/kg per injection. Mice receiving saline exhibited rapid tumor growth and all animals either died or had to be killed due to debilitating tumor growth within 20 days of the first treatment. Comparable results were obtained for mice administered empty liposomes (prepared identically to drug-containing liposomes) at a dose equivalent to the highest liposomal DOX dose (data not shown). In contrast, administration of DOX in either free or liposomal form caused a dose-dependent decrease in the tumor growth rate.

The relative antitumor activities of the var-

Table 2. Effect of free and liposomal doxorubicin on SC115 tumor growth[a].

Treatment group	Day 1 Tumor wt. (g) Mean ± S.D.	Day 8 Percent increase in mean tumor weight ± S.D. (Compared to day 1)	30-Day survivors[b]	Complete tumor regression[c]
Saline	0.7 ± 0.6	1283 ± 621	0/16	0/16
Free Dox 3.25 mg/kg	0.4 ± 0.3	1178 ± 740	1/14	0/14
Free Dox 6.5 mg/kg	0.9 ± 0.4	307 ± 201[d]	2/8	0/8
Lipodox 3.2 mg/kg	0.6 ± 0.5	743 ± 705	3/16	0/16
Lipodox 6.5 mg/kg	1.3 ± 0.6	135 ± 132[d,e]	6/9	1/9
Lipodox 13.0 mg/kg	0.9 ± 0.8	143 ± 150[d]	13/16[f]	4/16

[a]Mice bearing solid SC115 tumors were treated i.v. with the indicated doses of doxorubicin on days 1, 8 and 15.
[b]Non surviving animals either died or were terminated due to debilitating tumor growth (tumor weight > 7 g).
[c]Complete tumor regression was defined as a non-palpable tumor.
[d]Statistically different from saline control group ($P < 0.01$).
[e]Statistically different from free drug group at the equivalent dose ($P < 0.05$).
[f]Two mice were killed on day 22 due to severe ulceration at the tumor site even though the tumor was less than 1 g.

ious treatment groups were evaluated on the basis of percent increase in mean tumor weight, 30-day survival and complete tumor remission (Table 2). A DOX dose of 3.25 mg/kg per injection in free or liposomal form yielded tumor weight increases that were statistically comparable to control groups and no occurrence of complete tumor remission. However, 7% and 19% 30-day survival was observed for free and liposomal DOX, respectively, at this dose level. Increasing the dose of free DOX to 6.5 mg/kg per injection resulted in an increase in antitumor activity as evidenced by a 76% reduction in the tumor growth on day 8 as well as 25% 30-day survival. In comparison, liposomal DOX administered at 6.5 mg/kg per injection effected an 89% decrease in the tumor growth on day 8, 67% 30-day survival and 11% complete tumor remission. Statistical analysis indicated that inhibition of tumor growth for liposomal DOX was superior to equivalent doses of free drug (Table 2).

The ability to increase the DOX dose from the MTD of 6.5 mg/kg per injection for free drug to 13.0 mg/kg per injection in the less toxic liposomal form resulted in a dramatic inhibition of the tumor growth (Fig. 1 and Table 2). In addition, this treatment yielded a significant debulking of tumor load. For example, tumors reaching 3.4 g to 5.7 g within 8 days post first injection were reduced to less than 0.5 g over the full course of treatment (Fig. 1). Furthermore, 81% 30-day survival and 25% complete tumor regression indicated a substantial improvement of antitumor efficacy.

Discussion

The utility of carrier systems to enhance the therapeutic activity of anticancer agents can be accomplished by reducing drug-related toxicities to normal tissues, thereby allowing increased drug doses to be employed and/or by enhancing the antitumor potency of the drug. The present study suggests that the enhanced antitumor activity observed for liposomal DOX preparations utilized here is related to the ability of these carrier systems to increase tumor drug levels over an extended period of time. This improved delivery of DOX to the tumor occurs at doses equivalent to free drug as well as at doses greater than free drug. The former result contrasts another study employing a rat immunocytoma solid tumor

model [22] which demonstrated that liposome encapsulation results in reduced or equivalent DOX tumor uptake. One previous investigation has shown that increased antitumor activity of small (< 100 nm) liposomal DOX preparations correlates with increased drug levels in tumor cells [7]. However, in this tumor model the disease site resides in the liver, a known site of liposome accumulation. The results reported here provide clear indications that appropriately designed liposomal DOX systems [13] can be utilized to increase drug delivery to and activity against peripheral solid tumors.

Previous studies have demonstrated that small liposomes composed of suitable lipids exhibit extended circulation times and increased uptake into solid tumors [9]. This compares favorably with observations here where tumor liposome levels increased over 24 h, leading to approximately 5% of the administered liposome dose accumulating in the tumor. The results presented here suggest that such 'passive targeting' may lead to increased drug tumor levels. It should be noted, however, that plasma and tumor drug to lipid ratios indicate that a portion of initial tumor drug levels arises from DOX which has been released from liposomes in the circulation.

In summary, this study has demonstrated that liposomes exhibiting suitable physical properties can be used to increase the delivery of DOX to solid tumors. More importantly, such increases result in enhanced antitumor efficacy compared to that achievable with free DOX. The combination of increased tumor drug delivery and increased tolerated drug doses for liposomal DOX results in decreased tumor growth rate and reduction of tumor burden.

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