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# Lyophilized preliposomal formulation of the non-cross-resistant anthracycline annamycin: effect of surfactant on liposome formation, stability and size

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**Abstract** We report a method of preparing a submicron and stable liposome formulation of the non-cross-resistant anthracycline annamycin. The lipids were dimyristoylphosphatidyl choline (DMPC) and dimyristoylphosphatidyl glycerol (DMPG) at a 7:3 molar ratio and the optimal lipid:drug ratio was 50:1 (w/w). The selected formulation was a preliposome lyophilized powder that contained the phospholipids, annamycin, and Tween 20. The liposome suspension was obtained on the day of use by adding normal saline at 37 °C (1 ml/mg annamycin) and hand shaking for 1 min. The presence of Tween 20 was essential in shortening the reconstitution step (from $> 2$ h to 1 min), avoiding the early formation of free drug crystals, and reducing the median particle size by tenfold (from 1.5 μm to 0.15 μm) without destroying the liposome vesicles. At room temperature, the preliposome powder was chemically stable for $> 3$ months, and the liposome suspension was chemically and physically stable for $> 24$ h. The in vitro cytotoxicity of the formulation was equivalent to that of the same lipid composition prepared by the standard evaporation method. The results of the study indicate that small amounts of surfactant may be used to enhance the reconstitution step and reduce the size of liposome suspensions obtained from lyophilized preliposome powders. The formulation described is being used for ongoing clinical trials with liposomal annamycin.

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## Introduction

Anthracycline antibiotics, namely doxorubicin (Dox), daunorubicin and idarubicin, are among the most effective and widely used anticancer agents. However, their use is limited by side effects, mainly acute myelosuppression and chronic cardiotoxicity and natural or acquired drug resistance [16, 18]. Liposomes have been extensively studied by different investigators as carriers of this class of compounds. Liposome incorporation has been shown to reduce the cardiac, gastrointestinal, and vesicant side effects of Dox in animals and humans [5, 14]. Tumor-targeted formulations of liposomal Dox are now being evaluated in humans [3] and have been recently approved for the treatment of Kaposi's sarcoma by the Food and Drug Administration in the US. Other interesting studies have shown that multidrug resistance (MDR1), a common mechanism of acquired resistance to anthracyclines and other drugs, may be overcome in vitro and in vivo in bone marrow cells by using Dox encapsulated in liposomes containing certain lipids, such as cardiolipin or phosphatidylserine [15, 17].

An alternative approach to enhance the therapeutic index of this class of compounds is the use of new and potentially less toxic, more active analogs. Although a large number of analogs have been synthesized and studied, most of these efforts took place before MDR1 was described and well characterized. However, during the last few years, several subfamilies of anthracycline with non-cross-resistance properties have been described [1, 2, 13]. Because most of these anthracyclines are highly lipophilic and, therefore, not suitable for intravenous (i.v.) administration in water solutions, a delivery system is needed to facilitate their i.v. administration.

We have extensively explored the use of liposomes as carriers of lipophilic and non-cross-resistant anthracyclines. Our objectives were to: (1) select compounds that had a natural affinity for liposome membranes and were therefore easy to incorporate within the lipid membranes of a variety of liposomes and (2) use liposomes with optimal pharmacological properties (enhanced tumor-targeting properties) as a delivery system for the i.v. administration of these compounds [11]. Annamycin was selected as the leading compound possessing these characteristics. We have previously reported that liposomal annamycin is not cross-resistant with Dox in vitro or in vivo and is noncardiotoxic in mice [12, 22, 23].

Lyophilization of preformed liposomes or of the liposome constituents followed on the day of use by reconstitution with an aqueous solution to obtain the liposome suspension has been successfully explored by us and other investigators [8, 11]. Potential problems with formulations using preliposomal lyophilized powders are basically related to the difficulties encountered with the reconstitution step to obtain a reproducible liposomal suspension. Here we describe a modified lyophilization method for preparing a highly stable, easy to reconstitute submicron liposome suspension of the lipophilic anthracycline annamycin and we report on the crucial role played by a small amount of the surfactant Tween 20 in improving the characteristics of the formulation.

