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A Comparison of the Therapeutic Effects of Free and Liposomally Encapsulated Vincristine in Leukemic Mice

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Abstract—*The neurological toxicity of the Vinca alkaloids restricts their use in cancer chemotherapy. In this study we investigated the potential of liposomal encapsulation of one of these drugs, vincristine, to reduce this toxicity and hence increase the utility of this class of antimitotic agents.*

Plasma clearance studies for i.v. administration to NMRI mice, indicated that vincristine (VCR) included in liposomes (VCR-Lip) was removed more slowly from the plasma than was free VCR, and that whilst the liposomes were in the circulation most of the VCR remained associated with its liposomal carrier.

Early therapeutic trials of vincristine in liposomes, given intravenously to DBA/2 mice previously inoculated with P388 leukemic cells, demonstrated that considerable toxicity was caused by the lipid composition of the carrier (phosphatidylcholine, cholesterol and stearylamine). This toxicity was confirmed in in vitro *screening of empty liposomes of various composition, and from this study a liposome composition of very low toxicity was selected (phosphatidylcholine, cholesterol and phosphatidylserine).*

Further therapeutic trials with the drug entrapped in 'non-toxic' liposomes showed that liposomal encapsulation of Vinca alkaloids did not provide any advantage either in terms of reduced toxicity or in increased therapeutic index over the free drug for the treatment of lymphocytic leukemia.

INTRODUCTION

THE ANTI-MITOTIC agents of the Vinca alkaloid class, namely vinblastine and vincristine, are widely used in cancer chemotherapy. However, their use, particularly that of the more active vincristine, is restricted by their toxic effects on the central and autonomic nervous system [1].

Liposomes, phospholipid vesicles, have been proposed as drug carriers firstly because of their biodegradability and secondly because the flexibility of their composition may offer the possibility of selective cellular interactions. A large number of drugs, enzymes etc. have already been encapsulated in liposomes [2] and we had already shown [3] that both alkaloids could be entrapped in liposomes and were retained with only a slow leakage even in the presence of serum. In addition liposomally encapsulated drugs were accumulated by rat embryo fibroblasts but in a markedly different manner from that of the free drugs. We concluded that the liposomal complex initially adsorbed to the cell surface, then entered the cell probably by an endocytotic process; whilst the free drug entered by passive diffusion.

It was therefore decided to investigate whether or not encapsulation of these drugs in liposomes would reduce their toxicity whilst maintaining, or even improving, their therapeutic effect; since vincristine is more active but also more toxic it was chosen for this trial.

For the comparison of the two forms of vincristine against animal tumors we followed the protocol of the National Cancer Institute [4] and used mice inoculated with P388 leukemic cells, since from natural products screening *in vivo* it has become evident that whilst L1210 and P388 leukemias tend to

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Abbreviations: VCR, vincristine (Oncovin); VCR-Lip, vincristine entrapped in liposomes.

respond to similar materials, P388 cells are generally more sensitive to materials showing activity against both.

MATERIALS AND METHODS

Phosphatidylcholine (grade VE), cholesterol and phosphatidylserine were obtained from Sigma Chemical Co., St Louis, U.S.A.; and stearylamine from Koch Light Labs Ltd, Colnbrook, U.K. Vincristine sulphate (VCR) was purchased from E. Lilly Benelux, SA, Brussels, Belgium. 26-^{14}C Cholesterol (50 mCi/mmole, spec. act.) and G-^{3}H vincristine sulphate (2.10 Ci/mmole, spec. act.) were purchased from the Radiochemical Centre, Amersham, U.K. The scintillation fluid, Aqualuma SB, was obtained from Lumac System AC Basel, Switzerland. All other chemicals being reagent grade.

Liposome preparation

Liposomes containing vincristine were prepared according to the method described. Briefly phosphatidylcholine, cholesterol and either stearylamine or phosphatidylserine, in a molar ratio of 7:2:1, dissolved in chloroform, were evaporated under vacuum onto the walls of a glass flask. Vincristine, being predominantly lipophilic, was dissolved in chloroform and mixed with the lipids prior to evaporation, the radioactive labels, ^{14}C-cholesterol and ^{3}H-VCR being similarly incorporated. Liposomes were formed by adding physiologically iso-osmotic phosphate buffered saline (PBS) [0.8% (w/v) NaCl, 27 mM KCl, 7.9 $Na_2HPO_4.2\ H_2O$, 1.47 mM KH_2PO_4, pH 7.3], to the dried film, the mixture was then dispersed by shaking and subsequent sonication. Sonic disruption was carried out using a Branson Sonifier (75 W, 20 kHz, 27.5 mm probe) for 15 sec at 1 min intervals for a total sonication time of 2 min. The sonication was performed under a nitrogen atmosphere at 25°C. After sonication the suspension was kept under N_2 at 25°C for at least 2 hr to allow liposomes to form and seal [5].

