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Organ distribution and tumor uptake of annamycin, a new anthracycline derivative with high affinity for lipid membranes, entrapped in multilamellar vesicles*

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Abstract. Annamycin (Ann) is a lipophilic, non-cross resistant anthracycline antibiotic that is easily amenable to formulation in a wide variety of liposomal carriers. We studied the organ distribution and tumor uptake of Ann entrapped in multilamellar vesicles (L-Ann), free annamycin (F-Ann), and doxorubicin (DOX) in C57BL/6 mice bearing advanced subcutaneous B16 melanoma tumors. L-Ann was composed of DMPC: DMPG: Ann at a molar ratio of 7:3:0.7. Mean particle size was 1.88 ± 0.89 μm, and the entrapment efficiency was $93.08\% \pm 2.96\%$. F-Ann was prepared as a suspension (particle size ≤ 0.2 μm) in 10% DMSO. Drug levels were measured by fluorescence spectrometry after extraction with chloroform. The extraction ratio ranged between 60% and 90% for both drugs in most tissues. Compared with those of DOX, organ AUCs of L-Ann were threefold higher in plasma and brain, twofold higher in liver and kidney, sixfold higher in lung, ninefold higher in spleen, and tenfold higher in B16 tumors. Compared with F-Ann, organ AUCs of L-Ann were twofold higher in plasma, liver, and B16 tumors and were twofold lower in brain. Heart AUCs were similar with all three drugs. Higher tumor uptake was associated with a faster penetration and more prolonged retention of Ann in tumor tissue compared with those of DOX. The results obtained indicate significant differences in organ distribution between L-Ann and DOX as a result of the higher affinity of Ann for lipid membranes and the use of the liposomes as a delivery system. The potential clinical relevance of the increased uptake of L-Ann in B16 tumors, lung, and brain is being investigated.

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Introduction

The anthracycline antibiotic doxorubicin (DOX) is one of the most effective anticancer agents used [7]. DOX-containing combinations are routinely used as front-line therapy against human leukemia, lymphoma, breast carcinoma, osteosarcoma, and soft tissue sarcoma. The use of DOX in patients with sensitive tumors is, however, limited by acute myelosuppression, chronic cardiotoxicity, and the emergence of acquired resistance. The latter is believed to be due, at least in part, to the overexpression on the cell membrane of P-glycoprotein, which acts as an energy-dependent drug efflux pump [2]. In addition, most common human malignancies, such as colon and lung carcinoma, are naturally resistant to DOX, thus limiting its widespread use.

Liposomes have been extensively explored as carriers of DOX in an attempt to improve its therapeutic index by altering its pharmacokinetics and organ distribution [12, 14, 24, 25, 30, 31]. Preclinical studies have shown different liposomal DOX formulations to be less cardiotoxic than free DOX [4, 9, 18]. There are currently two different formulations of liposomal DOX in clinical trials [2, 13]. One of these formulations employs the so-called "Stealth" liposomes, which have been shown to protect the vesicles from phagocytosis by the reticuloendothelial system, to prolong significantly the plasma half-life, and to increase markedly drug uptake by subcutaneous murine tumors [3, 11, 15].

The ability of liposomes containing DOX to overcome multidrug resistance [26] in vitro has been reported for certain formulations containing cardiolipin or phosphatidylserine [8, 27]. Liposomes containing cardiolipin and phosphatidylinositol have also recently been reported to enhance accumulation of free DOX in multidrug-resistant cells [33]. Alternatively, overcoming DOX resistance by using analogues has been previously reported for 4′-I-doxorubicin [5], AD198 [23], 3′-hydroxydoxorubicin [29], and the different morpholino derivatives in vitro studies [1, 17, 19]. In the case of some of the morpholino derivatives