## Materials and methods

### Materials

Dimyristoyl phosphatidylcholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-[phosphor-*rac*-(1 glycerol)] (DMPG) were obtained as a dry powder from Nippon Fine Chemicals, Osaka, Japan. Annamycin, $>95\%$ pure, molecular weight (MW) 640.39 Da [20] was synthesized as previously described with slight modifications [6]. Tween 20, Tween 80, dimethyl sulfoxide (DMSO), chloroform and *t*-butyl alcohol were obtained from Aldrich Chemical Company, Milwaukee, Wis. Normal saline was obtained from Abbott Laboratories, North Chicago, Il.

### Liposome preparation

The lipid composition was in all cases DMPC : DMPG at a 7 : 3 molar ratio. Other ratios were explored but did not result in any significant advantage; therefore, only results with the 7 : 3 molar ratio are presented. Different total lipid : drug ratios (w/w) ranging from 10 : 1 to 100 : 1 were tested.

The lyophilization method used was adapted from a method we previously used for other compounds with modifications [11]. Briefly, a preliposomal powder was initially prepared by freeze-drying a solution of the different ingredients in *t*-butyl alcohol, and the liposome suspension was obtained by controlled hydration of the preliposomal powder on the day of use.

The procedure involved the following general steps:

1. *Preparation of preliposomal lyophilized powder* Annamycin was dissolved in DMSO (100 mg/ml). The lipids (DMPC : DMPG = 7 : 3 molar ratio) and surfactant (Tween 20 or Tween 80) were dissolved in *t*-butyl alcohol. The two solutions were mixed and filtered through a 0.22-μm pore filter of regenerated cellulose (Micro Filtration Systems) for sterilization. Aliquots of this solution containing 10 mg annamycin were placed in 25-ml lyophilization vials. The vial contents were frozen over dry ice in acetone and lyophilized for 48 h to remove all DMSO and *t*-butyl alcohol.
2. *Preparation of liposomal annamycin suspension* On the day of use, the preliposomal powder was reconstituted by adding saline prewarmed at 37 °C (1 ml saline per mg annamycin) to the lyophilized vial and hand shaking until all lyophilized material was in suspension.

### Liposome characterization

*Entrapment efficiency* The entrapment efficiency (%EE) was determined by the differential density centrifugation method previously described [21]. Annamycin leakage or dissociation from the liposomes was assessed by sequential determination of the %EE over time. The percentage drug leakage at $x$ h = %EE at time 0 − %EE at $x$ h.

*Size* The median liposome size and size distribution were measured in a Nicomp Submicron Particle Sizer, Model 370 (Nicomp Particle Sizing System, Santa Barbara, Calif.) using the Gaussian method. The size of the liposomes was also assessed by freeze-fracture electron microscopy by Dr. Larry Boni at Oncotherapeutics, Cranbury, N.J.

*Effect of surfactant* The effect of nonionic surfactant (Tween 20 and Tween 80) on liposome size and annamycin crystallization in the liposome suspension was assessed by fluorescence microscopy at different time-points after reconstitution. All preliposome preparations were made using the same procedure but different lipid : drug ratios or surfactant concentrations. The liposome suspension was stored at room temperature (25–26 °C) and an illuminance of 600–1000 Lux (60–100 foot-candle). Samples (2 μl) were taken from the bottom and middle portions of the reconstituted vials and analyzed at different time-points. The whole slides were screened twice. The average number of crystals per field was obtained from six randomly selected fields.

*Effect of lipid : drug ratio* Vials of preliposomal annamycin preparations using different lipid : drug ratios (with or without surfactant) were reconstituted. Drug crystallization was assessed every 10 min for 1 h, every hour from 1 to 6 h, and daily from day 1 to day 7 after reconstitution, by fluorescence microscopy as previously described.

