Antitumor Activity of Free and Liposome-entrapped Annamycin, a Lipophilic Anthracycline Antibiotic with Non-Cross-Resistance Properties

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ABSTRACT

The lipophilic-anthracycline antibiotic annamycin (Ann) was entrapped in liposomes of different size [median diameter: 1.64 μm, multilamellar liposomal Ann (L-Ann); 0.030 μm, small unilamellar Ann (S-Ann)] with >90% entrapment efficiency and tested in vitro against four pairs of sensitive and multidrug-resistant (MDR) tumor cell lines and in vivo by the i.v. route in five tumor models: advanced s.c. B16 melanoma; s.c. M5076 reticulosarcoma; lung metastases of Lewis lung carcinoma; and s.c. KB and KB-V1 xenografts in nude mice. Predetermined optimal doses of the different formulations were used and the results were compared with doxorubicin (Dox). In vitro, Ann, either in suspension in 10% dimethyl sulfoxide (F-Ann) (1 mg/ml) or entrapped in liposomes, was able to partially overcome resistance in all four pairs of sensitive and MDR KB, 8226, P388, and CEM cell lines (resistance indexes 63, 269, 333, and 356 for Dox versus 4, 5, 19, and 8.7 for L-Ann, respectively). In vivo, both F-Ann and liposome-entrapped Ann were slightly more effective than Dox in inhibiting the growth of advanced s.c. B16 melanoma tumors. L-Ann was markedly more effective than Dox and moderately more effective than F-Ann in prolonging the life span of animals bearing s.c. M5076 and lung metastases of Lewis lung carcinoma; and s.c. KB and KB-V1 xenografts, whereas all Ann formulations were markedly more effective than Dox in delaying the growth of s.c. KB-V1 (MDR) xenografts. In all in vivo experiments, S-Ann was consistently more effective than L-Ann and L-Ann was more effective than F-Ann. These results indicate that (a) Ann is more effective than Dox by the i.v. route against several tumor models and that MDR tumors are partially not cross-resistant to Ann both in vitro and in vivo, (b) liposomes enhance the in vivo antitumor properties of Ann, and (c) small liposomes are more effective than large liposomes in enhancing Ann antitumor activity.

INTRODUCTION

The anthracycline antibiotic Dox is one of the most widely used anticancer agents (1). However, lack of tumor specificity and natural or acquired resistance limit its clinical use. During the last few years, several anthracycline analogues with non-cross-resistance properties have been synthesized (2–8). However, the lack of cross-resistance was primarily shown in vitro or in P388/MDR leukemia in vivo (7). In addition, the significant cytotoxicity of these compounds against MDR cells is associated with their ability to induce DNA interstrand cross-linking or DNA alkylation (9, 10), which Dox does not induce, and some of these compounds do not inhibit topoisomerase II, which is considered a key biochemical effect of Dox (9). In contrast, the lipophilic anthracycline Ann is being studied in our laboratories as an anthracycline prototype with potential non-cross-resistance properties as a result of a marked affinity for lipid membranes, enhanced cellular uptake and retention, and different subcellular distribution rather than a different mechanism of action at the DNA level (11). Because of its hydrophobicity and marked affinity for lipid membranes, liposomes are a natural delivery system for this drug and offer the potential of enhancing its localization in tumor tissue by using vesicles with specific physicochemical characteristics.

Ann was designed based on the hypothesis that the affinity of anthracyclines for P-glycoprotein is determined, at least in part, by the basicity of the sugar moiety (8). Ann has four well-defined structural changes compared with Dox (Fig. 1). Removal of the amino group at position 3' in the sugar portion and replacement by a hydroxyl group has been previously shown (a) to confer partial lack of cross-resistance without altering the interaction with topoisomerase II (8) and (b) to reduce cardiotoxicity (8). Demethylation at position 4 of the aglycone portion confers an increased lipophilicity and potency, as in the case of idarubicin, an anthracycline derivative currently approved for the treatment of acute leukemia in humans (12). The presence of iodine at position 2' results also in an increased lipophilicity and affinity for lipid membranes (11) and can probably increase the stability of the glycosidic bond. Axial orientation of the iodine atom is critical for preservation of high biological activity (13). Inversion of the hydroxyl group configuration at position 4' has been suggested to confer a reduced cardiotoxicity (14).