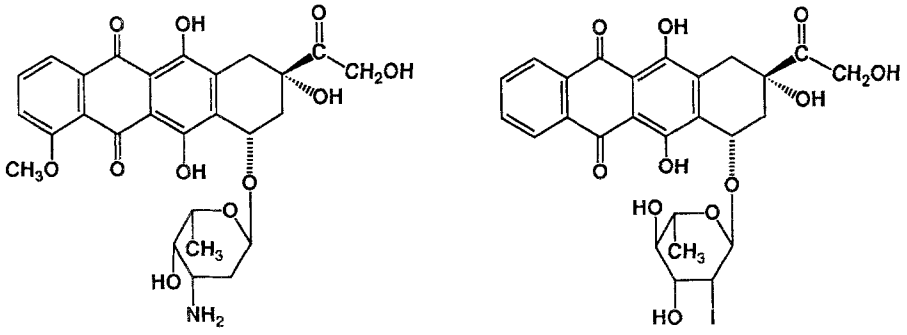

Fig. 1. Chemical structure of DOX and Ann

[17] and hydroxyrubicin [29], in vivo overcoming of resistance has also been reported.

We have explored during the past few years the use of anthracycline derivatives with a high affinity for lipid membranes in an attempt to overcome multidrug resistance by altering significantly their cellular uptake and subcellular distribution, thus avoiding interaction with P-glycoprotein. Among more than 20 lipophilic anthracyclines tested, we selected Ann (Fig. 1) for in-depth biological testing and mechanistic studies to prove our hypothesis. We have previously reported that Ann is partially non-cross-resistant with DOX in different cell lines in vitro (unpublished data). Because of its lack of solubility in water, Ann cannot be administered in standard aqueous solutions. We are, therefore, exploring the use of different types of liposomes as vehicles for the administration of this compound. In previous studies, we showed that Ann entrapped in multilamellar liposomes was more effective and about 2 times more potent than free DOX against L1210 leukemia and M5076 reticulosarcoma in vivo [28]. As part of the preclinical evaluation of Ann, it was important to explore the role of the liposome carrier in altering the organ distribution and tumor uptake of the drug. We present here the formulation studies, organ distribution, and tumor uptake of Ann entrapped in multilamellar liposomes (L-Ann), free Ann (F-Ann), and DOX in mice bearing advanced B16 melanoma tumors.

Materials and methods

L-Ann formulation. Dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) were obtained from Avanti Polar Lipids, Inc. Alabaster, Alabama. Ann was synthesized in our laboratories as previously described [28] and purified to a level of $\geq 98\%$. Doxorubicin HCl (DOX) for injection was purchased from Ben Venue Laboratories, Bedford, Ohio.

Multilamellar liposomes were prepared by standard evaporation methods [32]. The drug-to-lipid ratio was 1:15 (w/w). The DMPC : DMPG molar ratio was 7 : 3. Ann and the lipid components were dissolved in chloroform: methanol (5 : 1 v/v), and the organic phase was evaporated in a rotary evaporator under reduced pressure at 42° C. A thin lipid film was obtained. Normal saline was added (2 ml/mg Ann) and the liposome suspension was obtained by rotation of the flask at 30–40 rpm for 1.5 h at 30–35° C and subsequently shaking it for 30 min at 30° C in a mechanical shaker (Versa-Bath S Model 224, Fisher Scientific, Pittsburgh, Pa.). The liposome suspension was sonicated in a bath sonicator (Model G1125P1T, Lab. Supplies, Hicksville, N. Y.) for 30 s and passed through a 5-μm syringe filter. The final Ann concentration was determined by fluorometry and adjusted to 0.5 mg/ml.

The entrapment efficiency (%EE) was determined by measuring both the liposome-bound drug and the initial total amount of Ann in the liposomal preparation. Two 200-μl aliquots of the initial L-Ann preparation were transferred to two centrifuge tubes containing 1.8 ml Histopaque-1077 (H-1077; Sigma, St. Louis, Mo.) each. One of the tubes was centrifuged at 1000 rpm for 5 min. Ten or 20 μl of the liposomal drug on the top of the tube was removed and the Ann concentration determined (liposome-bound drug). The other aliquot was vortexed and the Ann concentration quantitated (total drug).