*Chemical stability of annamycin* The chemical stability of annamycin in the lyophilized powder or the liposome suspension was assessed by assaying the amount of intact annamycin remaining over time. The quantitative determination of annamycin was performed by high-performance liquid chromatography using fluorescence detection (excitation wavelength 485 nm, emission wavelength 532 nm) as previously described [20].

*Physical stability of the liposomes* The physical stability of the liposomes was assessed by determining at different time-points after formation of the liposome suspension, the percentage of annamycin leakage, the changes in liposome size distribution and median size, and the *de novo* appearance of free annamycin crystals or liposome aggregates as described above.

## Results

### Effects of lipid : drug ratio and the addition of surfactant

No problems were encountered in obtaining clear solutions of the lipids and annamycin in the DMSO and *t*-butyl alcohol mixture described previously before the addition of surfactant and the freeze-drying step. The preliposome lyophilized powders obtained were homogeneously flaky and orange in appearance. Neither the lipid : drug ratio nor the addition of surfactant appeared to change the physical appearance of the powders.

The hydration/reconstitution step and the appearance of the liposome suspensions obtained from the preliposome lyophilized powders using lipid : drug ratios of 10 : 1 to 25 : 1 without surfactant presented some problems. Even using saline warmed at 37 °C, prolonged hand shaking (up to 2 h) was required to obtain a homogeneous suspension without gross aggregates. At the completion of the reconstitution step, typical multilamellar liposome suspensions without the presence of free drug crystals or liposome aggregates were observed under the microscope. However, within minutes, free annamycin crystals appeared and had become very significant 6 h after reconstitution. In an attempt to address these limitations, we first explored the effect of the lipid : drug ratio on the time of appearance of free-annamycin crystals and on liposome size in the absence of surfactant. The results are illustrated in Fig. 1a. The appearance of free annamycin crystals was markedly delayed by increasing the lipid : drug ratio to above 25 : 1. However, the median liposome size was also increased (from about 1.6 μm at a ratio of 50 : 1 to about 6 μm at a ratio of 100 : 1). Although no crystals were observed in preparations using a lipid : drug ratio of 100 : 1, the amount of lipid required to administer the projected therapeutic doses in humans and the median liposome size were both deemed to be excessive and constitute major shortcomings to this approach. As a result, a lipid : drug ratio of 50 : 1 was selected for further formulation optimization studies.

We subsequently studied the effect of the presence of the surfactant Tween 20 and Tween 80 on liposome size distribution and the formation of annamycin crystals at 6 h after reconstitution of the preliposome powder. Amounts of surfactant from 0.5 to 4.0 mg/mg annamycin were tested. The lipid : drug ratio was kept constant at 50 : 1 in these experiments. The results are illustrated in Fig. 2. Both Tween 20 and Tween 80 completely prevented the formation of free annamycin crystals at 6 h after reconstitution in a dose-dependent fashion. No free annamycin crystals were observed in formulations containing $\geq 1.7$ mg Tween 20 or $\geq 3.0$ mg Tween 80 per mg annamycin. Both surfactants also reduced the median liposome size in a dose-dependent fashion. This effect was comparable for both agents and amounts of either surfactant $\geq 3.0$ mg/mg annamycin resulted in significant liposome destruction as shown by the appearance of a population of small ($< 50$-nm) particles consistent with micelles. The presence of either surfactant was also found to shorten the time needed to reconstitute the preliposome lyophilized powder to 1 min. The minimum amount of surfactant completely preventing drug crystallization without liposome disruption was 1.7 mg Tween 20 per mg annamycin.

Next, the effect of the lipid : drug ratio on the time of free annamycin crystallization in formulations containing the optimal amount of surfactant was studied. The results are illustrated in Fig. 1b. The lowest lipid : drug ratio delaying drug crystallization for $> 48$ h was