We have previously shown that P388/Dox (15) and KB-V1 cells are partially not cross-resistant to Ann suspended in 10% DMSO saline solution in vitro, as a result of its markedly increased drug accumulation and retention in resistant cells compared with Dox. Pharmacokinetics and drug distribution studies of Ann entrapped in multilamellar vesicles (mean particle size, 1.64 μm) in mice showed a higher uptake of L-Ann in s.c. B16 melanoma tumors (3-fold), lung (6-fold), and brain (3-fold) compared with Dox (16). In initial in vivo antitumor activity studies, L-Ann was found to be more effective than Dox in the treatment of L1210 leukemia and liver metastases of M5076 reticulosarcoma (11). The objectives of the current study were to assess the ability of Ann and its large (L-Ann) and small (S-Ann) liposomal formulations to overcome multidrug resistance in vitro and in vivo, to investigate the in vivo relevance of the different organ distribution of Ann for the treatment of s.c. or lung tumors, and to determine the effect of liposome size on the antitumor activity of Ann. Our results indicate that all four MDR cell lines studied display partial lack of cross-resistance to Ann in vitro; that both L-Ann and S-Ann are more effective than Dox in the treatment of lung tumors, s.c. tumors,
and tumors expressing the MDR phenotype in vivo; and that S-Ann is more effective than L-Ann.

**MATERIALS AND METHODS**

**Free Ann and Dox**

Ann was synthesized and characterized as reported previously with modifications (17). Free Ann was suspended in 10% DMSO and 90% normal saline (1 mg/ml stock solution; F-Ann) and Dox (Cetus Corporation, Emeryville, CA) dissolved in normal saline. Ann and Dox concentrations were determined by HPLC (18, 19) and adjusted to 0.4 and 1.0 mg/ml, respectively.

**Liposomal Ann**

Dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). The composition of all liposomal Ann formulations used was dimyristoylphosphatidylcholine: dimyristoylphosphatidylglycerol:Ann in a molar ratio of 70:30:7. All liposomal suspensions were prepared in normal saline.

Multilamellar vesicles containing Ann (L-Ann) were prepared by the thin lipid film hydration method (16). Briefly, Ann and the lipid components, at the desired molar ratio, were dissolved in chloroform:methanol (5:1, v/v) and placed in a round-bottomed flask. The organic solvents were evaporated in a rotary evaporator under reduced pressure, and a film containing the lipids and the drug was obtained. Normal saline was added and liposomes were formed by rotating the flask at 30-40 rpm for 120 min at 35°C. The resulting suspension was sonicated in a bath sonicator for 30 s and passed through a 5-μm syringe filter. The total Ann concentration was determined by HPLC and adjusted to 4.0 mg/ml.

Small unilamellar liposomes containing Ann (S-Ann) were obtained by sonicating L-Ann 3 times for 3 min each time using a probe sonicator (Micronson; Heat Systems, Farmingdale, NY) at 35-37°C. S-Ann was used within 2 h of preparation.

The percentage of entrapment efficiency was determined by measuring both the liposome-associated and total Ann amounts in the liposome suspension by differential density centrifugation, as reported previously (16).

The size distribution of the liposome suspensions was determined by the dynamic light scattering method using a submicron particle sizer (Nicomp model 370; Nicomp Particle Sizing Systems, Santa Barbara, CA).

The physical (vesicle) stability of liposomal Ann preparations was determined by sequential analysis of the particle size distribution using the submicron particle sizer. The chemical stability of Ann, both F-Ann or liposomal Ann was determined by HPLC. Drug standards in saline (0.4 and 1.0 mg/ml) were prepared, kept in transparent sealed vials, and exposed to a light intensity of 50 foot candles at 25°C. At different time points, triplicate samples were taken and the Ann concentration was determined by HPLC.

