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# The Effect of Liposome (Phospholipid Vesicle) Entrapment of Actinomycin D and Methotrexate on the *In Vivo* Treatment of Sensitive and Resistant Solid Murine Tumours*

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**Abstract**—*When tested in mice bearing the Ridgway osteosarcoma (ROS) the activity of methotrexate entrapped in small cationic liposomes was increased, both in terms of tumour growth inhibition and toxicity for normal tissues. Conversely, actinomycin D entrapped in small cationic liposomes lost its cytotoxic activity, both against the tumor and normal tissues. In addition, liposome-entrapped actinomycin D proved ineffective therapeutically against a new ROS tumour subline in which resistance to free drug had been derived* in vivo; *moreover, this resistance appeared to have resulted from failure to retain drug rather than through impaired drug uptake. These results, and those obtained from tissue distribution studies using free and liposome-entrapped actinomycin D, suggested that (a) liposome entrapment modifies the pharmacokinetics and hence activity of these drugs by acting as a slow release system rather than by providing a means of selective delivery to tumours, and (b) the use of liposome-entrapped actinomycin D to overcome drug resistance acquired* in vivo *may be inappropriate.*

## INTRODUCTION

LIPOSOMES are bilayered phospholipid vesicles which were originally developed as model membranes [1], but which have attracted considerable attention in recent years as potential carriers of cytotoxic drugs for selective delivery to tumours [2–5]. An enhanced therapeutic effect has been claimed for several of them [6–11], and two groups have demonstrated prolonged survival of ascites-tumour-bearing mice following treatment with liposome-entrapped actinomycin D (AD) [6, 7]. In one of these studies [6], a reduction in host toxicity was clearly demonstrated, comparing free and entrapped drug, but the effect of entrapment on the anti-tumour activity of AD was not assessed in either study. Papahadjopoulos *et al.* [12] have reported that transport-defective AD-resistant Chinese hamster cells can be rendered sensitive *in vitro* by the use of liposome-entrapped AD, but there is no evidence that AD resistance can be overcome *in vivo* by this method.

Kosloski *et al.* [11] have shown that methotrexate (MTX)-resistant solid murine tumours respond to liposome-entrapped MTX *in vivo* using a regimen which was ineffective when MTX was given as free drug, although Kimelberg and Atchison [13], using an L1210 ascites tumour model, reported that liposome-entrapped MTX increased host toxicity but did not enhance the drug's antitumour activity as assessed by comparative survival times. Most of the other positive therapeutic studies relate to entrapped cytosine arabinoside, and overall the evidence to date supports the hypothesis that the observed therapeutic effects of liposome entrapment result not from any selective delivery of drug to tumours, but rather from the delay in plasma clearance which has been demonstrated for several liposome-entrapped drugs [14–16].

This study therefore examined three major areas: (a) the influence of liposome entrap-

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ment of AD and of MTX on their anti-tumour activity and host toxicity over a wide range of doses; (b) the effect of liposome entrapment of AD on drug uptake in host tissues and in both sensitive and resistant solid tumours; (c) the therapeutic efficacy of liposome-entrapped AD *in vivo* in the treatment of an AD-resistant solid tumour.

The mouse tumour model chosen was the Ridgway osteogenic sarcoma (ROS) which is particularly sensitive to free AD, exhibiting an orderly dose–response curve for the drug [17, 18]. The parent tumour is relatively resistant to free MTX, but for the studies involving resistance to AD, a new subline of the ROS tumour, with AD resistance acquired *in vivo*, was derived.

## MATERIALS AND METHODS

*Lipids and drugs*

Egg phosphatidylcholine (PC) was prepared by the method of Dawson *et al.* [19]. Cholesterol (C) (Grade 99+%) was purchased from Sigma Chemical Co., stearylamine (S) (*N*-octadecylamine) from K & K Laboratories. Mannitol-free actinomycin D (used for liposome entrapment) was generously supplied by Merck, Sharp & Dohme Ltd.; for studies using free AD, Cosmegen (Lyovac), was purchased from the same company. Tritium-labelled AD (The Radiochemical Centre, Amersham) was supplied in 50% aqueous ethanol with a specific activity of 11–16 mCi/mg. Significant metabolism of actinomycin D following injection into mice does not occur [20], and measurements of tritium label are therefore taken to represent intact drug in all *in vivo* studies. Methotrexate (Lederle Laboratories, Cyanamid, U.K.) was supplied as the sodium salt, containing no preservatives, and this was used for liposome entrapment and for free drug studies. For the processing of radioactive samples, scintillation fluid was prepared containing 15 g butyl PPD (Sigma Chemical Co.) and 250 mg of dimethyl POPOP (Koch Laboratories) in 2.5 l of toluene (BDH Chemicals Ltd.) then diluted 2:1 with Triton X-100 (Sigma Chemical Co.). All other chemicals were of reagent grade.

*Preparation of liposome-entrapped drugs*

Stock lipid solutions (10 mg/ml) containing PC:C:S in the molar ratio of 18:4:5 were prepared in chloroform, stored at −20°C in an atmosphere of nitrogen and used throughout these studies for preparation of liposomes. AD entrapped in liposomes was prepared by dissolving measured quantities of the drug in aliquots of lipid solution together with trace amounts of [$^3$H]-actinomycin D; the solution was then subjected to rotary evaporation under vacuum in a round bottomed flask at 37°C. The dried lipid film was dispersed with sterile phosphate-buffered saline (P.B.S.) (Dulbecco's modified, calcium and magnesium-free, Gibco Biocult) in an atmosphere of nitrogen taking 2 ml of buffer for 100 mg of total lipid. The suspension was then subjected to sonication for a total of 15 min in an ice-bath, using a probe system (Heat Systems, W225-R) with a 12 mm tip and a 50% pulsed input; this produced a relatively homogenous preparation of small liposomes (estimated mean diameter 70 nm by electron microscopy). Entrapped AD was separated from free drug by Sephadex G-50 column chromatography (K 15/30 column, Pharmacia), eluting with sterile PBS, and the percentage of entrapment was measured by radioactive counting of the liposome fraction which was eluted in the void volume (approximately 12 ml). Counting of tritium was performed on a Packard tricarb liquid scintillation counter with external standardisation and a counting efficiency of 40–60%. A starting ratio of total lipid:AD of 100:1 gave the highest efficiency of entrapment at 15.3% ±1.4 (S.E.M. of 14 preparations) and this was generally employed for tissue distribution studies. For therapeutic studies, lower rates of entrapment but more concentrated drug-containing liposomes were obtained by using a lipid:AD ratio of 100:5 or 100:10. For some studies, further concentration of AD-containing liposomes was performed by ultrafiltration using an Amicon cell and XM100A membrane to produce a solution of entrapped drug containing up to 400 μg of AD/ml, and 5 μg of AD/mg of lipid.