The %EE was calculated with the following formula:

$$\%\ \text{EE} = \frac{\text{Liposome-bound drug}}{\text{Total initial drug}} \times 100$$

The mean vesicle size and size distribution of L-Ann were determined by disc centrifugation method. L-Ann samples were diluted 100 times in normal saline and injected into a BI-DCP particle sizer (Brookhaven Instrument, Long Island, N.Y.). The disc speed was 1500 rpm, and the centrifugation time 2 h.

Liposome morphology was assessed by scanning electron microscopy. The liposome samples were fixed at room temperature with Karnovsky's fixative, pH 7.5, for at least 30 min. The liposome particles were rinsed in 0.125 M sodium cacodylate buffer for three 30-min periods and were post-fixed in 2% OsO_4 in the same buffer for 30 min at room temperature. After three more rinses in sodium cacodylate buffer, the liposomes were dehydrated in a graded series of 80% to 100% ethanol, transferred to Peldri II for critical dehydration, and placed in a vacuum desiccator for 24 h. After sublimation of the Peldri, the liposomes were mounted onto stubs and sputter-coated with 200 Å of gold: palladium (80 : 20, w/w), in a Hummer VI (Technics, Springfield, Va.) and examined in a Hitachi Model S520 scanning electron microscope.

F-Ann and DOX. F-Ann was prepared as a suspension in 10% dimethyl sulfoxide (DMSO) and 90% normal saline. When observed under the fluorescent microscope, the F-Ann preparation showed no drug crystals, thus indicating that the particle size of F-Ann was <0.2 μm.

DOX was dissolved in normal saline. Final F-Ann and DOX concentrations were adjusted to 0.5 mg/ml and 1.0 mg/ml, respectively.

Drug determinations. Ann and DOX concentrations were determined by fluorescence spectrophotometry using a MPF-44A Perkin-Elmer spectrofluorometer (Perkin-Elmer, Norwalk, Conn.). For Ann, the excitation wavelength was 470 nm, and the emission wavelength 565 nm; for DOX, the excitation wavelength was 470 nm, and the emission wavelength 585 nm. Standard curves of fluorescence versus drug concentration for Ann and DOX were used to calculate drug concentration in the samples. The sample solvent used was chloroform: methanol (9 : 1, v/v).