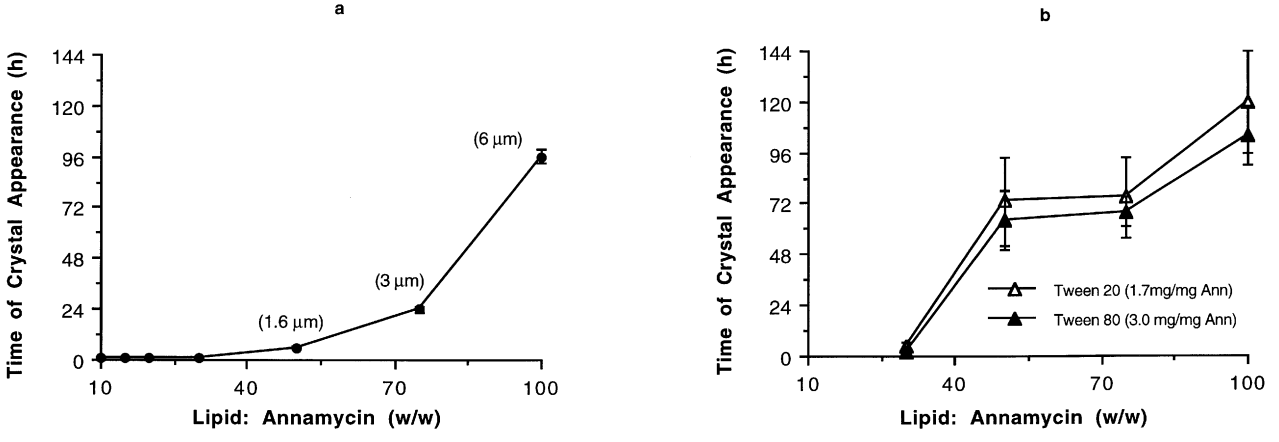


**Fig. 1a** Effect of lipid : annamycin weight ratio on the time of appearance of free annamycin crystals and the median liposome size in the absence of surfactant. The numbers in parentheses are the mean size of the liposome particles. **b.** Effect of lipid : annamycin weight ratio on the time of appearance of free annamycin crystals in the presence of optimal amounts of surfactant. The size of all reconstituted liposomes with Tween 20 was 50–350 nm and of those with Tween 80 was 20–600 nm measured using a Nicomp sizer after removal of the crystals

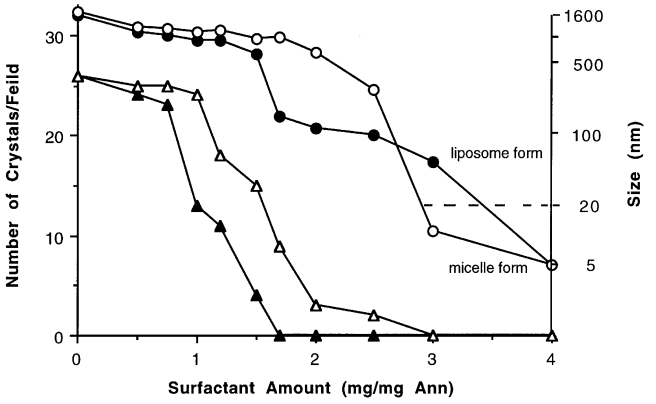


**Fig. 2** Effect of the presence of Tween 20 (*closed symbols*) and Tween 80 (*open symbols*) on the number of free annamycin crystals (*triangles*) and the median liposome size (*circles*) 6 h after reconstitution of the preliposome powder (lipid : annamycin ratio 50 : 1)

50 : 1; however, drug crystallization occurred within a few hours when a lipid : drug ratio of 30 : 1 was used.

As a result of all these studies, the formulation containing 1.7 mg Tween 20 per mg annamycin and a lipid : drug ratio of 50 : 1 was selected for further characterization studies. The characteristics of this formulation were:

1. A time of reconstitution of 1 min
2. No free annamycin crystals at 24 h
3. A median liposome suspension size of 150 nm
4. No micelles present at 24 h

All characterization studies were performed in vials containing 10 mg annamycin, 500 mg DMPC/DMPG at a 7 : 3 molar ratio, and 17 mg Tween 20.

## Characterization of the selected liposomal annamycin formulation

*Optical microscopy evaluation* Table 1 compares the results observed in three different batches of liposomal annamycin that contained Tween 20 with one control batch that did not contain Tween 20. In the Tween 20-containing batches, neither free annamycin crystals nor sediment were observed before 72 h, although crystals were observed in two batches at 96 h. In the control batch, crystals and sediment were observed at 6 h.