Free VCR was separated from that entrapped in liposomes by a modification of the method described by Fry *et al.* [6]. Plastic 5 ml syringes (B-D plastipak), containing two filters, with their ends closed off, were filled with prepared mixture of Sephadex G50 medium, activated charcoal and Dextran T 70 in weight ratio of 100:5:0.05, such that their entire volume was packed with swollen gel. A stock of such packed syringes were stored at 4°C ready for use. To prepare the syringes for a liposome-free drug separation, the ends were unblocked, the syringes inserted into polyallomer 7.5 cm × 1.6 cm centrifuge tubes and centrifuged at 600 ***g*** for 10 min (Searle Jouan E96 centrifuge), the run-up time being less than 15 sec. Approximately 2 ml of the liposome solution was then carefully added to the top of the Sephadex column, allowing the uppermost part of the column to reswell. The syringes were then placed in polyallomer centrifuge tubes and centrifuged at 600 ***g*** for 10 min. The liposomes collected at the bottom of the centrifuge tube whilst the free drug was retained on the column within the syringes. By this method 92% of the original lipid was recovered in the liposomes as determined either from ^{14}C-cholesterol counting or direct phospholipid assay [7]. The liposomes thus prepared were routinely sampled and examined for lipid oxidation by the method of Klein [8], none was detected within the sensitivity limits (0.01%) of the method. The efficiency of entrapment, being defined as the percentage of the original amount of drug which remained associated with the liposomes, was routinely 10–11%. VCR was found to leak out, or become dissociated from, these liposomes at a rate of $<0.1\%$/hr, the rate being unaffected by the presence of 50% serum. After incubation in serum for 30 hr at 37°C, the ratio of ^{14}C-cholesterol to phospholipid was virtually identical to the original ratio prior to incubation.

Plasma clearance

NMRI mice (Animalerie of Heverlee) of average weight 25 ± 0.5 g were given free or liposomally encapsulated vincristine by tail vein injection; for each time point three mice were anaesthetized, the inguinal region opened and blood drawn rapidly from the severed femoral artery. Blood samples were placed in cold Eppendorf microcentrifuge tubes (No. 3924) containing 100 μl of EDTA as anticoagulant, shaken then centrifuged at 9500 ***g*** for 5 min in a Hettich Mikro rapid centrifuge. Aliquots of plasma were taken for separate ^{3}H and ^{14}C determination using a Packard Tri-Carb sample oxidizer. The zero time points were calculated from the injected concentrations of VCR and lipid on the basis of an average blood volume per mouse of 0.8 ml.