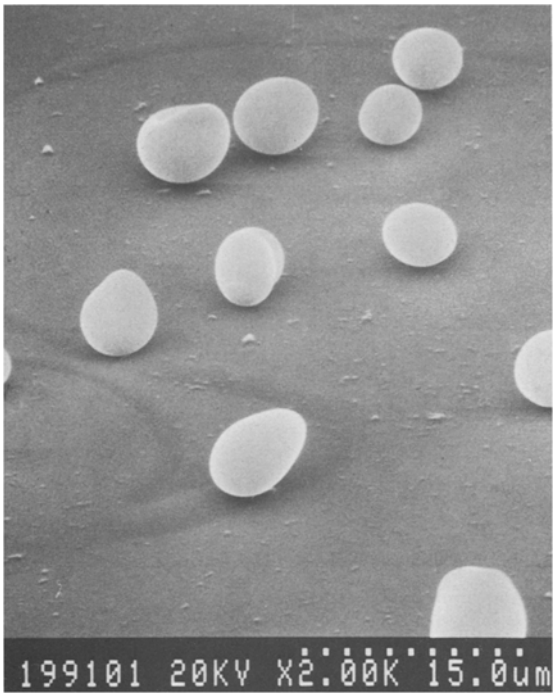

Fig. 2. Scanning electron micrograph of multilamellar liposomes containing Ann

Biodistribution. Male C57BL/6 mice 7 to 8 weeks of age were purchased from Harlan Sprague Dawley (Indianapolis, Ind.). Animals were inoculated subcutaneously with 4×10^6 viable B16 cells (in 200 µl normal saline). Ten days after tumor inoculation, animals were divided into 3 groups with 12 animals per group. Animals in each group were injected with 5 mg/kg of L-Ann, F-Ann, or DOX. At 3 min, 1 h, 6 h, and 24 h after administration, 3 mice in each group were killed by exsanguination. The brain, heart, kidney, liver, lung, spleen, and tumor were resected and a plasma sample obtained. The plasma sample (300 µl) was dissolved in 1 ml of normal saline. A volume of normal saline equal to 6 or 20 times the tissue sample weight was added, and the tissues were homogenized. Drugs were extracted from plasma and tissues with 5 ml of chloroform. The chloroform layer was collected, dried, and dissolved in methanol. Ann and DOX concentrations in these samples were determined by the fluorescence method described above and corrected for their respective extraction ratios. Extraction ratios for each organ and plasma were calculated by dividing the extracted amount of Ann or DOX by the amount of drug added. Extraction ratios are shown in Table 1.

Background fluorescence was determined in plasma, organs, and tumors from three non-treated animals using the same procedure described above. The average background fluorescence in each organ was subtracted from the determinations obtained in treated animals.

Calculation of AUC. Organ and plasma AUCs were calculated as the sum of the areas between every 2 time points (from 0 to 24 h).

Statistical analysis. Differences in biodistribution and AUCs were analyzed for statistical significance with a Student's *t*-test.

Results

Physical properties of L-Ann

The size of L-Ann liposomes was 1.88 ± 0.89 µm as measured with the disc centrifugation method. Scanning electron microscopy studies of the vesicles were consistent with these results (Fig. 2).

The %EE from three separate determinations was $93.08 \pm 2.96\%$. The gradient centrifugation method was used to separate the liposome bound Ann from F-Ann. In preliminary studies, we observed that 97–100% F-Ann in H-1077 is recovered at the bottom of the tube after centrifugation, while 95–99% L-Ann in the same conditions is recovered at the top. No crystals of free drug were identified in the L-Ann preparation either by fluorescence microscopy or scanning electron microscopy.

Organ and tumor distribution

Figure 3 shows the differences in levels of L-Ann, F-Ann, and DOX in plasma (a), liver (b), kidney (c), heart (d), brain (e), lung (f), spleen (g), and tumor (h).

Plasma levels with L-Ann were about two- to fivefold higher than with F-Ann or DOX until 6 h ($P < 0.05$). The liposome carrier increased the plasma levels of Ann by about twofold until 6 h ($P < 0.05$). Between L-Ann and F-Ann no significant differences in plasma levels were observed at 24 h, while L-Ann levels were still significantly higher than those of DOX at 24 h ($P < 0.05$).

The highest tissue levels for all drugs were observed in the liver. Liver levels of L-Ann were about threefold higher than those of F-Ann at 3 min and 1 h ($P < 0.01$). However, at 6 h, liver annamycin levels were similar in both groups. Liver levels of DOX were similar to those of F-Ann after 1 h. Peak kidney levels after injection of L-Ann were about 50% higher than after injection of F-Ann ($P < 0.05$, at 3 min). Kidney levels of F-Ann were significantly higher than those of DOX at all time points ($P < 0.05$ and/or $P < 0.01$). At 1 h and thereafter, kidney levels were similar in animals treated with L-Ann and F-Ann. Heart levels were similar in all three groups, except at 1 h after injection, when heart levels of F-Ann were about two- to threefold higher than those of L-Ann or DOX ($P < 0.05$). There were detectable levels of Ann in the brain after the injection of F-Ann and L-Ann. Generally, brain levels with F-Ann and L-Ann were about two- to fivefold higher than those with DOX ($P < 0.05$). After liver and kidney, the lung was the organ with highest Ann levels. Lung levels with F-Ann and L-Ann were similar ($P = 0.1$) and three- to tenfold higher than those of DOX at all time points