In vitro cytotoxicity against P388, P388/Dox, KB, and KB-V1 cells was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay as reported previously (25). Exponentially growing P388 or P388/Dox cells (1 x 10⁶/ml) were exposed to various concentrations of L-Ann and Dox at 37°C for 72 h. In the case of KB and KB-V1 cells, cells were seeded in 96-well microplates (10⁴/well) and then exposed to different concentrations of drugs for 4 h at 37°C. Following removal of drugs, cells were washed twice with cold phosphate-buffered saline and reincubated in drug-free medium for 72 h. For both pairs of cells, cell survival at 72 h was measured by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye.

In vitro cytotoxicity against 2256, 2226/R, CEM, and CEM/Vbl cells was assessed by using a growth inhibition assay. Exponentially growing cells were seeded in triplicate at 3 × 10⁵ cells/ml in 24-well titer plates containing various concentrations of drugs. Control cells without drug were run in parallel. Cell numbers were determined by hemocytometry in the presence of trypan blue on the seventh day and expressed as a percentage of control.

The 50% inhibitory concentration was defined as the drug concentration resulting in 50% cell survival compared with control and was calculated by extrapolation of the percentage of cell survival data obtained at each different drug concentration tested.

**In Vivo Antitumor Activity**

All animals used in this study were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN).

**Advanced s.c. B16 Melanoma.** B16 melanoma has been used as a screening system for anthracycline analogues (26). In a previous study, we determined the s.c. B16 tumor drug levels achieved after the administration of Ann, L-Ann, and Dox (16). In the present study, we selected this tumor model to study the correlation between tumor drug levels and antitumor activity.

Male C57BL/6 mice (7-8 weeks old) were inoculated s.c. with 2 × 10⁶ viable B16 cells. Ten days later (tumor size about 5 x 5 mm), tumor-bearing animals were divided into five groups of eight mice each. Treatment consisted of a single i.v. injection of F-Ann, L-Ann, S-Ann, or Dox via tail vein. The dose of Ann was 5 mg/kg in all groups and that of Dox was 10 mg/kg. These doses were selected from preliminary dose-response experiments, in which it was found that the optimal doses of Ann and Dox in this strain of mice are 4-5 and 8-10 mg/kg, respectively. Higher doses resulted in toxic deaths. Seven days after drug administration, the animals were sacrificed by exsanguination. The tumors were carefully resected and weighed. The %TGI was calculated as:

\[
\%\text{TGI} = \left(1 - \frac{\text{Mean tumor wt of treatment group}}{\text{Mean tumor wt of control group}}\right) \times 100
\]

**s.c. M5076 Reticulosarcoma.** M5076 cells metastasize exclusively to the liver after i.v. or s.c. inoculation. We have previously reported that L-Ann is markedly more effective than Dox in the treatment of experimental liver metastases of M5076 reticulosarcoma (11). In the present study, we used this tumor model as a second type of s.c. tumor. Treatment was started 4 days after
s.c. tumor inoculation when only microscopic tumors are present at the site of inoculation. The end point was survival.

Male C57BL/6 mice (7–8 weeks old) were inoculated s.c. with 6 × 10^3 MS076 cells. Four days later, animals were divided in five groups of six mice each and treated i.v. with 4 mg/kg F-Ann, L-Ann, or S-Ann or 10 mg/kg Dox. Survival of all animals was recorded for a period of 90 days. The %ILS was calculated as

\[
\text{%ILS} = \left( \frac{\text{Mean survival of treatment group}}{\text{Mean survival of control group}} - 1 \right) \times 100
\]

Animals alive at the termination of the experiment were counted as deaths for the purpose of the %ILS calculation. This experiment was repeated using 12 mice in each treatment group and 30 mice in the control group.

LLE. Dox has limited activity against lung metastases of LLC (7). This tumor model was selected for the present study to investigate whether the higher lung uptake of Ann correlates with an increased antitumor activity.

