Biochemical Pharmacology, Vol. 33, No. 4, pp. 698–700, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

Effect of cholesterol content of liposomes on the encapsulation, efflux and toxicity of adriamycin

(*Received* 7 *February* 1983; *accepted* 26 *August* 1983)

Adriamycin (ADR) is an important chemotherapeutic agent with a broad spectrum of activity against human malignancies [1, 2]. The cytostatic and cytocidal effects of ADR are suggested to be due to intercalation with DNA [1], production of free radicals during intracellular metabolism [3], and interaction with the plasma membrane [4]. A major drawback in the clinical use of ADR is the cardiotoxicity associated with repeated courses of therapy [2]. To circumvent the problem of cardiotoxicity and possibly improve the antitumor activity, studies have been carried out comparing the toxicity and antitumor activity of conjugates of ADR with DNA [5, 6] and with liposome-encapsulated drug [7]. Liposomes have received considerable attention, due to their versatility [8], with studies on liposome-encapsulated ADR suggesting a reduction in cardiotoxicity with possible improvement in antitumor activity [9, 10].

Due to the significant interaction of ADR with phospholipids [11, 12] and the dose- and schedule-dependent toxicity of ADR [13, 14], in the present study using anionic liposomes we have determined: (a) the effect of lecithin:cholesterol (L:C) ratio of the liposomal lipid bilayer on ADR encapsulation and efflux; (b) toxicity *in vivo* of single high doses of liposome-encapsulated ADR with similar doses of the free drug administered in various schedules; and (c) antitumor activity *in vitro* and *in vivo* of free and liposome-encapsulated ADR [15].

Materials and methods

Stock solutions of L-α-lecithin (from egg-yolk, type VIIE), dicetylphosphate and cholesterol (Sigma Chemical Co., St. Louis, MO) in chloroform were stored under nitrogen at −20°. The lipid composition for encapsulating adriamycin hydrochloride (NSC 123127) in anionic liposomes was L-α-lecithin–cholesterol–dicetylphosphate in molar ratios of 1:0.1:0.14, 1:0.5:0.14 and 1:1:0.14. Lipids in the required molar ratios were evaporated to dryness under vacuum, and the lipid film was dispersed in 4 ml of 0.85% sodium chloride solution containing ADR. Sonication of the liposomes for 10 min under nitrogen in an ultrasonic cleaner (Ultramet II, Fisher Scientific Co., Medford, MA) was followed by repeated washing of the liposomes (three times) with 10 vol. of 0.85% sodium chloride and centrifugation at 100,000 *g* for 45 min. An aliquot of the reconstituted liposomes in 0.85% sodium chloride was dissolved in chloroform–methanol (1:9, v/v) to release encapsulated ADR, and the fluorescence intensity of the solution was measured at excitation and emission wavelengths of 470 and 585 nm respectively. Drug concentration was computed from a standard curve for adriamycin hydrochloride.

For *in vitro* efflux studies, liposomes containing encapsulated ADR were diluted (4–5 μmoles phospholipids/ml) in RPMI 1640 tissue culture medium with or without 50% fetal bovine serum (Grand Island Biological Co., Grand Island, NY) and incubated at 37°. Duplicate 1-ml aliquots obtained immediately following dilution (0 hr) and subsequently at 24, 48, 96 and 120 hr were diluted 8-fold with 0.85% sodium chloride, the liposomes were recovered by centrifugation at 100,000 *g* for 45 min, and ADR content was determined fluorimetrically as described earlier.

For *in vivo* toxicity studies, female DBA/2 mice (18–22 g) in groups of eight were treated as follows: (a) single i.p. dose of 20 mg ADR/kg in 0.85% sodium chloride or encapsulated in anionic liposomes with an L:C ratio of 1:0.1, (b) multiple i.p. doses of 5 mg ADR/kg, QD × 4 in 0.85% sodium chloride, (c) continuous tail vein infusion of 5 mg ADR per kg per 24 hr for 96 hr using the method of Paul and Dave [16]; and (d) 0.85% sodium chloride (control mice). The mortality of mice was recorded daily, and changes in body weight were determined at weekly intervals over a 60-day period.