Table 1. Extraction recovery of Ann and DOX in plasma and tissues[a]

	Brain	Heart	Kidney	Liver	Lung	Plasma	Spleen	Tumor
Ann	86.6±1.4	81.0±1.3	60.2±3.1	86.6±2.5	68.0±3.5	64.4±1.1	59.2±0.9	65.3±2.1
DOX	83.8±1.3	74.8±2.1	56.3±3.2	71.4±2.3	89.1±2.7	71.7±1.7	48.5±1.2	77.8±2.3

[a] Animals: C57BL/6 mice. Tumor: subcutaneous B16 melanoma. Extraction ratios were obtained by dividing the amount of Ann extracted by the amount of Ann added. Results are mean ± standard deviation in percentage ($n = 3$)

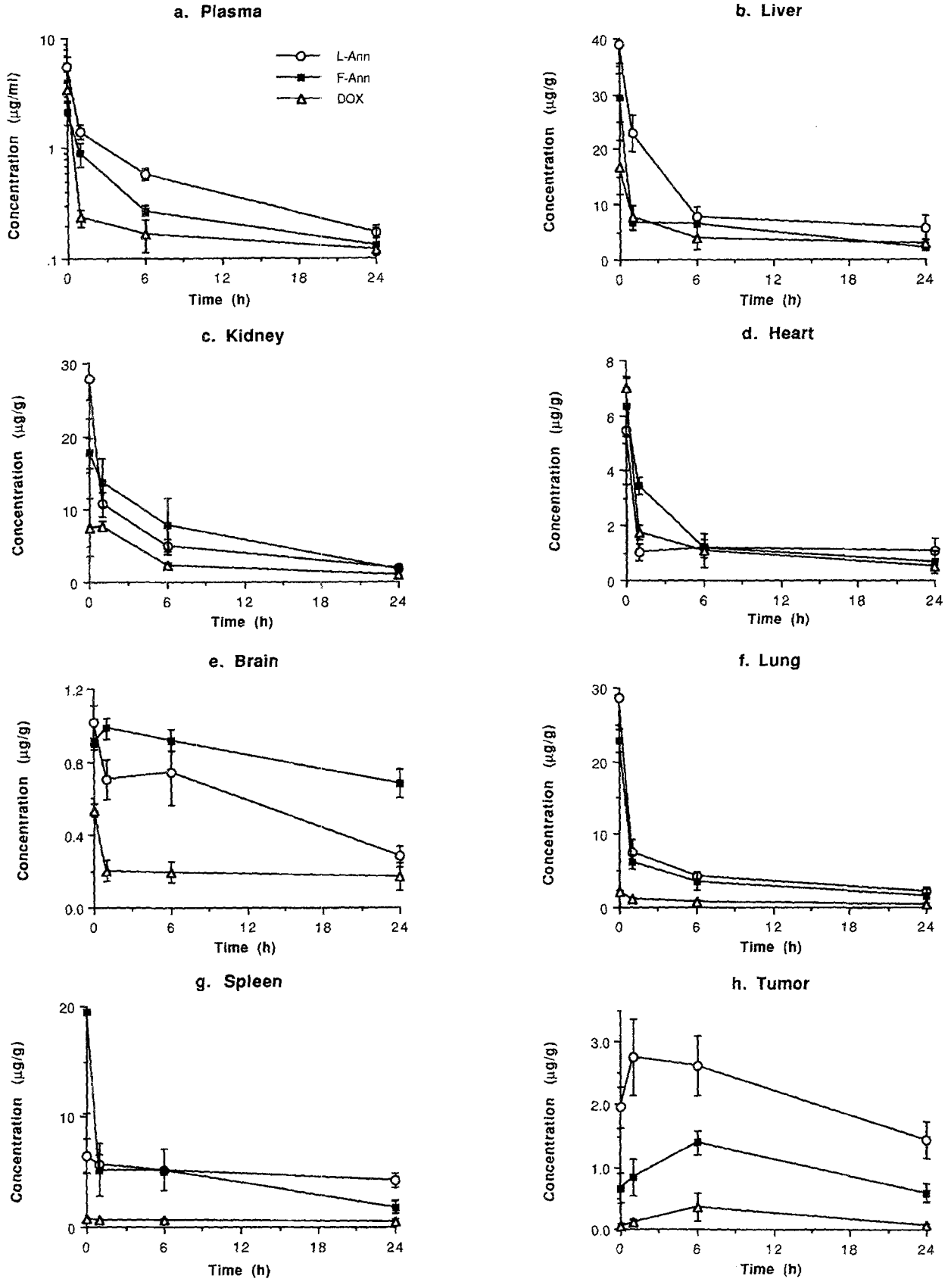

Fig. 3. Distribution of L-Ann (-○-), F-Ann (-■-), DOX (-△-) in plasma(a), liver(b), kidney(c), heart(d), brain(e), lung(f), spleen(g), and subcutaneous B16 tumor (h) after i. v. administration (5 mg/kg of each drug). Each data was obtained from three mice. The error bars are standard deviations

Table 2. AUCs of L-Ann, F-Ann, and DOX

Drug	Plasma	Brain	Heart	Kidney	Liver	Lung	Spleen	Tumor
L-Ann	15.1±3.5*, **	13.7±2.9**	29.1±9.9	121.7±22.2**	229.7±51.1*, **	106.6±17.7**	118.6±30.6**	52.2±10.0*, **
F-Ann	8.0±1.9	20.0±7.0***	33.2±6.7	155.7±60.1	127.7±16.1	84.6±27.6***	100.9±20.1***	24.2±4.5***