For liposome entrapment of MTX, aliquots of stock lipid solution (PC:C:S in the molar ratio 8:4:5) were rotary evaporated as above, and the lipid film was dispersed with a 50 mg/ml solution of MTX in sterile P.B.S. in an atmosphere of nitrogen. The suspension was then sonicated as above to produce small liposomes, and entrapped MTX was separated from free drug by a similar process of Sephadex G-50 column chromatography. The concentration of MTX in liposomes was estimated spectrophotometrically, measuring absorbance at 300 mM together with that of a standard MTX solution on a Unicam (SP800) ultraviolet spectrophotometer.

The efficiency of entrapment of MTX prepared by this method was 2.0% ±0.2% (S.E.M. of 6 preparations). For some studies, concentration of liposome solution was performed by ultrafiltration, to produce a preparation of liposomes containing 2500 μg of MTX/ml and 20 μg of MTX/mg of lipid.

For other studies, empty liposomes were prepared from the same stock solutions as above using the same method but dispersing the lipid film with sterile P.B.S. alone. Prior to use, all liposome preparations, drug-containing or empty, were passed through 0.22 μ sterile filters (Acrodisc, Gelman) into evacuated sterile vials, from which aliquots were taken and injections performed within an hour of preparation of liposomes.

*The tumour model*

The AD sensitive Ridgway osteogenic sarcoma (ROS), kindly supplied by Dr. F. M. Schabel, Jr. (Southern Research Institute, Alabama), was maintained by serial subcutaneous passage of 200 mg tumour fragments through female inbred AKR mice weighing 20–30 g. All mice were bred at Charing Cross Hospital Medical School Animal Unit, and mice of either sex were used for therapeutic testing. All animals were housed in plastic cages in conditions of heating, light, humidity and feeding which were kept constant throughout the studies. Tumours were suitable for use 10–12 days after implantation, at a size of 1–1.5 g, and these were generally used for all therapeutic experiments.

The AD resistant ROS subline was derived *in vivo* by successive passages of tumour fragments in the phase of regrowth after repeated treatments with free AD, starting at a dose of 0.25 μg/g (given 4 times at 14 day intervals) and progressively increasing doses in successive generations. After passaging tumour through seven generations of treated mice, the tumour generally continued to grow despite treatment with 0.8 μg/g i.v. (the $LD_{50}$ of free AD). This resistant ROS subline was maintained in the same inbred AKR mice as were used for the sensitive tumour.

*Procedure for therapeutic studies*

*Actinomycin D.* Groups of sensitive and resistant tumour-bearing mice (six per group) were treated with single intravenous (tail vein) injections of either free or liposome-entrapped AD (0.2–0.4 ml) at spaced doses, increasing by a factor of 2 in each case. Free drug was diluted in sterile water to a concentration of 50 μg/ml and given at doses from 0.125 to 2.0 μg/g body weight; liposome-entrapped AD, prepared as above, was given at doses of 0.125–8.0 μg/g body weight. Mice were weighed and caliper measurements of tumour were taken on the day of treatment, and on the fifth and tenth post-treatment days. The tumour measurements [longest diameter ($a$) and perpendicular height ($b$)] were all performed by the same observer and were used to derive a measure of tumour volume from the formula

$$\frac{a \times b^2,}{2}$$

and this has generally been equated with tumour mass (in g) (Dr. F. M. Schabel, Jr., personal communication).

*Methotrexate.* Groups of AD-sensitive tumour-bearing mice (5–7 per group) were treated with single intravenous injections of either free or liposome-entrapped MTX in a volume of 0.2–0.4 ml. Free drug was diluted with sterile water and given at doses from 5 to 160 μg/g body weight. Entrapped MTX, prepared as above, was given at doses from 5 to 40 μg/g body weight. Treated animals were weighed and tumours measured as described previously.

In addition, two groups of AD-sensitive tumour-bearing mice (6 per group) were treated with single intravenous injections of empty liposomes only, prepared as above, at doses of either 12.5 or 40 mg of total lipid, and their response was followed by the methods outlined above. These doses were chosen as equivalent to the average and the maximum doses of lipid received by mice receiving liposome-entrapped drug (AD or MTX).