Results and discussion

The effect of the L:C ratio on ADR encapsulation is shown in Table 1. At an L:C ratio of 1:0.1, encapsulation of ADR (50%) was nearly twice that at L:C ratios of 1:0.5 and 1:1 (26–27%). In a preliminary study with drug-free liposomes, the binding/association of ADR at L:C ratios of 1:0.1, 1:0.5 and 1:1 was 21, 8 and 5% respectively. Although the binding/association of ADR with the liposomal lipid bilayers could be a function of both lipid and electrostatic binding [11], the results on the effect of L:C ratio (see Table 1) suggest that the enhanced encapsulation at low molar ratios of cholesterol is due primarily to the extent of lipid binding, since the electrostatic binding may be unaffected by cholesterol.

The *in vitro* efflux of ADR from liposomes with different L:C ratios is shown in Fig. 1. It is interesting to note that, unlike the cholesterol-dependent efflux of Ara-C observed in our earlier study [17], the efflux of ADR from liposomes was not significantly dependent on the cholesterol content. However, differences in the retention of ADR by liposomes with different L:C ratios were more prominent in the absence of serum. The amount of ADR retained by liposomes treated with or without serum was significantly different only at and after 96 hr ($P < 0.01$). The retention of ADR by liposomes treated without serum was not significantly different at 48 and 96 hr, but was significantly less than at 24 hr and significantly more than at 120 hr ($P < 0.05$). In liposomes incubated with serum, the amount of ADR retained at 48 hr was significantly more than at 96 and 120 hr, but significantly less than at 24 hr ($P < 0.01$). Since the incorporation of cholesterol in lecithin membranes reduces chain mobility and permeability to entrapped species [18, 19], it appears from the present results that incorporation of a high amount of cholesterol decreases encapsulation of ADR, possibly due to reduced intercalation and binding of ADR to less fluid bilayers [20].

Table 1. Effect of lecithin:cholesterol ratio on adriamycin encapsulation

Lecithin:cholesterol ratio	% Adriamycin encapsulated
1:0.1	50 ± 4*
1:0.5	27 ± 3†
1:1.0	26 ± 5

* Significantly different from L:C ratios of 1:0.5 and 1:1 ($P < 0.01$).

† Not significantly different from L:C ratio of 1:1.

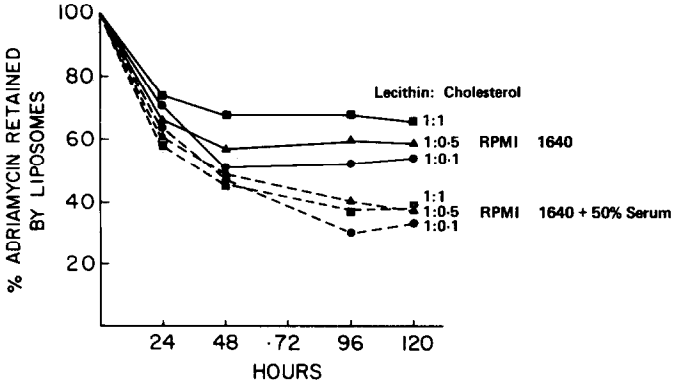

Fig. 1. Efflux of adriamycin *in vitro* from liposomes containing lecithin–cholesterol–dicetylphosphate in molar ratios of 1:0.1:0.14 (●), 1:0.5:0.14 (▲), 1:1:0.14 (■) and incubated in RPMI 1640 tissue culture medium with (-----) and without (——) 50% fetal bovine serum at 37°.