DOX	5.4±1.3	4.7±1.6	25.9±11.0	61.2±10.0	100.3±38.0	17.6±3.9	13.2±5.7	5.3±2.8

Results are mean ± standard deviation ($n = 3$). AUC (μg × h/g or ml) represent values recorded over a 24-h period.
* $P < 0.01$ between L-Ann and F-Ann
** $P < 0.01$ between L-Ann and DOX
*** $P < 0.01$ between F-Ann and DOX

Table 3. Tumor : organ drug ratios

	Tumor: liver				Tumor: plasma				Tumor: heart			
	0.05 h	1 h	6 h	24 h	0.05 h	1 h	6 h	24 h	0.05 h	1 h	6 h	24 h
L-Ann	0.05	0.12	0.33	0.25	0.36	1.94	4.54	8.54	0.36	2.66	2.24	1.32
F-Ann	0.02	0.12	0.22	0.27	0.31	0.93	5.19	4.64	0.10	0.24	1.16	0.91
DOX	0.002	0.02	0.09	0.03	0.01	0.61	2.13	0.62	0.005	0.07	0.33	0.14
L-Ann/DOX	25	6.0	3.7	8.3	36	3.2	2.1	14	72	39	6.8	9.4
F-Ann/DOX	10	6.0	2.4	9.0	31	1.6	2.4	7.5	20	3.5	3.5	6.5
L-Ann/F-Ann	2.5	1.0	1.5	0.9	1.2	2.1	0.9	1.8	3.6	11	1.9	1.5

($P < 0.01$). Spleen levels with F-Ann and L-Ann were also three- to tenfold higher than those of DOX at all time points ($P < 0.01$).

Levels of Ann in subcutaneous B16 melanoma tumors were 2 to 3 times higher after injection of L-Ann than F-Ann ($P < 0.01$), showing that liposome delivery enhanced tumor uptake of Ann. Tumor L-Ann levels were 5 to 8 times higher than those of DOX.