*Entrapment efficiency* The %EE was $> 95\%$ (mean 97.3%) in the three Tween 20-containing batches (Table 1).

*Particle size* The median particle size and size distribution were determined in the three Tween 20-containing batches (Table 1) and found to be very reproducible: 145, 154, and 140 nm. This size was also confirmed by freeze-fracture electron microscopy studies (Fig. 3).

**Table 1** Physical stability of liposomal annamycin formulation. The formulation was reconstituted in the addition of 10 ml normal saline at 37 °C to vials containing 10 mg annamycin and 100 mg lipid followed by 1 min hand shaking. The formulation was stored at room temperature ($26 \pm 1$ °C) under an illuminance of 600–1000 Lux. *Batches A, B and C* formulations with Tween 20, *control* formulation without Tween 20. The appearance time of the crystals and sediment was determined using optical and fluorescence microscopy

| Batch | Storage time | Entrapment efficiency (%EE) | Size (nm) | Crystals/ sediment appearance time (h) |
|---|---|---|---|---|
| A | 0 | $99.0 \pm 1.3$ | $145 \pm 76$ | 96–168 |
| | 24 | $97.2 \pm 1.4$ | $142 \pm 65$ | |
| B | 0 | $97.1 \pm 1.5$ | $154 \pm 67$ | $> 168$ |
| | 24 | $96.5 \pm 1.2$ | $147 \pm 45$ | |
| C | 0 | $95.9 \pm 0.8$ | $140 \pm 87$ | $> 168$ |
| | 24 | $95.4 \pm 1.2$ | $141 \pm 57$ | |
| Control | 0 | $87.2 \pm 3.4$ | $1540 \pm 650$ | $< 6$ |
| | 24 | $74.2 \pm 5.1$ | $1780 \pm 820$ | |

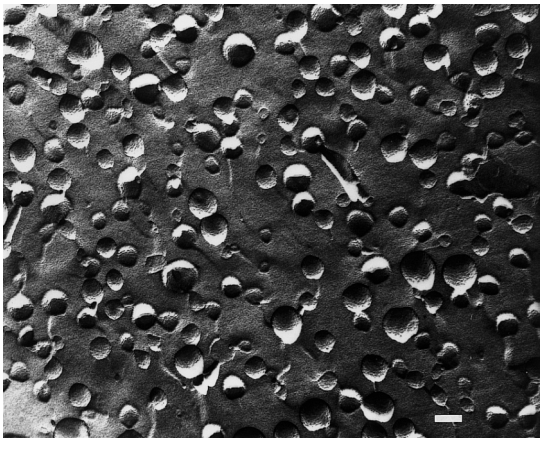

**Fig. 3** Freeze-fracture electron micrograph of the liposomal annamycin suspension. Size range 40–180 nm (*scale bar* 100 nm)

*Stability studies* The Chemical stability of annamycin in the preliposome lyophilized powder was tested in four different batches of lyophilized annamycin at room temperature and an illuminance of 600–1000 Lux. No significant reduction in annamycin content was detected during 4 months in any of the batches tested. Six different batches of reconstituted liposomal annamycin suspension (three samples per batch) were tested under the same conditions as above. No significant reduction in annamycin content was detected during 96 h in any of the batches.

Table 1 shows the changes in%EE and median size of three different batches of liposomal annamycin kept at room temperature and an illuminance of 600–1000 Lux for 24 h. The drug leakage was between 0.5% to 1.8% during this period of time, and the change in median size was less than 5 nm.