LLC cells were kept in vivo as s.c. tumors in male C57BL/6 × DBA2 F, (hereafter called B6D2F,) mice. LLC cells for a treatment experiment were obtained from an s.c. tumor after homogenization with normal saline (1:10, w/v). The suspension was filtered in a cell strainer (Falcon, Lincoln Park, NJ) and the cell number was adjusted to 5 × 10^6 viable cells/ml. LLC cells (3.6 × 10^6/mouse or 1 × 10^6/mouse) were inoculated via tail vein to 7-week-old male B6D2F, mice. Eighteen days after inoculation, the animals were divided into five treatment groups of 10 to 12 mice each and one control group of 21 mice. At the time of therapy, <1-mm tumor nodules and 1-2-mm tumor nodules were found on the lung surface of low-inoculum and high-inoculum animals, respectively. A single dose of F-Ann, L-Ann, S-Ann (4 mg/kg), or Dox (10 mg/kg) was injected via the tail vein into animals in each treatment group. The survival of all animals was recorded during an observation period of 60 days (high inoculum) or 110 days (low inoculum). Animals alive at the termination of the experiment were counted as deaths for the purpose of the %ILS calculation.

s.c. KB and KB-V1 (MDR) Human Xenografts. These tumor models were used to study the ability of Ann to overcome MDR in vivo.

Nude mice [nu/nu, male, 7–8 weeks old] were inoculated s.c. with 1.6 × 10^6 cultured KB or KB-V1 cells. When the tumor reached a size of approximately 15 × 20 mm, tumors were resected, homogenized, suspended in normal saline, and used for the inoculation of mice for the therapeutic experiments. Therefore, all experiments were performed using cells obtained at the time of the first in vivo passage. For the therapeutic experiments reported in this paper, the inoculum was 0.4 × 10^6 KB cells/mouse or 5.8 × 10^6 KB-V1 cells/mouse. Treatment was started when tumors reached a diameter of approximately 5 mm (on day 28 for KB tumors and on day 16 for KB-V1 tumors). Treatment consisted of 3 weekly i.v. injections of F-Ann, L-Ann, S-Ann, or Dox via tail vein on days 28, 35, and 42 (KB) or days 16, 23, and 30 (KB-V1). Because nude mice are able to tolerate higher doses of Ann and Dox than imbed animals, the optimal doses of Ann and Dox determined in preliminary dose-response experiments were 4 mg/kg/injection (total dose, 12 mg/kg) and 6 mg/kg/injection (total dose, 18 mg/kg), respectively. Higher doses caused significant weight loss or death. Two perpendicular tumor diameters \((a, b)\) were measured weekly by calipers in cm. Tumor weight was calculated as

\[a \times b^2 \div 2\]

and expressed in g. The slope of the linearly simulated tumor growth curve (tumor weight versus time) was used to determine the tumor growth rate. %TGI was calculated as indicated above. These experiments were repeated three times using five animals per group each time.

Statistical Analysis

Differences in %TGI and survival time were analyzed for statistical significance using Student’s t test.

RESULTS

Physical Properties of L-Ann and S-Ann

The particle size of L-Ann and S-Ann suspensions was about 1.64 ± 0.01 (SD) and 0.03 ± 0.01 μm, respectively, as measured with the dynamic light scattering method. The percentage of entrapment efficiency was >90% (93.08 ± 2.96% for L-Ann and 96.4 ± 2.05% for S-Ann) in at least three different preparations, in agreement with results previously reported (16). Both F-Ann and liposome-entrapped Ann were very stable at room temperature (>95% 6 months after preparation). The size distribution of L-Ann did not significantly change in 6 months; however, in the case of S-Ann, a significant proportion (about 5%) of large particles (>800 nm) was found 48 h after preparation. Consequently, all S-Ann preparations were used within 2 h of preparation.

In Vivo Cytotoxicity

Table 1 shows the cytotoxicity as means of at least 3 experiments with L-Ann, F-Ann, and Dox against four pairs of sensitive and MDR tumor cell lines. The cytotoxicity of L-Ann and F-Ann was similar to that of Dox against the sensitive cell lines. As expected, all four resistant counterparts were highly resistant to Dox; resistance indexes were 63 for KB versus KB-V1, 33 for P388 versus P388/Dox, 356 for CEM versus CEM/Vbl, and 269 for 8226 versus 8226/R. In contrast, the resistance indexes for L-Ann were 15 to 50 times lower, at 4, 19, 8.7, and 5, respectively. The resistance indexes of F-Ann were similar to those of L-Ann. These results indicate that all MDR cell lines tested display partial lack of cross-resistance to Ann, both free and liposome entrapped.