Therapeutic trials

DBA/2 mice (Animalerie Iffa Credo) of

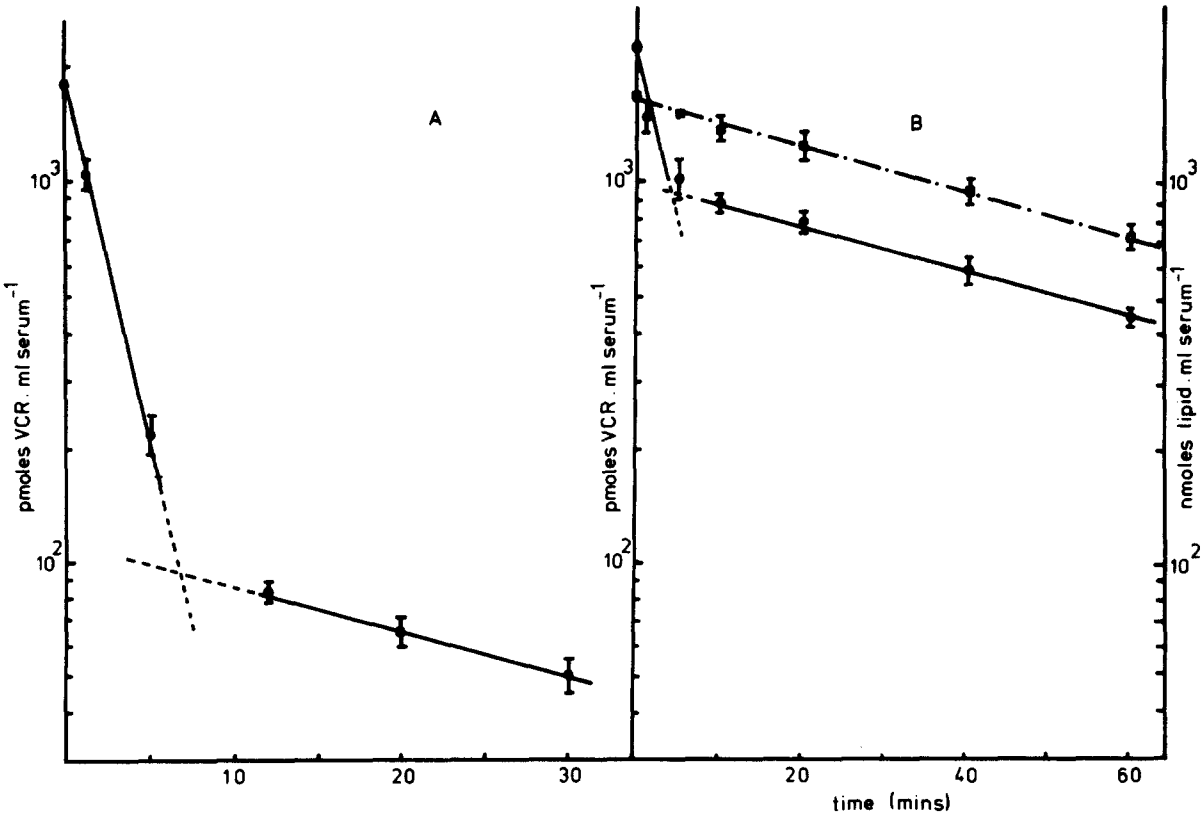

Fig. 1. The plasma clearance of free and liposomally encapsulated vincristine after i.v. administration to NMRI mice. (A) Free vincristine (2 nmole/25 g *mouse) injected into the tail vein at time zero, the dose being adjusted for each mouse, three mice were used for each time point. At the times indicated the mice were anesthetised and blood drawn from the severed femoral artery as described, and the concentration of* ^{3}H-*VCR remaining in the serum determined. (B) Vincristine* (2.4 nmole/25 g *mouse) encapsulated in liposomes composed of phosphatidylcholine, cholesterol and strearylamine* (7:2:1 *mole ratio) injected into the tail vein at time zero. Total lipid injected corresponds to* 1.6 μmole/25 g *mouse. The procedure was the same as described in (A).*

average weight 20 ± 1.0 g were i.v. inoculated with 2×10^4 P388 cells on day 0. Liposomally encapsulated or free VCR, at the concentrations stated, was given by tail vein injection beginning on day 1 and thereafter according to the dosage schedule adopted. The mice were weighed daily at the same time of day throughout the trial.

RESULTS

The plasma clearance of free VCR and VCR encapsulated in liposomes composed of PC:CHOL:SA in NMRI mice is shown in Fig. 1. The clearance of the free drug (Fig. 1A) may be considered as being biphasic, with an initial fast phase of $T_{\frac{1}{2}} = 1$ min 50 sec followed later by a slower phase of $T_{\frac{1}{2}} = 26$ min 18 sec. The clearance of entrapped VCR, as monitored by ^{3}H-VCR, could also be approximated to being biphasic, again with a fast phase of $T_{\frac{1}{2}} = 1$ min 45 sec but followed almost immediately by a much slower phase of $T_{\frac{1}{2}} = 50$ min 1 sec. The clearance of liposomes, as indicated by the label ^{14}C-cholesterol, showed only a single slow phase of $T_{\frac{1}{2}} = 50$ min 30 sec; and the extrapolation of the linear plot to zero time gave an intercept value which was almost identical to that calculated from the dilution of the original injected concentration by the total blood volume.