The survival of DBA/2 mice treated with 20 mg ADR/kg of the free drug in various schedules or after encapsulation in liposomes is shown in Fig. 2. The results from this study can be summarized as follows. Single (20 mg/kg) and multiple (5 mg/kg, QD × 4) i.p. doses of free adriamycin were toxic, and all mice were dead within 40 days following treatment. Mice in these groups showed a 10–15% loss in body weight after treatment and never recovered to pretherapy weights. In contrast, no mortality was observed in 60 days in mice treated with a similar dose of ADR (20 mg/kg) as a single i.p. injection encapsulated in liposomes (L:C = 1:0.1) and as a continuous 96-hr tail vein infusion of the free drug. Although a 10% loss in body weight following treatment was observed in these groups, recovery to pretherapy weights occurred by the second week and weight gain at later time intervals was similar to untreated controls. In a separate study (data not shown), the toxicity of a single i.p. dose of ADR (20 mg/kg) encapsulated in liposomes with L:C ratios of 1:0.5 and 1:1 was similar to the long-term study with liposomes at an L:C ratio of 1:0.1.

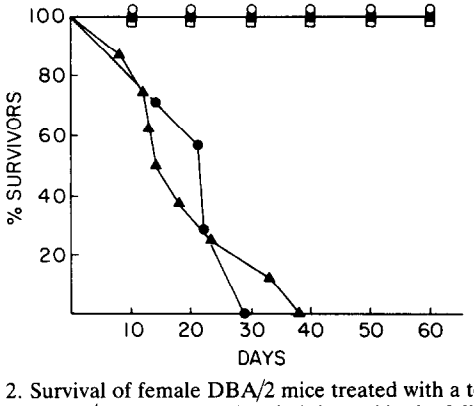

Fig. 2. Survival of female DBA/2 mice treated with a total dose of 20 mg/kg of adriamycin administered in the following schedules: (a) control (○); (b) single i.p. dose of free drug (▲); a similar single i.v. dose of free adriamycin resulted in a 100% mortality of mice occurring between day 11 and day 52 post-treatment; (c) multiple (5 mg/kg, QD × 4) i.p. doses of free drug (●); similar multiple i.v. doses of free adriamycin resulted in a 75% mortality of mice within 60 days, with deaths occurring between day 54 and day 60; (d) 96-hr continuous infusion of the free drug via tail vein (□); and (e) single i.p. dose of adriamycin encapsulated in anionic liposomes with an L:C ratio of 1:0.1 (■).

In spite of the known higher toxicity for i.p. administered free ADR [1], the lack of mortality with a single high dose of liposome-encapsulated ADR is certainly interesting. Similar reduced toxicity with i.v. administered single high doses of ADR in liposomes has been observed [21]. Various studies with liposome-encapsulated ADR have demonstrated reduced toxicity and altered plasma and tissue pharmacokinetics of ADR due to encapsulation [7, 9, 21]. Since an infusion could simulate the slow ADR release from liposomes, the observed reduced toxicity with i.p. administered high dose ADR in liposomes is suggested to be due to slow release of ADR in the peritoneal cavity. The toxicity of ADR is well documented to be a function of dose and schedule of administration [1, 2, 14]. Legha *et al.* [14] in a recent study have clearly demonstrated the relationship of ADR-induced cardiotoxicity in patients as a function of cumulative dose and peak plasma level based on duration of infusion. Studies are underway to compare the toxicity, antitumor activity, and the plasma, tissue and tumor pharmacokinetics of the parent drug and metabolites, in normal and solid-tumor-bearing mice treated with comparable high doses of ADR administered either as infusion or after encapsulation in liposomes in order to establish the superiority of either form of drug delivery in chemotherapy.