In Vivo Antitumor Activity

Advanced s.c. B16 Melanoma. F-Ann was slightly, although significantly, more effective than Dox in inhibiting the growth of s.c. B16 melanoma tumors (Table 2; %TGI 65.0 versus 57.0, P < 0.05). L-Ann and F-Ann had similar antitumor activity in this model (%TGI 67.6 versus 65.0, P > 0.10) whereas S-Ann was significantly more effective (%TGI 74.4, P < 0.05). These results suggest some correlation between the observed antitumor effect and the previously reported higher drug levels achieved in s.c. B16 melanoma tumors with the administration of F-Ann or L-Ann than with that of Dox (16) and an advantage for the use of small liposomes to deliver Ann.

Table 1 In vitro cytotoxicity of L-Ann and F-Ann against different sensitive and MDR tumor cell lines (means of at least 3 experiments)

<table>
<thead>
<tr>
<th>Drug</th>
<th>KB</th>
<th>KB/MDR</th>
<th>KB/Dox</th>
<th>P388</th>
<th>P388/Dox</th>
<th>CEM</th>
<th>CEM/Vbl</th>
<th>8226</th>
<th>8226/Dox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dox</td>
<td>3.2</td>
<td>202</td>
<td>63</td>
<td>0.015</td>
<td>0.35</td>
<td>4.5</td>
<td>1600</td>
<td>356</td>
<td>2.6</td>
</tr>
<tr>
<td>L-Ann</td>
<td>8.4</td>
<td>34</td>
<td>40</td>
<td>0.018</td>
<td>0.35</td>
<td>19</td>
<td>8.0</td>
<td>70</td>
<td>2.0</td>
</tr>
<tr>
<td>F-Ann</td>
<td>7.8</td>
<td>37</td>
<td>47</td>
<td>0.022</td>
<td>0.35</td>
<td>16</td>
<td>13.0</td>
<td>110</td>
<td>8.0</td>
</tr>
</tbody>
</table>

a ID50, 50% inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
b Four-day drug incubation, MTT assay.
c 72-h drug incubation, MTT assay.
d Seven-day drug incubation, cell number.
s.c. M5076 Tumors. Table 2 shows the mean %ILS values obtained in 2 different experiments. F-Ann and its liposomal formulations significantly increased the life span of animals bearing s.c. M5076 reticulosarcoma tumors whereas Dox had a minimal effect. Both liposomal formulations were more effective than F-Ann, and S-Ann was more effective than L-Ann (%ILS 19.4 for Dox, 45.6 for F-Ann, 63.2 for L-Ann, and 74.4 for S-Ann; P < 0.01 between any two groups). These results confirm the lack of activity of Dox against this tumor model as reported previously (11), indicate a marked activity of Ann against this tumor model, and suggest again a superiority of small liposomes.

LLCs. In the experiment using a low tumor cell inoculum, all Ann formulations significantly increased the life span of animals bearing LLCs whereas Dox was ineffective (Table 2). Both liposomal formulations were more effective than F-Ann, and the use of small liposomes as carriers of Ann further enhanced its antitumor activity (%ILS 2.4 for Dox, 39.2 for F-Ann, 73.5 for L-Ann, and 87.8 for S-Ann; P < 0.01 between any two groups). Long-term survivors were observed in all Ann groups: 8 of 10 in S-Ann, 7 of 10 in L-Ann, and 4 of 10 in F-Ann (Fig. 2a). An autopsy of these animals did not reveal tumor in the lungs. No long-term survivors were observed in the Dox or control groups.