Figure 2 shows the effect of i.v. administration of free and entrapped VCR on mice, inoculated with P388 leukemic cells. In Fig. 2A, a total dose of 2.87 mg/kg of free VCR given on day 1 and 2, gives some therapeutic effect, but as can be seen from the large weight loss, is very toxic (LD_{50} in mice = 3.0 mg/kg [9]). However a similar dosage (2.82 mg/kg) when given in liposomal form causes the death of almost all animals [mean survival time (MST) for VCR-liposomes = 3.0 days, for free VCR = 13.0 days and for controls = 11.2 days]. Figure 2B shows the effect of a lower dose (1.6 mg/kg) given over a greater interval, the liposomal form of VCR again causing deaths the day after injection and no

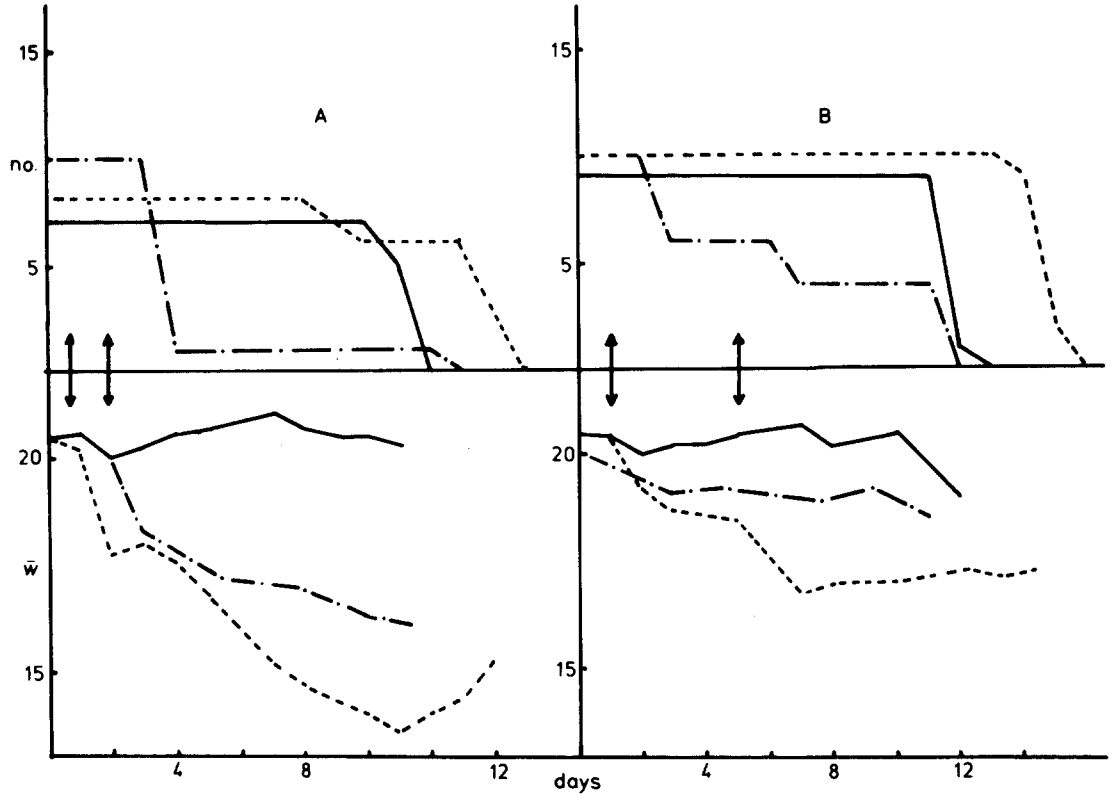

Fig. 2. A comparison of the therapeutic effect of free and liposomally encapsulated vincristine on DBA/2 mice inoculated with 2×10^4 *p 388 leukemic cells on* day 0. *The liposomes were composed of phosphatidylcholine, cholesterol and stearylamine in a mole ratio of* 7:2:1. (*A*) (———) *controls;* (- - - - -) *free VCR* (1.44 mg/kg *given i.v. on* days 1 *and* 2); (— · — · —) *VCR in liposomes* (1.42 mg/kg *given i.v. on* days 1 *and* 2). *Total lipid dose corresponds to* 4.2 g/kg. *The upper part of the figure shows the number of survivors each day at the time of weighing, whilst the lower part shows their average weight.* (*B*) (———) *controls;* (- - - - -) *free VCR* (0.8 mg/kg *given i.v. on* days 1 *and* 5); (— · — · —) *VCR in liposomes* (0.8 mg/kg *given i.v. on* days 1 *and* 5). *Total lipid dose corresponds to* 1.51 g/kg. *The figure is as described for* (*A*).

animals surviving longer than the controls (MST for VCR-liposomes = 6.3 days, for free VCR = 14.4 days and for controls = 11.5 days).