*Procedure for tissue distribution studies*

Groups of AD-sensitive and AD-resistant tumour-bearing mice were treated with single intravenous injections of 0.5 μg/g of AD containing a tracer amount of [$^3$H]-actinomycin D, either in the free form or entrapped in liposomes (prepared as described previously). At time intervals from 3 hr to 7 days after treatment, heparinised blood samples were obtained by retro-orbital puncture from anaesthetised animals which were then killed, and tissues were removed, as listed in Tables 1 and 2. These were weighed and digested overnight in 4 vol 33% KOH; then 0.2 ml aliquot of each digest was neutralized with 1.0 ml of 1.5 M HCl, 10 ml of scintillation fluid was added, and tritium levels in samples were counted after a 48-hr delay to allow for decay of chemiluminescence. Plasma samples and bone marrow samples (obtained by flush-

*Table 1. Tissue distribution of [$^3$H]-actinomycin D following injection in free form into AD-sensitive ROS tumour-bearing AKR mice*

| Organ | 3 hr | 24 hr | 48 hr | 72 hr | 7 days |
|---|---|---|---|---|---|
| Liver | 5.63 ± 1.43 (6) | 0.69 ± 0.07 (6) | 0.20 ± 0.02 (6) | 0.22 ± 0.03 (6) | 0.08 ± 0.03 (5) |
| Spleen | 15.36 ± 2.07 (6) | 8.55 ± 1.30 (6) | 4.08 ± 0.26 (6) | 3.21 ± 0.39 (6) | 0.29 ± 0.04 (5) |
| Kidney | 9.69 ± 1.10 (6) | 1.28 ± 0.13 (6) | 0.35 ± 0.04 (6) | 0.36 ± 0.05 (6) | 0.10 ± 0.03 (5) |
| Lung | 6.84 ± 0.61 (6) | 1.63 ± 0.17 (6) | 0.51 ± 0.04 (6) | 0.40 ± 0.08 (6) | 0.10 ± 0.01 (5) |
| Small intestine | 10.49 ± 1.20 (6) | 1.61 ± 0.16 (6) | 0.51 ± 0.06 (6) | 0.43 ± 0.07 (6) | 0.07 ± 0.03 (5) |
| ROS tumour | 4.66 ± 0.40 (6) | 5.96 ± 0.50 (7) | 5.27 ± 0.74 (7) | 4.82 ± 0.23 (6) | 1.46 ± 0.30 (5) |
| Muscle | 2.26 ± 0.27 (6) | 0.33 ± 0.05 (6) | 0.17 ± 0.04 (6) | 0.15 ± 0.02 (6) | 0.03 ± 0.01 (6) |
| Bone marrow* | 3.56 ± 0.45 (5) | 1.37 ± 0.18 (5) | 0.38 ± 0.09 (5) | 0.15 ± 0.06 (5) | — |
| Plasma† | 1.04 ± 0.10 (6) | 0.44 ± 0.18 (7) | 0.10 ± 0.03 (7) | 0.08 ± 0.02 (6) | — |

Results expressed as percentage of injected dose per g of tissue (corrected for blood content) with standard error of mean and number of animals per group in parentheses.

*Results expressed as percentage of injected dose in whole bone marrow, estimated by the method of Rahman *et al.* [40].

†Results expressed as percentage of injected dose in estimated whole plasma volume.

*Table 2. Tissue distribution of liposome-entrapped [$^3$H]actinomycin D following injection into AD-sensitive ROS tumour-bearing AKR mice*

| Organ | 3 hr | 24 hr | 48 hr | 72 hr | 7 days |
|---|---|---|---|---|---|
| Liver | 11.06 ± 1.11 (5) | 10.03 ± 1.25 (6) | 9.07 ± 0.71 (5) | 6.83 ± 1.05 (5) | 5.56 ± 0.93 (6) |
| Spleen | 3.30 ± 0.36 (5) | 3.94 ± 0.25 (6) | 4.46 ± 0.35 (5) | 2.49 ± 0.35 (5) | 1.73 ± 0.34 (6) |
| Kidney | 3.40 ± 0.52 (5) | 2.19 ± 0.20 (6) | 2.24 ± 0.22 (5) | 1.44 ± 0.19 (5) | 1.15 ± 0.20 (6) |
| Lung | 2.70 ± 0.60 (5) | 1.73 ± 0.45 (6) | 1.35 ± 0.12 (5) | 1.18 ± 0.20 (5) | 0.56 ± 0.08 (6) |
| Small intestine | 3.17 ± 0.34 (5) | 1.57 ± 0.21 (6) | 1.37 ± 0.21 (5) | 0.98 ± 0.13 (5) | 0.60 ± 0.08 (6) |
| ROS tumour | 2.12 ± 0.40 (5) | 2.00 ± 0.19 (6) | 1.45 ± 0.14 (5) | 0.69 ± 0.06 (5) | 0.44 ± 0.08 (6) |
| Muscle | 0.60 ± 0.05 (5) | 0.50 ± 0.04 (6) | 0.60 ± 0.08 (5) | 0.41 ± 0.05 (5) | 0.38 ± 0.05 (6) |
| Bone marrow* | 1.26 ± 0.32 (5) | 0.57 ± 0.14 (6) | 0.55 ± 0.16 (5) | 0.34 ± 0.08 (5) | — |
| Plasma† | 26.77 ± 2.29 (5) | 1.38 ± 0.11 (6) | 0.73 ± 0.03 (5) | 0.40 ± 0.04 (5) | 0.11 ± 0.02 (6) |

Results expressed as percentage of injected dose per g of tissue (corrected for blood content) with standard error of mean and number of animals per group in parentheses.

*Results expressed as percentage of injected dose in whole bone marrow, estimated by the method of Rahman *et al.* [40].

†Results expressed as percentage of injected dose in estimated whole plasma volume.

ing through both tibia with normal saline were similarly treated by digestion with equal volumes of 33% KOH. In all cases, corrections for any reduction in counting efficiency due to colour quenching were made using automatic external standardization.

In order to correct tissue drug concentrations for drug present in blood in each of the tissues, an estimate was made of blood content of all tissues (including tumour) using isologous $^{51}$Cr-labelled red cells [2]. In practice, a significant correction applied only in the case of liver, spleen, kidney and lung samples taken 3 hr after the injection of entrapped AD.