The antitumor activity against L1210 and P388 mouse leukemia based on cell kill (measured by trypan blue dye exclusion) and perturbations in cell cycle traverse measured by flow cytometry after a 24-hr treatment with 0.01, 0.1, 1.0 and 5.0 μg/ml of ADR encapsulated in liposomes with L:C ratios of 1:0.1, 1:0.5, and 1:1 was similar to identical doses of ADR in solution. In DBA/2 mice bearing i.p. L1210 (early treatment, day 1) and P388 (late treatment, day 5) mouse leukemia as ascites and treated with 4 mg/kg of free or liposome (L:C = 1:1)-encapsulated ADR, the survival times (increase in life span of approximately 50–80%) were similar. The lack of potentiation in antitumor activity with liposome-encapsulated ADR has also been observed by Rahman *et al.* [9] and Olson *et al.* [21], although in a study by Forssen and Tökés [10] improved antitumor effects with encapsulated ADR were observed. Since both experimental and clinical studies with bolus vs multiple and infusion doses of ADR indicate only reduced toxicity and essentially equivalent antitumor effects [13, 14], the lack of enhanced antitumor activity with liposome-encapsulated ADR is not surprising.

The results from this study can be summarized as follows: (a) Unlike a polar drug like cytosine arabinoside, the encapsulation efficiency of adriamycin in anionic liposomes containing cholesterol was significantly greater (~2-fold higher) at an L:C ratio of 1:0.1 than at L:C ratios of 1:0.5 and 1:1. However, the pattern of efflux of adriamycin *in vitro* from liposomes at these different L:C ratios was not

markedly different; (b) The toxicity of a single i.p. dose of 20 mg ADR/kg (LD_{50} for single i.v. dose of free drug) encapsulated in anionic liposomes was comparable to a similar dose of the free drug administered as a 96-hr tail vein infusion. Based on the mortality of mice treated with a 20 mg/kg dose of free adriamycin, i.p. and i.v., either as single or divided bolus injections, the reduced toxicity observed with ADR encapsulated in the anionic liposomes with no selective targeting properties is probably due to slow drug release *in vivo*; (c) In preliminary studies with L1210 and P388 mouse leukemia *in vitro* and *in vivo*, the antitumor activity of ADR was not found to be compromised or potentiated after encapsulation in liposomes; and (d) Due to the variable interaction of drugs with liposomal lipid bilayers, future studies should be directed toward systematically evaluating the effect of lipid composition on encapsulation of a drug, and quantitatively comparing liposome vs infusion doses of the drug, to understand the potential for such methods of drug delivery in cancer chemotherapy. Further, due to the differences in antitumor activity of parent drug and metabolites of the anthracyclines [22, 23], pharmacokinetic studies of metabolite formation following treatment with slow drug release carriers and as infusion will be important.

Acknowledgements—The authors would like to thank Dr. George Williams and Ms. Julie MacMillan, Department of Biostatistics, Cleveland Clinic Foundation, Cleveland, OH, and Ms. Susan Hilsenbeck, Comprehensive Cancer Center, Miami, FL, for statistical analysis of the data. The excellent secretarial assistance of Ms. Catherine Reinhard is gratefully acknowledged. Supported by the Cleveland Foundation, Public Health Service Grant CA14395, and American Cancer Society Grant IN-51U.

Research Division
Cleveland Clinic Foundation
Cleveland, OH 44106, U.S.A.
RAM GANAPATHI*

Department of Oncology
University of Miami School of Medicine
Miami, FL 33101, U.S.A.
AWTAR KRISHAN

* Address all correspondence to: Ram Ganapathi, Ph.D., Research Division, Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44106.

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Biochemical Pharmacology, Vol. 33, No. 4, pp. 700–702, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

Structure–function relationships in the inhibition of synaptosomal dopamine uptake by phencyclidine and analogues: potential correlation with binding site identified with [^{3}H]phencyclidine

(*Received* 21 *June* 1983; *accepted* 14 *September* 1983)

Phencyclidine (PCP) exhibits psychotomimetic, anaesthetic, analgesic, stimulant and depressant effects which probably involve a variety of neuropharmacological mechanisms. Biochemical and electrophysiological studies have shown that PCP interacts with a series of functional neuronal receptors. These receptors include the muscarinic cholinergic receptor, the μ-opiate receptor [1], the Na^+ and K^+ channels [2] and the nicotinic receptor [3]. PCP also