Table 2 summarizes the AUCs of the three drugs in plasma, tissues, and tumor. Compared with F-Ann, the L-Ann AUC was twofold higher in the plasma, liver, and tumor ($P < 0.05$). On the other hand, the brain AUC with L-Ann was almost twofold lower. Compared with DOX, L-Ann resulted in a marked increase in the AUC of all organs ($P < 0.01$) except heart: the plasma and brain AUC was 3 times higher, the liver and kidney AUC 2 times, the lung AUC 6 times, and the spleen and tumor AUC 10 times.

Tumor : liver, tumor : plasma, and tumor : heart ratios are shown in Table 3. The tumor : liver ratios of L-Ann and F-Ann were similar and about 3–25 and 2–10 times higher than those of DOX at the different time points. The tumor : plasma ratios of L-Ann and F-Ann were similar and 2–30 times higher than those of DOX. Tumor : heart ratios of L-Ann were about 2–10 times higher than those of F-Ann and 7–70 times higher than those of DOX. Tumor uptake indexes ([tumor: organ of L-Ann or F-Ann]/[tumor: organ of DOX or F-Ann]) are summarized in Table 3. The indexes of L-Ann and F-Ann were highest at early time points, thus suggesting that L-Ann and F-Ann penetrated tumor much faster. L-Ann : DOX and F-Ann : DOX indexes were significantly decreased at 1 and 6 h but increased slightly by 24 h, thus suggesting a more prolonged tumor retention of L-Ann and F-Ann. L-Ann : F-Ann indexes were much lower than those of L-Ann or F-Ann : DOX, but still higher than 1 at most time points, especially in tumor : heart. This suggests that besides allowing for the i.v. injection of Ann, the liposomes also improved its organ distribution.

To ascertain whether tumor size had an effect on tumor drug uptake, we plotted the relationship between tumor weight and tumor drug levels. L-Ann resulted in the highest tumor drug levels. Tumor drug levels were independent of tumor size in all the cases (linear regression coefficients <0.6).

Discussion

Our study indicates that the organ and tumor distribution of the lipophilic anthracycline annamycin entrapped in multilamellar vesicles (L-Ann) differs significantly from that of DOX and, in some cases, from that of F-Ann. Several-fold differences between L-Ann and DOX were observed in lung, B16 tumor, and brain. These differences, which may be therapeutically relevant, can in some cases be attributed to the lipophilic nature of Ann and, in other cases, to the liposomes used as delivery system.

Differences in organ distribution between L-Ann and F-Ann were minor compared with the differences between F-Ann and DOX, which suggests that the organ distribution of Ann is basically driven by its intrinsic lipophilicity and that the liposome carrier is a suitable vehicle for its intravenous administration without altering significantly its basic properties.

Lung levels and AUC of both F-Ann and L-Ann were approximately six- to tenfold higher than those of DOX. No significant differences were observed between lung uptake of F-Ann and L-Ann. It is well known that arrest of multilamellar liposomes in the lung vasculature occurs at early time points after administration and lasts for only a

few hours. In a study of a lipophilic cisplatin analogue, we have previously reported that entrapment in multilamellar vesicles resulted in lung drug levels that were several-fold higher up to 6 h after administration [21]. Rahman et al. have shown that lung levels of DOX are not significantly affected by encapsulation of the drug in small unilamellar vesicles, which are approximately 10 times smaller in diameter than the multilamellar vesicles used for Ann [21]. Because of the small particle size of F-Ann (≤ 0.2 μm) and the similar and prolonged lung levels after administration of L-Ann and F-Ann, the higher lung levels of Ann noted in our current study are probably related to Ann's high affinity for lung tissue, although aggregation of F-Ann particles in the plasma cannot be ruled out. This finding may be relevant for the treatment and prophylaxis of lung tumors or metastases.