## RESULTS

*Actinomycin D*

The therapeutic toxicity data on the treatment of the sensitive ROS tumour with free and liposome-entrapped AD are summarised in Fig. 1. Also illustrated is the mean tumour weight at 10 days of 9 control untreated mice. It is clear that the cytotoxic activity both against tumour and normal tissue has been greatly reduced by virtue of entrapment. With respect to antitumour activity, the minimum effective dose (inhibition to 10% of control tumour weight) of free drug is 0.3 μg/g, whereas entrapped AD in doses up to 8 μg/g fails to inhibit tumour growth to less than 68% of untreated tumours. With respect to host toxicity, the $LD_{50}$ of free AD is 0.8 μg/g, whereas at the highest dose of entrapped drug (8 μg/g) survival of animals is 83%. Treatment with empty cationic liposomes (12.5 or 40 mg lipid) resulted in tumour growth inhibition from 69 to 78% of control tumour weight (see legend to Fig. 1) and 100% animal survival. It is therefore probable that the minor tumour growth inhibition seen with all doses of entrapped AD can be attributed to the carrier rather than the drug, particularly in view of the *in vitro* evidence using both EMT mammary sarcoma cells [21] and human and rat bladder cancer cells [22] which showed that charged liposomes, including those containing stearylamine, were capable at high concentrations of inhibiting tumour cell growth.

The tissue distribution data from mice bearing the AD-sensitive ROS tumour following injection with free or liposome-entrapped AD are given in Tables 1 and 2. It is evident that for all tissues, excluding liver but including tumour, the initial (3 hr) drug concentration is reduced by about 3-fold following injection of entrapped AD compared to free drug administration, despite a 25-fold increase in the

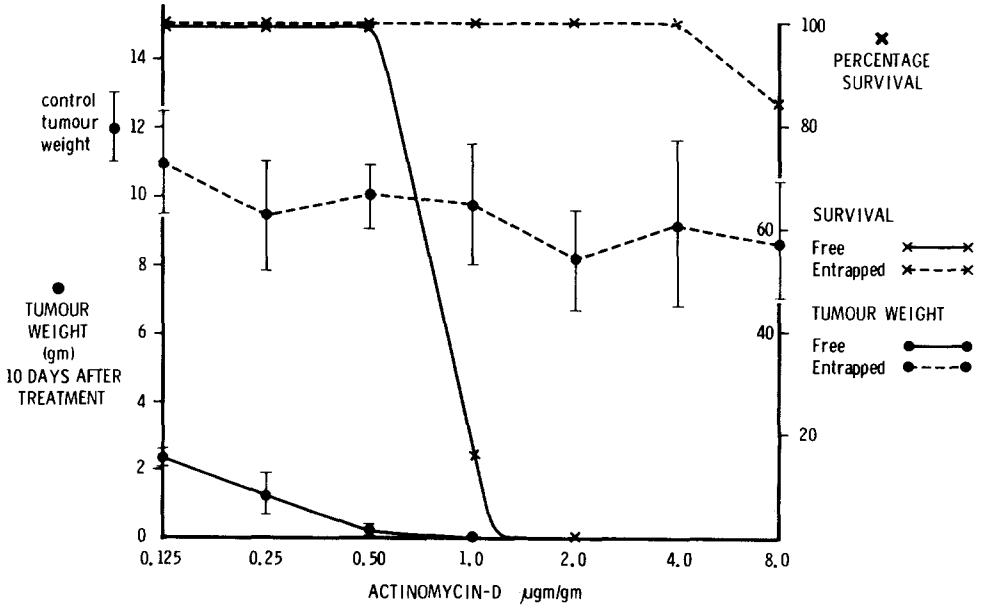


*Fig. 1. Therapeutic-toxicity study of free and liposome-entrapped actinomycin D in parent (drug-sensitive) ROS tumour. Mean tumour weights with standard errors are shown (6 mice per treated group, 9 mice in control untreated group). In addition, tumour weights 10 days after treatment with empty cationic liposomes were 9.43 g ± 1.0 (6 mice) and 8.15 g ± 0.62 (6 mice) at doses of 12.5 and 40 mg of lipid, respectively (see text for details).*

plasma drug level at that time. In addition, it is apparent that when the drug is given in liposomes the drug concentrations measured in all tissues except tumour at 48 hr, 72 hr and 7 days after injection exceed those obtained with free drug. With respect to the ROS tumour, at all points the concentrations following injection of free AD exceed those following injection of entrapped drug. Conversely, AD uptake by the liver is markedly enhanced as a result of liposome entrapment, and hepatic drug concentrations remain at an appreciable level throughout the 7-day period.

The therapeutic toxicity data on the treatment of mice bearing the AD-resistant ROS subline are presented in Table 3. It is clear that liposome-entrapped AD is therapeutically ineffective against this subline, the results of treatment being essentially similar to those obtained in the sensitive tumour using entrapped drug. The tissue distribution data using free and entrapped AD in the resistant tumour are given in Tables 4 and 5, and comparison with the data in Tables 1 and 2 indicates that in all tissues other than tumour, drug concentrations are similar in the two sublines, implying a similar method of drug handling. However, a major difference is seen in tumour drug concentrations following injection of free AD in the sensitive and resistant tumours, as illustrated in Fig. 2. It is evident that initial (3 hr) levels are similar, and that the major point of difference between the two sublines is a rapid and consistent fall in AD concentrations in the resistant tumour, compared to the sensitive line in which the drug is apparently retained efficiently.