In the experiment using a high tumor cell inoculum, similar trends were observed (%ILS 6.9 for Dox, 18.7 for F-Ann, 26.7 for L-Ann, and 57.6 for S-Ann; P < 0.01 between any Ann group and Dox and between S-Ann and any other group). Survival curves are shown in Fig. 2b. Autopsy studies showed that the lungs of all dead animals were fully occupied by tumor and no macroscopic tumors were seen in all other organs.

s.c. KB and KB-V1 Xenografts. All drugs displayed significant and similar antitumor activity against s.c. KB xenografts as shown by the tumor growth curves (Fig. 3a) and %TGI values (Table 2; %TGI 76.6 for Dox, 78.6 for F-Ann, 83.2 for L-Ann, and 87.8 for S-Ann; P < 0.01 between any treatment group and control). S-Ann was, again, the most effective formulation (S-Ann versus F-Ann or Dox, P < 0.05).

Fig. 2b shows the tumor growth curves obtained from three different experiments in nude mice bearing KB-V1 xenografts. In contrast with the studies in mice bearing KB xenografts, all Ann formulations were markedly effective in delaying the growth of s.c. KB-V1 xenografts, whereas Dox had only a moderate effect (%TGI 43.8 for Dox, 82.3 for F-Ann, 86.4 for L-Ann, and 92.6 for S-Ann; Dox versus all Ann formulations, P < 0.01) (Fig. 2b; Table 2). The activity of L-Ann was similar to that of F-Ann, but S-Ann was significantly more effective than F-Ann and L-Ann (P < 0.01 and P < 0.05, respectively).

The KB-V1 tumor growth rate was obtained from the slope of the simulated tumor growth curve. The mean tumor growth rate of animals treated with Dox was not significantly different from that of the control group, whereas the mean tumor growth rates of F-Ann, L-Ann, and S-Ann groups were 7-, 8-, and 19-fold lower than that of control and 4-, 5-, and 11-fold lower than that of animals treated with Dox (tumor growth rates in mg/day: 206 ± 71 for control; 120 ± 52 for Dox; 30 ± 12 for F-Ann; 25 ± 11 for L-Ann; and 11 ± 9 for S-Ann). Differences between any Ann group and Dox or control are statistically significant at P < 0.01.

DISCUSSION

The results of our study indicate that the lipophilic anthracycline antibiotic Ann is more active than Dox in several in vivo murine tumor models, that MDR tumors are partially not cross-resistant to Ann in...
The tumor weights were estimated as described in “Materials and Methods.” Data for KB xenografts represent mean ± SD (bars) of one experiment (5 mice/group). Data for KB-V1 xenografts represent mean ± SD (bars) of three independent experiments (5 mice/group each experiment).

Fig. 3. Tumor growth curves of s.c. KB (a) or KB-V1 (b) xenografts in nude mice treated with three doses of 4 mg/kg F-Ann ( ), L-Ann ( ), or S-Ann ( ), or 6 mg/kg Dox ( ), and equal volume of saline as control ( ). Arrowheads, treatment days. The tumor weights were estimated as described in “Materials and Methods.” Data for KB xenografts represent mean ± SD (bars) of one experiment (5 mice/group). Data for KB-V1 xenografts represent mean ± SD (bars) of three independent experiments (5 mice/group each experiment).

vitro and in vivo, and that Ann’s antitumor activity is enhanced by using liposomes as delivery system, particularly small unilamellar vesicles.

The in vivo lack of cross-resistance properties of Ann may be clinically relevant for the treatment of human tumors that express the MDR phenotype. It is interesting to note that the lack of cross-resistance was more apparent in vivo than in vitro in the case of the KB/KB-V1 cells. Although several anthracycline analogues have been reported to have non-cross-resistance properties, the in vivo data supporting such properties are extremely limited (7). In addition, some of these analogues act as alkylating agents and do not inhibit topoisomerase II (9, 10), and it is likely that they are cytotoxic to MDR cells as a result of their fundamentally different mechanism of cytotoxicity.