B16 tumor drug levels and AUC were several-fold higher in the animals treated with F-Ann than in those treated with DOX. The use of liposomes as a delivery system for Ann further increased by twofold B16 tumor drug uptake. As a result, tumor levels were about 7- to 53-fold higher in animals treated with L-Ann than in animals treated with DOX, and the tumor AUC was about tenfold higher. The tumor drug uptake results obtained with L-Ann suggest that both an increased affinity (penetration and retention) of the drug for tumor tissue and a liposome-enhanced delivery (through intact liposomes or plasma lipoprotein-bound drug) may play a role. Because B16 tumors are very well vascularized, direct arrest of liposomes in the capillary bed might explain the enhancement of tumor uptake by the liposomes. However, in similar studies with the lipophilic platinum compound NDDP, no differences were found in B16 tumor drug levels after the administration of NDDP entrapped in multilamellar liposomes and cisplatin [20]. The fact that drug uptake of L-Ann, F-Ann, and DOX by B16 tumors was independent of the tumor size suggests that the vascularization of small and large tumors is similar.

Tumor:plasma, tumor:liver, and tumor:heart drug levels were several-fold higher for L-Ann than DOX. The greatest differences between the tumor:organ ratios of L-Ann and DOX were observed at early and late time points, thus suggesting an enhanced diffusion, as well as a prolonged retention of Ann in the tumor. Enhanced passive tumor drug targeting has also been reported with the use of "Stealth" liposomes and other formulations as carriers of DOX [16] and daunorubicin [10]. It would appear that enhanced tumor drug uptake may be obtained either by using long circulating liposomes that avoid reticuloendothelial system (RES) recognition or by the use of compounds with high tissue permeation properties as a result of their high affinity for cell membranes, thus also partially avoiding localization in the RES by a different mechanism.

Annamycin, both free and liposome entrapped, was detected in brain at low levels, although several-fold higher than those achieved with DOX. Liposome entrapment reduced somewhat brain drug levels compared with F-Ann. P-glycoprotein has been suggested to play a role in the blood-brain barrier transport system [6]. Ann has been shown to be non-cross-resistant with DOX in cells that overexpress P-glycoprotein [22]. It appears, therefore, that transport across the blood-brain barrier and overcoming of multidrug resistance might be related to a decreased interaction of the drug with the drug efflux systems.

Differences in other organs were also found. However, because of their lower magnitude, a major therapeutic impact appears unlikely. Plasma levels of F-Ann were several-fold higher than those of DOX and were increased only by twofold by liposome entrapment. This may be due to the fact that both F-Ann and L-Ann appear to associate quickly with plasma lipoproteins in studies ongoing in our laboratory. Liver levels of Ann were somewhat higher than those of DOX and were increased by two- to threefold by liposome entrapment at several time points. No major differences in heart drug levels were observed between the three agents tested. Changes in organ distribution of lipophilic drugs as a result of liposome entrapment have not been extensively studied in the past, mainly because of problems in obtaining acceptable free formulations of many of these compounds. In a previous study with a lipophilic platinum compound, we observed that liposome entrapment resulted in 2- to 3-fold increases in plasma and liver levels, as in the case of Ann, and 10- to 30-fold increases in lung and spleen levels, which were not observed in the present study [21].

In summary, this initial study of the organ distribution of the lipophilic anthracycline Ann entrapped in liposomes has shown markedly increased tumor, lung, spleen and brain uptake compared with DOX whereas heart levels were similar for both agents. The different distribution pattern appears to be mainly related to a higher affinity of both forms of annamycin for membrane structures but also to the particular pharmacology of multilamellar liposomes. In addition, a possible reduced interaction of Ann with P-glycoprotein might explain the enhanced brain uptake. The therapeutic relevance of our results is being investigated in several animal tumor models. As suggested by the work of other investigators [10, 16, 27], the use of liposomes with different chemical and physical properties is also being explored to further modify the organ distribution pattern, enhance tumor uptake, and optimize the therapeutic index of this agent.

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