Preliminary experiments with empty liposomes of the same composition given i.v. were found to cause weight loss and even death of the animals and that if different lipids were used less toxic effects were observed. A study of the effects of coincubation of liposomes with cultured leukemic cells and fibroblasts was undertaken and showed that the presence of certain lipids, even in small amounts, could be toxic to both cells, whilst others were toxic to only one cell type, and still others were non-toxic [10].

Using this information VCR was then encapsulated in liposomes of the non-toxic mixture PC:CHOL:PS (mole ratio 7:2:1), the plasma clearance (not shown) was not changed by this modification.

Figure 3 shows the effect of administration of this liposome drug complex as compared to free VCR on mice inoculated with P388 cells. In Fig. 3A. a low total dose (0.9 mg/kg) divided over days 1, 5 and 9 produces little toxicity and a similar therapeutic effect for either form (MST for VCR-liposomes = 12.7 days, for free VCR = 12.1 days and for controls = 10.7 days). When almost twice the dose is given over the same period (Fig. 3B), toxicity of free VCR begins to be apparent, and whilst less toxicity is observed for the liposomal form, some therapeutic effect is lost (MST for VCR-liposomes = 11.8 days, for free VCR = 13.4 days and for controls = 11.0 days). Repeat experiments confirmed the observation that at low dosages VCR in liposomes was as effective as free VCR for therapy and slightly less toxic, but that at higher doses although possibly the toxicity remained less, much of the therapeutic benefit was lost.

DISCUSSION

The plasma clearance of free VCR could be described as biphasic with components of $T_{\frac{1}{2}}$ about 2 min and about 26 min, these results are similar to those found for vinblastine [11]. The clearance of VCR in liposomes