## Discussion

Here we report on the effect of small amounts of the surfactant Tween 20 in improving the characteristics of the liposome formulation of the lipophilic anthracycline antibiotic annamycin, which is currently undergoing clinical evaluation and was selected from more than 30 lipophilic anthracyclines because of its lack of cross-resistance properties, its promising biological activity in different animal models, and its ease of formulation in liposomes as a result of its high affinity for lipid membranes [10, 23]. However, when the development of a lyophilized preliposomal annamycin formulation amenable to large-scale manufacturing was attempted, problems associated with the length of the reconstitution step and the early appearance of annamycin crystals in the liposome suspension were encountered. The addition of a precise amount of Tween 20 and the use of a specific lipid : drug weight ratio resulted in a complete resolution of these limitations. Furthermore, the presence of Tween 20 decreased by about tenfold the median size of the liposomes obtained. This is a potentially important advantage, because smaller liposomes are not taken up as avidly by the reticuloendothelial system and are, therefore, more likely to remain in circulation for prolonged periods of time, thus increasing their chance of crossing the endothelial barrier and reaching the tumor tissue. In preclinical studies, we have consistently observed that annamycin incorporated in small liposomes (50–200 nm in diameter) is more active in vivo against different tumor models than annamycin incorporated in large multilamellar liposomes ($>$ 1000 nm in diameter) [22].

The findings reported here are new and potentially important because they could be applied to the development of liposome formulations of other lipophilic compounds. The amount and type of surfactant needed may vary, however, for each drug. In the case of annamycin, the amount and type of surfactant appear to be crucial, because higher amounts of Tween 20 were found to disrupt the liposomes, whereas lower amounts did not completely avoid drug crystallization. In the case of Tween 80, the amounts needed to completely prevent drug crystallization were found to cause significant liposome disruption.

The addition of Tween 80 has been previously reported to enhance the stability and%EE of liposomes [4, 7, 19] but not to decrease the median size of the liposomes, not to reduce the hydration time or the crystallization of entraped water-insoluble drugs. The mechanisms by which the presence of optimal amounts of surfactant can enhance the hydration step, decrease free drug crystallization formation, and reduce the size of the liposomes are unknown and open to speculation. The hydrocarbon tail of Tween 20 may be able to penetrate the lipid bilayer, thus leaving the polyethylene oxide groups on the surface of the liposomes, which may decrease liposome fusion and may consequently decrease lipid and annamycin exchange upon collision of the liposome particles, as previously suggested [7]. This would prevent the association of annamycin molecules and, therefore, the formation of free drug crystals. Alternatively, Tween 20 may only prevent the free association of annamycin molecules when the aqueous solution is added to the lyophilized preliposome powder to form the liposomes, thus decreasing the chance of free drug crystal formation. The first mechanism appears more likely, because no crystals were observed immediately after reconstitution in the absence of Tween 20, indicating that drug crystallization is a phenomenon that occurs a few hours after initiation of the lipid hydration process.

The liposomal annamycin preparation described in this paper is being used in clinical trials currently in progress at M.D. Anderson Cancer Center. Its cytotoxicity has been tested in vitro against KB cells and compared with the preparation obtained using the evaporation method that we have previously reported [12]. The cytotoxicity of both preparations is equivalent (data not shown). Subacute mouse toxicity studies have also been performed and have shown that the liposomal preparation obtained by the evaporation method is about twice as toxic as the one obtained from the preliposomal powder containing Tween 20 ($LD_{50}$ 8 mg/kg vs 15 mg/kg) [23]. This difference is probably caused by the different size of the preparations. Finally, the liposomal annamycin formulation described in this report has been found to be noncardiotoxic in mice using a variation of the Bertazzolli test [23].

The presence of Tween 20 can induce allergic reactions in patients. Tween 80 is currently an ingredient in different parenteral formulations approved for clinical use in the US (for example etoposide, multivitamins). Tween 80 is also an ingredient in the current formulation of the new antitumor agent taxotere [9], which is currently under intensive investigation. The amount of Tween 80 needed to deliver a therapeutic dose of taxotere has been calculated to be about 30–50 times higher than the amount of Tween 20 that will be administered with the projected therapeutic dose of liposomal annamycin. Still, allergic reactions secondary to the presence of Tween 20 are a potential side effect of liposomal annamycin, and premedication to avoid such a reaction may be needed in the ongoing clinical trials.

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