Unlike the other anthracyclines that have shown activity against MDR cells, Ann belongs to the family of the 3’-hydroxylated anthra
cyclines (8). These compounds are topoisomerase II inhibitors like Dox and are not alkylating agents. We have previously studied the cellular pharmacology of F-Ann and L-Ann and concluded that their lack of cross-resistance appears to be related to a markedly increased accumulation and retention in MDR cells compared with Dox, probably as a result of an increased drug cellular uptake, a different subcellular distribution, and a decreased P-glycoprotein-mediated drug efflux (15). Whether such decreased efflux is due to a decreased availability of Ann for interaction with the efflux pump or to an intrinsic reduced affinity of Ann for the efflux pump is unknown. The relatively high Ann accumulation in resistant cells explains its ability to induce DNA double-strand breaks and, secondarily, cell death, thus overcoming the resistance.

From our in vitro and in vivo results, it is evident that the ability of Ann to partially overcome MDR is not mediated by the liposomes used. In contrast, Dox encapsulated in certain types of liposomes, containing cardiolipin or phosphatidylserine, has been reported to partially overcome MDR in several in vitro systems by different investigators (27–34). A direct interaction of these liposomes with P-glycoprotein has been suggested as the mechanism involved. We are currently exploring the use of some of these liposomes as carriers of Ann in an attempt to further decrease its resistance index and optimize its therapeutic efficacy against MDR tumors.

The high lipophilicity of Ann determines not only a different uptake and distribution at the cellular level but also a different tissue distribution after i.v. administration (16). Particularly interesting are the previously reported results of several-fold higher levels of Ann, both free and liposome-entrapped forms, than Dox after administration of equimolar doses in s.c. B16 melanoma tumors, lung parenchyma, and brain, while heart levels are similar (16). In the current study, we tried to investigate whether some of these changes correlate with differences in antitumor activity. The results show similar trends for tissue or tumor levels and antitumor activity.

In the case of B16 melanoma, we previously found that drug levels achieved with L-Ann and F-Ann were about 7- and 3-fold higher than with Dox, respectively. Since L-Ann and F-Ann, in the current study, were moderately more effective than Dox in delaying the growth of advanced s.c. B16 melanoma tumors, a certain degree of correlation between tumor drug levels and antitumor activity can be concluded. Results obtained in the treatment of animals with early s.c. M5076 tumors were much more striking, since Dox was ineffective in delaying the growth of the tumors and prolonging the life span of the animals, whereas both F-Ann and L-Ann had a marked antitumor activity.

Antitumor activity and organ drug levels also followed parallel trends in animals with LLCs. Lung drug levels with F-Ann and L-Ann were about 6-fold higher than with Dox. Both F-Ann and L-Ann were markedly effective in prolonging the life span of the animals with lung metastases of LLC, whereas Dox was ineffective. However, L-Ann was more effective than F-Ann, even though the lung drug levels achieved with these two preparations are similar. Interestingly, enhanced activity against lung LLC has been reported previously for other non-cross-resistant anthracyclines (7).

Because of its extreme lipophilicity, Ann cannot be administered i.v. in a solution form. The use of liposomes is a logical approach to address the in vivo delivery of Ann. In the present study, large and small liposomes were tested. We consistently observed a higher antitumor activity with Ann entrapped in large liposomes than with Ann suspension in 10% DMSO and with Ann entrapped in small liposomes than in large liposomes. The superiority of small liposomes over large liposomes was not surprising, since it has been observed by other investigators with different hydrophilic anthracyclines (35). Pharmacokinetics and organ distribution studies comparing large and small liposomes are now in progress to study the relationship among antitumor activity, plasma half-life, and tissue drug levels achieved with both formulations.

In summary, the current study, although mainly descriptive, provides compelling evidence that liposome-entrapped Ann has a markedly different spectrum of antitumor activity than Dox. This characteristic may be relevant to the treatment of human cancer, particularly of tumors that express the MDR phenotype. Extensive work is in progress to identify the optimal liposomal Ann formulation. Liposome entrapment offers the potential of reducing Ann cardiotoxicity, as
shown with other anthracyclines, and of further enhancing Ann tumor targeting, as shown with long-circulating liposomes as carriers of a variety of antitumor agents (36). Because of its exquisite affin Ity for lipid membranes, Ann is easily amenable to formulation in a wide variety of liposomes. Such versatility is expected to facilitate ongoing formulation efforts.

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