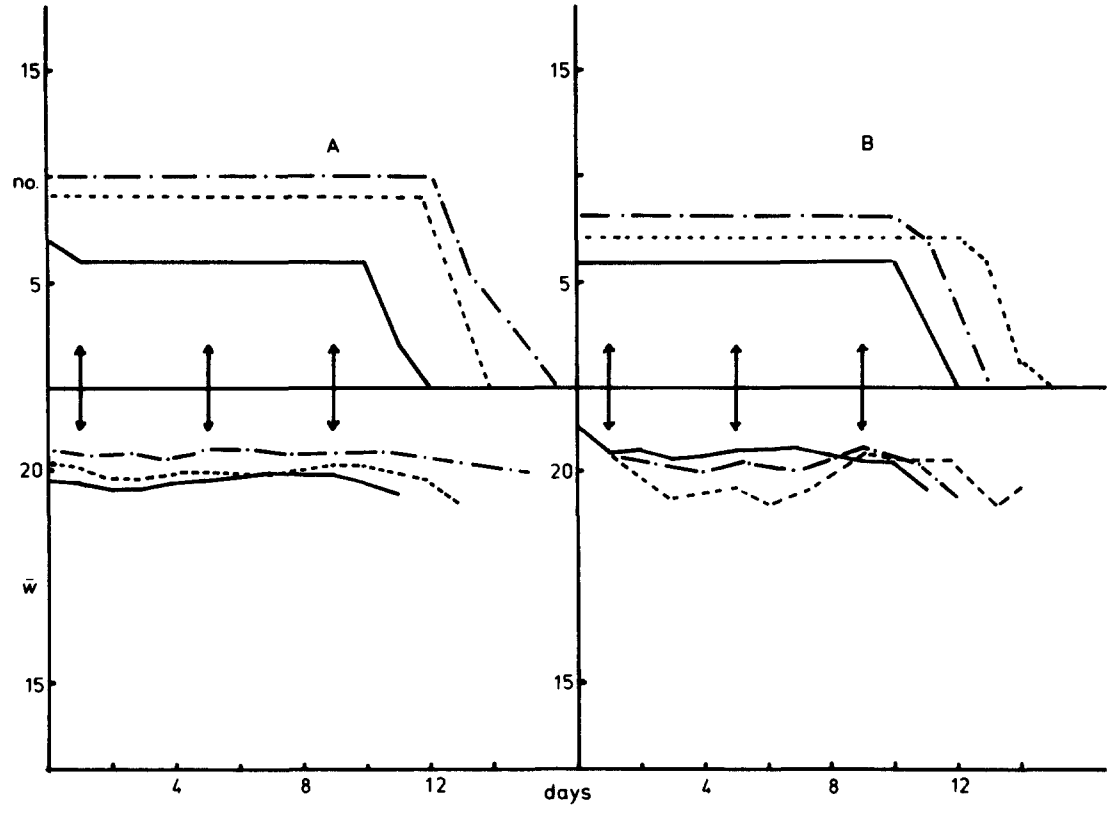

Fig. 3. A comparison of the therapeutic effect of free VCR and VCR encapsulated in liposomes (composed of phosphatidylcholine, cholesterol and phosphatidylserine in a mole ratio 7:2:1) on DBA/2 mice inoculated with 2×10^4 *P* 388 *leukemic cells on* day 0. *(A)* (————) *controls;* (-----) *free VCR* (0.3 mg/kg *given i.v. on* days 1, 5 *and* 9); (—·—·) *VCR in liposomes* (0.3 mg/kg *given i.v. on* days 1, 5 *and* 9). *Total lipid dose corresponds to* 0.85 g/kg; *figure description as given in Fig.* 2*(A). (B)* (————) *controls;* (-----) *free VCR* (0.6 mg/kg *given i.v. on* days 1, 5 *and* 9); (—·—·) *VCR in liposomes* (0.6 *mg/kg given i.v. on* days 1, 5 *and* 9). *Total lipid dose corresponds to* 1.62 g/kg.

(Fig. 1B) was different in that although a fast phase of $T_{\frac{1}{2}}$ about 2 min was observed, the bulk of VCR was cleared much more slowly, $T_{\frac{1}{2}}$ about 50 min, and in addition the clearance of liposomes as indicated by ^{14}C measurements showed only a single slow phase of $T_{\frac{1}{2}}$ about 50 min. There has been considerable discussion [12] firstly as to whether liposomes remain as intact carriers in the blood and secondly whether ^{14}C-cholesterol is a good marker for the liposomal membrane since it can exchange with other membrane lipids and lipoproteins [13]. We are unable to prove conclusively that the liposomes used here are not broken up in the plasma, but suggest that since the clearance plot of ^{14}C-cholesterol gives an extrapolated value for the zero time concentration which corresponds to that calculated for the dilution of the original liposome concentration, only two hypotheses can be advanced. Either the liposomes are ruptured almost instantaneously, or that they remain relatively intact.

We would suggest that the liposomes remain relatively intact for the following reasons; firstly incubation of these liposomes in the presence of 50% serum for 30 hr as described in the Materials and Methods section showed no indication of breakdown of the liposome structure as determined by the cholesterol phospholipid ratio, and secondly the bulk of VCR is cleared out at the same rate as that of the cholesterol marker. The finding that a small amount of drug is cleared at the same rate as for free VCR is not surprising since we have previously shown [3] in culture experiments that a small proportion of VCR is always liberated from liposomes as soon as coincubation commences. This portion probably represents the fraction of VCR which is adsorbed onto the liposome surface but then desorbs in the presence of serum proteins, platelets etc to which the vinca alkaloids are known to bind [1].

The severe toxicity observed for VCR in liposomes composed of PC:CHOL:SA (Fig. 2) was shown to be due to the particular lipid composition, since when the mixture was changed to PC:CHOL:PS (Fig. 3) no toxicity was observed.

Only at low doses was liposomally encapsulated VCR as therapeutically effective as the free form. At higher doses, some of the therapeutic effect, compared to the free form, was lost and from the weight measurements a likely explanation would be that this loss may be associated with the beginning of some toxicity of the liposome–drug complex. Higher drug dose, in the latter case, is associated with higher lipid concentration, and since it was previously shown [10] that even this second liposome composition became toxic *in vitro* at high concentrations, then the onset of toxicity *in vivo* may be related to a cumulative effect of the drug and the liposomal lipids.

Such speculation concerning the dose dependency of therapeutic effect apart, we concluded from this study that liposome encapsulation of vincristine does not offer any advantage in the treatment of lymphocytic leukemia.

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