*Methotrexate*

The therapeutic-toxicity data on the treatment of tumour-bearing mice with free and entrapped MTX are given in Fig. 3. It is clear that, in contrast to AD, the cytotoxic effects of MTX both in terms of antitumour activity and host toxicity have been enhanced by liposome entrapment. Expressing the therapeutic index (T.I.) in this relatively re-

*Table* 3 (*a*). *Therapeutic-toxicity study using free actinomycin D in the treatment of resistant ROS subline*

| Dose (μg/g body wt) | Mean tumour wt (g) on day of treatment (S.E.M.) | Mean tumour wt (g) 10 days after treatment (S.E.M.) | No. of survivors at 10 days / No. of treated animals |
|---|---|---|---|
| 0 | 1.28 (0.20) | 11.70 (1.26) | 6/6 |
| 0.125 | 1.20 (0.14) | 7.71 (1.96) | 6/6 |
| 0.25 | 1.27 (0.22) | 6.69 (0.91) | 7/7 |
| 0.5 | 1.27 (0.22) | 5.39 (0.48) | 7/7 |
| 1.0 | 1.59 (0.18) | — | 0/6 |

*Table* 3 (*b*). *Therapeutic-toxicity study using liposome-entrapped actinomycin D in the treatment of resistant ROS subline*

| Dose (μg/g body wt) | Mean tumour wt (g) on day of treatment (S.E.M.) | Mean tumour wt (g) 10 days after treatment (S.E.M.) | No. of survivors at 10 days / No. of treated animals |
|---|---|---|---|
| * | 1.31 (0.09) | 9.77 (0.64) | 6/6 |
| 0.125 | 1.21 (0.20) | 10.19 (1.35) | 6/6 |
| 0.25 | 1.10 (0.19) | 10.75 (2.54) | 6/6 |
| 0.5 | 1.22 (0.18) | 10.77 (2.54) | 6/6 |
| 1.0 | 1.38 (0.18) | 9.33 (1.85) | 6/6 |
| 2.0 | 1.17 (0.24) | 10.10 (2.81) | 6/6 |
| 4.0 | 1.21 (0.28) | 9.53 (2.59) | 5/5 |
| 8.0 | 1.39 (0.37) | 9.39 (3.43) | 5/5 |

*Empty liposomes equivalent to 12.5 mg of lipid.

*Table 4. Tissue distribution of free [$^3$H]actinomycin D following injection into AD-resistant ROS tumour-bearing AKR mice*

| Organ | 3 hr | 24 hr | 48 hr | 72 hr | 7 days |
|---|---|---|---|---|---|
| Liver | 5.05 ± 0.59 (7) | 0.63 ± 0.09 (6) | 0.22 ± 0.07 (5) | 0.12 ± 0.01 (5) | 0.04 ± 0.01 (5) |
| Spleen | 12.78 ± 0.54 (7) | 7.86 ± 0.72 (6) | 3.73 ± 0.40 (5) | 1.40 ± 0.16 (5) | 0.10 ± 0.01 (5) |
| Kidney | 7.67 ± 0.79 (7) | 1.23 ± 0.20 (6) | 0.32 ± 0.06 (5) | 0.18 ± 0.03 (5) | 0.05 ± 0.03 (5) |
| Lung | 5.63 ± 0.33 (7) | 1.51 ± 0.18 (6) | 0.50 ± 0.10 (5) | 0.22 ± 0.03 (5) | 0.04 ± 0.01 (5) |
| Small intestine | 6.87 ± 0.70 (7) | 1.37 ± 0.15 (6) | 0.38 ± 0.05 (5) | 0.19 ± 0.02 (5) | 0.06 ± 0.02 (5) |
| ROS tumour | 4.84 ± 0.43 (9) | 2.96 ± 0.45 (6) | 1.54 ± 0.20 (6) | 0.85 ± 0.12 (6) | 0.13 ± 0.04 (5) |
| Muscle | 1.51 ± 0.22 (7) | 0.38 ± 0.10 (6) | 0.10 ± 0.02 (6) | 0.08 ± 0.02 (5) | 0.02 ± (−) (5) |
| Plasma* | 0.74 ± 0.12 (7) | 0.16 ± 0.03 (6) | 0.07 ± 0.02 (6) | 0.03 ± 0.01 (6) | — |

Results expressed as percentage of injected dose per g of tissue (corrected for blood content) with standard error of mean and number of animals in parentheses.

*Results expressed as percentage of injected dose in estimated whole plasma volume.

*Table 5. Tissue distribution of liposome-entrapped [$^3$H]actinomycin D following injection into AD-resistant ROS tumour-bearing AKR mice*

| Organ | 3 hr | 24 hr | 48 hr | 72 hr | 7 days |
|---|---|---|---|---|---|
| Liver | 11.94 ± 1.22 (4) | 9.70 ± 0.98 (4) | 8.0 ± 0.55 (4) | 6.14 ± 0.22 (4) | 4.64 ± 0.41 (4) |
| Spleen | 4.10 ± 0.42 (4) | 3.99 ± 0.56 (4) | 2.32 ± 0.14 (4) | 2.42 ± 0.14 (4) | 1.66 ± 0.32 (4) |
| Kidney | 4.36 ± 0.47 (4) | 2.77 ± 0.25 (4) | 1.75 ± 0.06 (4) | 1.03 ± 0.12 (4) | 1.03 ± 0.16 (4) |
| Lungs | 2.70 ± 0.44 (4) | 1.57 ± 0.19 (4) | 1.07 ± 0.06 (4) | 0.67 ± 0.07 (4) | 0.55 ± 0.03 (4) |
| Small intestine | 3.30 ± 0.24 (4) | 1.63 ± 0.32 (4) | 0.91 ± 0.04 (4) | 0.61 ± 0.04 (4) | 0.56 ± 0.03 (4) |
| ROS tumour | 1.77 ± 0.05 (4) | 1.75 ± 0.21 (4) | 1.01 ± 0.05 (4) | 0.81 ± 0.06 (4) | 0.35 ± 0.03 (4) |
| Muscle | 0.95 ± 0.33 (4) | 0.56 ± 0.06 (4) | 0.48 ± 0.06 (4) | 0.31 ± 0.02 (4) | 0.34 ± 0.03 (4) |
| Plasma* | 22.72 ± 2.60 (4) | 1.82 ± 0.19 (4) | 0.67 ± 0.07 (4) | 0.47 ± 0.04 (4) | 0.24 ± 0.02 (4) |

*See notes to Table 4.

sistant tumour as $LD_{50}/ED_{20}$ (dose which inhibits tumour growth to 20% of controls), the T.I. for free MTX is 0.5 and for entrapped MTX is 0.6, indicating no significant overall change in its therapeutic efficacy.

## DISCUSSION

The marked contrast in the effect of liposome entrapment on the therapeutic efficacies of AD and of MTX in this tumour model strongly supports the view that the modification in the pharmacokinetic behaviour and tissue distribution of the drugs is the major underlying factor. As illustrated by the data on the distribution of liposome-entrapped AD, this effect for all organs except liver is 2-fold, resulting in both reduced peak concentration and prolonged drug exposure. With respect to hepatic drug levels, the results indicating en-

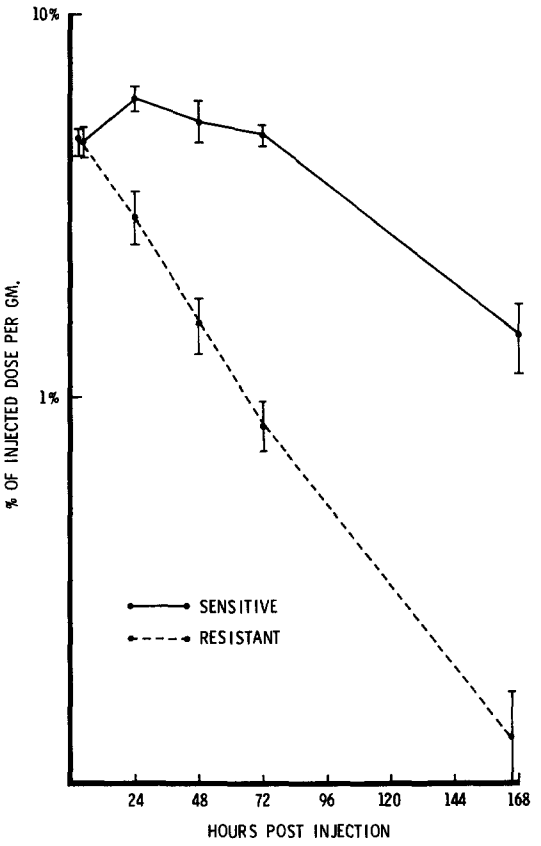


*Fig. 2. ROS tumour levels of [$^3$H]-actinomycin D following injection in free form in sensitive and resistant tumour sublines (results corrected for blood content).*

For a phase non-specific drug such as AD, the predicted effect of this modification in pharmacokinetics would be to reduce its activity [23]. Indeed, previous studies using the ROS tumour have already indicated a clear therapeutic advantage of a single large dose of AD over a multi-dose daily regime [24] Conversely, for phase-specific drugs such as MTX, the prolongation of drug exposure resulting from liposome entrapment would be predicted to result in a non-specific enhancement of its cytotoxicity [25, 26]. Prolongation of MTX exposure has also been demonstrated to increase its effectiveness in resistant tumour cells *in vitro* [27] and *in vivo* [28] (using the drug as a water–oil–water emulsion to provide slow release). For those tumours exhibiting apparent 'natural' drug resistance to MTX [29] through inappropriate scheduling, this increase in the duration of exposure may be a crucial factor in achieving a satisfactory response.

Recent studies from this laboratory [30] have indicated that AD entrapped in liposomes is released from the vesicles to a considerable extent when in the circulation, possibly by a process of liposome disruption involving the interaction of liposomal phosphatidylcholine with plasma components such as high density lipoproteins [31]. It is inevitable, therefore,

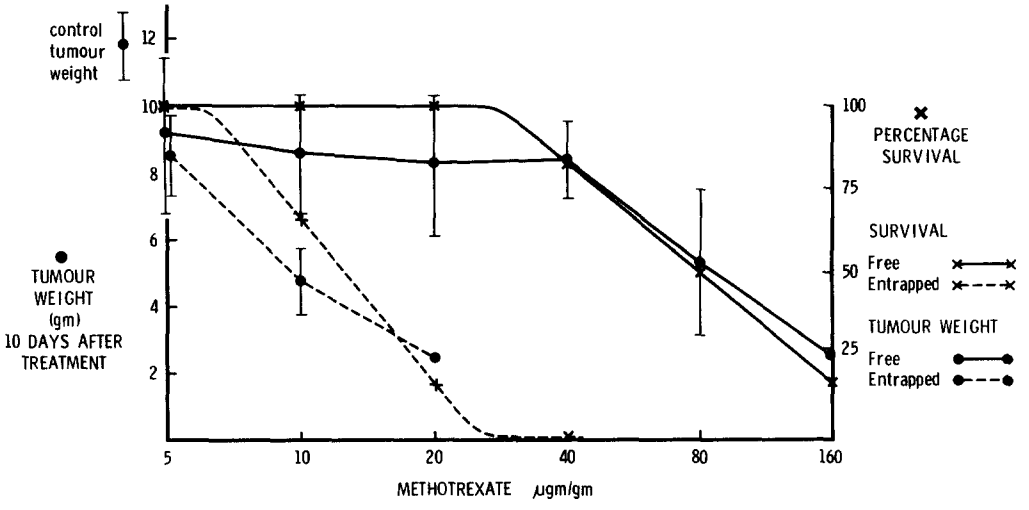


*Fig. 3. Therapeutic-toxicity study of free and liposome-entrapped methotrexate in parent ROS tumour. For details of groups treated with empty liposomes, see legend to Fig. 1 and text.*

hanced uptake and prolonged retention of AD injected in the entrapped form suggest that this organ might act as a depot from which drug is slowly released to give the extended exposure at low concentrations in other sites. that tumour cells will be exposed to the drug in the free form, and it remains uncertain as to whether any initially entrapped drug in fact gains access to tumour cells in intact liposomes following injection.

The suggestion has previously been made that negatively-charged liposomes might localise in the vicinity of tumours to a greater degree than those bearing a positive charge [32], but when AD entrapped in negatively-charged liposomes (phosphatidylcholine:cholesterol:phosphatidic acid in the molar ratio of 18:5:2) was injected into ROS tumour-bearing mice, the 24-hr tumour drug concentration [1.24% ±0.05 (5)] was in fact lower than that achieved using positively-charged liposomes. In a further attempt to improve the tumour uptake of entrapped drug, Kupffer cell blockade of the liver was induced using dextran sulphate [33]; however, tumour drug concentrations 3 hr after injecting entrapped AD under these circumstances were not significantly different from those seen previously (2.49% ±0.43 [6]).

The data indicating therapeutic inactivity of entrapped AD in the AD-resistant ROS subline emphasises the problems of translating positive *in vitro* results [12] obtained with liposome-entrapped drugs to the *in vivo* situation. Moreover, the observation that the resistance to AD acquired *in vivo* in this model appears to be due to a failure of drug retention rather than impaired drug uptake calls into question the basis for the use of liposomes in overcoming this type of resistance.

As previously pointed out by Inaba and Johnson in studies using P388 leukemia cell lines, [34], the mechanism which appears to be responsible for tumour cell AD resistance may vary according to the method chosen to derive that resistance, and they emphasise that when *in vivo* rather than *in vitro* methods of selection are used, failure of drug retention becomes an important factor in determining AD resistance. This group [35] and others [36, 37] have presented evidence of enhanced outward transport of anthracyclines in drug-resistant tumour cells which show cross-resistance with AD (and with vincristine) and it is conceivable, therefore, that a common mechanism permitting active drug efflux from resistant cells exists for a group of drugs.

In summary, the data presented here support the contention that the role of liposome entrapment in cancer chemotherapy is largely that of a system for gradual release of drug, probably from a depot site such as the liver. The observed therapeutic effect may then vary according to the mechanism of action of the entrapped drug, while an additional factor governing the rate of release from liposomes may be the physical characteristics of the drug concerned. Recently, Juliano and Stamp [38] have reported that non-polar drugs such as AD are released from liposomes *in vitro* more rapidly than polar drugs such as MTX. At present, the biological significance of these observations is not clear, but it is conceivable that the marked differences in therapeutic effects of entrapment of the two drugs may to some extent be related to contrasting rates of leakage from liposomes in the circulation.

As pointed out by Rustum *et al.* [39] with reference to entrapment of cytosine arabinoside, comparisons are now required between liposome-entrapped drug and infusions of free drug, which may already provide a more controllable means of achieving the same therapeutic effect. Further progress in the field of liposome entrapment of cytotoxic drugs will depend on the development of liposomes which remain stable in the circulation and which can be relied upon to gain access to tumour cells in order to achieve preferential drug delivery.

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## REFERENCES

1. A. D. Bangham, M. W. Hill and N. G. A. Miller, Preparation and use of liposomes as models of biological membranes. In *Methods in Membrane Biology.* (Edited by E. D. Korn) Vol. 1, p. 1. Plenum Press, New York (1974).
2. R. L. Juliano, The role of drug delivery systems in cancer chemotherapy. In *Membranes and Neoplasia.* (Edited by V. Marchesi) p. 21. Alan Liss, New York (1976).
3. J. H. Fendler and A. Romero, Liposomes as drug carriers. *Life Sci.* **20,** 1109 (1977).
4. G. Gregoriadis, Targeting of drugs. *Nature (Lond.)* **265,** 407 (1977).
5. H. K. Kimelberg and E. G. Mayhew, Properties and biological effects of liposomes and their uses in pharmacology and toxicology. *C.R.C. crit. Rev. Toxic.* **6,** 25 (1978).

6. Y. E. Rahman, E. A. Cerny, S. L Tollaksen, B. J. Wright, S. L. Nance and J. F. Thomson, Liposome encapsulated actinomycin D: potential in cancer chemotherapy. *Proc. Soc. Exp. Biol. (N.Y.)* **146,** 1173 (1974).
7. G. Gregoriadis and E. D. Neerunjun, Treatment of tumour-bearing mice with liposome-entrapped actinomycin D prolongs their survival. *Res. Commun. Chem. Path. Pharmacol.* **10,** 351 (1975).
8. R. J. Rutman, C. A. Ritter, N. G. Avadhani and J. Hansel, Liposomal potentiation of the anti-tumor activity of alkylating drugs. *Cancer Treatment Rep.* **60,** 617 (1976).
9. E. Mayhew, D. Papahadjopoulos, Y. M. Rustum and E. Dave, Inhibition of tumour cell growth *in vitro* and *in vivo* by 1-β-D arabinofuranosylcytosine entrapped within phospholipid vesicles. *Cancer Res.* **36,** 4406 (1976).
10. T. Kobayashi, T. Katoaka, S. Tsukagoshi and Y. Sakurai, Enhancement of anti-tumour activity of 1-β-D arabinofuranosylcytosine by encapsulation in liposomes. *Int. J. Cancer* **20,** 581 (1977).
11. M. J. Kosloski, F. Rosen, R. J. Milholland and D. Papahadjopoulos, Effect of lipid vesicle (liposome) encapsulation of methotrexate on its chemotherapeutic efficacy in solid rodent tumors. *Cancer Res.* **38,** 2848 (1978).
12. D. Papahadjopoulos, G. Poste, W. J. Vail and J. L. Biedler, Use of lipid vesicles as carriers to introduce actinomycin D into resistant tumor cells. *Cancer Res.* **36,** 2988 (1976).
13. H. K. Kimelberg and M. A. Atchison, Effects of entrapment in liposomes on the distribution, degradation and effectiveness of methotrexate *in vivo*. *Ann. N. Y. Acad. Sci.* **308,** 395 (1978).
14. G. Gregoriadis, Drug entrapment in liposomes. *FEBS Lett.* **36,** 292 (1973).
15. H. K. Kimelberg, Differential distribution of liposome-entrapped [$^3$H]-methotrexate and labelled lipids after intravenous injection in a primate. *Biochim. biophys. Acta (Amst.)* **448,** 531 (1976).
16. R. L. Juliano and D. Stamp, Pharmacokinetics of liposome-entrapped anti-tumor drugs. *Biochem. Pharmacol.* **27,** 21 (1978).
17. H. S. Schwartz, J. E. Sodergren, S. S. Sternberg and F. S. Philips, Actinomycin D: effects of Ridgway osteogenic sarcoma in mice. *Cancer Res.* **26,** 1873 (1966).
18. W. R. Laster, Ridgway osteogenic sarcoma—a promising laboratory model for special therapeutic trials aganst an advanced-staged, drug-sensitive animal tumour system. *Cancer Chemother. Rep.* **5,** 151 (1975).
19. R. M. C. Dawson, Studies on the hydrolysis of lecithin by a *Penicillium motatum* phospholipase B preparation. *Biochem. J.* **70,** 559 (1958).
20. H. Weissbach, B. Redfield, T. O'Conner and M. A. Chirigos, Studies on the disposition of actinomycin D—$^3$H in virus infected and tumor bearing mice. *Cancer Res.* **26,** 1832 (1966).
21. J. K. Dunnick, R. F. Kallman and J. P. Kriss, Lipid vesicle interaction with EMT-6 tumour cells and effect on subsequent cell growth. *Biochem. biophys. Res. Commun.* **73,** 619 (1976).
22. B. C. Behrens, N. Yanamoto, R. R. Brown and G. T. Bryan, Cytotoxicity of liposomes towards cultured human and rat bladder cancer cell lines. *Proc. Amer. Ass. Cancer Res.* **20,** 160 (1979).
23. H. E. Skipper, F. M. Schabel, L. B. Mellett, J. A. Montgomery, L. J. Wilkoff, H. H. Lloyd and R. W. Brockman, Implications of biochemical, cytokinetic, pharmacologic and toxicologic relationships in the design of optimal therapeutic schedules. *Cancer Chemother. Rep.* **54,** 431 (1970).
24. W. M. Galbraith and L. B. Mellett, Disposition of [$^3$H]actinomycin D in tumour-bearing mice. *Cancer Chemother. Rep.* **59,** 1061 (1975).
25. H. M. Pinedo and B. A. Chabner, Role of drug concentration, duration of exposure, and endogenous metabolites in determining methotrexate cytotoxicity. *Cancer Treat. Rep.* **61,** 709 (1977).
26. I. D. Goldman, Effects of methotrexate on cellular metabolism: some critical elements in the drug–cell interaction. *Cancer Treat. Rep.* **61,** 549 (1977).
27. J. H. Goldie and S. I. Harrison, Comparison of the cytotoxicity of methotrexate and aminopterin on methotrexate resistant murine lymphoma cells *in vitro*. *Europ. J. Cancer* **14,** 55 (1978).
28. C. J. Benoy, R. Schneider, L. A. Elson and M. Jones, Enhancement of the cancer chemotherapeutic effect of the cell phase-specific agents, methotrexate and cytosine arabinoside when given as a water–oil–water emulsion. *Europ. J. Cancer* **10,** 27 (1974).

29. M. LANE, General mechanisms of clinical resistance to cancer chemotherapy. *Biochem. Pharmacol. Suppl.* **2,** 83 (1974).
30. S. B. KAYE and B. E. RYMAN, The *in vitro* fate of liposome-entrapped actinomycin D and its therapeutic effect in a solid murine tumour. *Biochem. Soc. Trans.* **8,** 107 (1980).
31. G. SCHERPHOF, F. ROERDINK, M. WAITE and J. PARKS, Disintegration of phosphatidylcholine liposomes in plasma as a result of interaction with high density lipoproteins. *Biochim. biophys. Acta (Amst.)* **542,** 296 (1978).
32. V. J. RICHARDSON, K. JEYASINGH, R. F. JEWKES, B. E. RYMAN and M. H. N. TATTERSALL, Possible tumour localisation of $^{99}$Tc-labelled liposomes: Effect of lipid composition, charge and liposome size. *J. nucl. Med.* **19,** 1049 (1978).
33. J. W. B. BRADFIELD, R. L. SOUHAMI and I. E. ADDISON, The mechanism of the adjuvant effect of dextran sulphate. *Immunology* **26,** 383 (1974).
34. M. INABA and R. K. JOHNSON, Decreased retention of actinomycin D as the basis for cross-resistance in anthracycline-resistant sublines of P388 leukemia. *Cancer Res.* **37,** 4629 (1977).
35. M. INABA, H. KOBAYASHI, Y. SAKURAI and R. K. JOHNSON, Active efflux of daunorubicin and adriamycin in sensitive and resistant sublines of P388 leukemia. *Cancer Res.* **39,** 2200 (1979).
36. T. SKOVSGAARD, Mechanisms of resistance to daunorubicin in Ehrlich ascites tumour cells. *Cancer Res.* **38,** 1785 (1978).
37. K. DANO, Active outward transport of daunomycin in resistant Ehrlich ascites tumour cells. *Biochim. biophys. Acta (Amst.)* **323,** 466 (1973).
38. R. L. JULIANO and D. STAMP, Interaction of drugs with lipid membranes. Characteristics of liposomes containing polar or non-polar antitumour drugs. *Biochim. biophys. Acta* **586,** 137 (1979).
39. Y. RUSTUM, C. DAVE, E. MAYHEW and D. PAPAHADJOPOULOS, Role of liposome type and route of administration in the anti-tumour activity of liposome-entrapped 1-$\beta$-D-arabinofuranosylcytosine against mouse L1210 leukemia. *Cancer Res.* **39,** 1390 (1979).
40. Y. E. RAHMAN, W. R. HANSON, J. BHAPUCHA, E. J. AINSWORTH and B. N. JAROSLOW, Mechanisms of reduction of anti-tumour toxicity by liposome encapsulation. *Ann. N.Y. Acad. Sci.* **308,** 325 (1978).