Eur J Cancer Clin Oncol Vol. 17, No. 7, pp 809–817, 1981.
Printed in Great Britain.

0014-2964/81/070809-09 $02.00/0

Inability of Liposome Encapsulated 1-β-D-Arabinofuranosylcytosine Nucleotides to Overcome Drug Resistance in L1210 Cells*

YOUCEF M. RUSTUM,† ERIC MAYHEW,‡ FRANCIS SZOKA‡ and JOEL CAMPBELL†

†*Department of Experimental Therapeutics and Grace Cancer Drug Center*, ‡*Department of Experimental Pathology, Roswell Park Memorial Institute*, 666 *Elm Street, Buffalo, NY* 14263, *U.S.A.*

Abstract—*The uptake, metabolism and antitumor activity of* 1-β-D-*arabinofuranosylcytosine nucleotides encapsulated in liposomes were investigated in* L1210 *cells sensitive and resistant to the drug. These studies were carried out in order to determine whether encapsulation of the presumed active component of the drug, namely, arabinofuranosylcytosine* 5′ *triphosphate will convert resistant cells to sensitive. The results indicate that liposome entrapment of Ara-CMP and Ara-CTP did not result in increased delivery of these metabolites into* L1210 *cells. The amount of nucleotide found intracellularly following an* in vitro *incubation of encapsulated drugs with* L1210 *cells did not exceed the level expected from a simple extracellular breakdown of liposome releasing their contents with subsequent uptake and phosphorylation of the released nucleoside. Further evidence for lack of enhancement of drug uptake by liposome encapsulation were obtained when the rate of incorporation of TdR into DNA of* L1210/*Ara-C was not significantly affected by Ara-CTP encapsulation in liposome. The data presented herein also demonstrated that the* in vivo *sensitivity of* L1210 *cells resistant to Ara-C could not be modified by encapsulation of Ara-CTP in liposomes. The results strongly suggest that encapsulation of drugs unmodified or untargeted liposomes will not be able to overcome drug resistance related to transport defect and/or deletion of intracellular activating enzyme(s).*

INTRODUCTION

PHOSPHOLIPID vesicles (liposomes) are, at present, the subject of a considerable amount of effort to determine their utility as a drug delivery system [1–6]. There are, however, only a few instances where liposome entrapped drugs have been shown to have advantages compared to the use of free drug at equimolar doses [7–9] including data showing the markedly altered activity of cytosine arabinoside (Ara-C) when trapped in liposomes [7–11].

To provide further information concerning the efficacy of Ara-C entrapped in liposomes and to evaluate the potential utility of entrapped Ara-CTP for the purpose of overcoming resistance to Ara-C, studies were carried out in L1210 sensitive and resistant to Ara-C using liposomes containing entrapped nucleotides.

Accepted 12 February 1981.
*This work was supported in part by Project Grant CA-18420, Program Grant CA-13038, and Contract CM-77118.

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; CHOL, cholesterol; PCA, perchloric acid; KOH, potassium hydroxide; Ara-C, arabinofuranosylcytosine; Ara-CMP, Ara-CDP and Ara-CTP, the 5′ mono, 5′ di-and 5′-triphosphates of Ara-C, respectively; Ara-UMP, arabinofuranosyluridine 5′ monophosphate; L1210/0, and L1210/Ara-C leukemia L1210 cells sensitive and resistant to Ara-C, respectively; HPLC, high pressure liquid chromatograph; HEPES/MOPS, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid/morpholinopropane sulfonic acid; REV, reverse evaporation phase vesicles; SUV, sonicated unilamellar vesicles.

MATERIALS AND METHODS

Lipids

Phosphatidylcholine (PC) [12] and phosphatidylserine (PS) [13] were prepared as described previously and were repurified [11] on a Waters high pressure liquid chromatograph (HPLC) (LC-500) using a pre-pak-500 silica column with chloroform–methanol–water

60:30:4 as solvent. Cholesterol (A. B. Fluka, Switzerland) was recrystallized once from methanol. All lipids were greater than 99% pure as assayed by thin layer chromatography and stored in chloroform under nitrogen at 50°C.

Liposomes

Two basic types of liposomes were prepared: sonicated unilamellar liposomes (SUV) prepared by standard sonication methods [14] and larger liposomes prepared by a reverse phase evaporation procedure (REV) [15].

Ara-C or derivatives were dissolved in 0.15M phosphate buffered saline at a concentration of $3 \times 10^{-1} - 5 \times 10^{-3}$M and the pH adjusted, if necessary, to 7. A trace amount of labeled drug was added ($\sim 0.1\ \mu$Ci/ml).

Lipid mixtures were prepared in different ratios for the preparation of liposomes of different compositions. The liposomes used in the present studies had the following lipid ratios (a) PS:PC, 1:4; (b) PS:PC:CHOL, 1:4:5.

SUV

In a standard preparation, 50–200 μmol of the lipid mixture was evaporated to dryness under nitrogen and reduced pressure. The dried lipid was then suspended in the drug containing solution (usually at 60 μmol lipid/ml) and vortexed at 37°C for 10–15 min under nitrogen. The suspension was then sonicated in a closed tube under nitrogen in a sonicator bath (Lab-Supplies, Co., Model T-80-80-IRS) at 37°C for 1 hr. Liposomes prepared in this way were checked by Freeze Fracture electron microscopy and found essentially free of multilamellar liposomes. Sonication did not affect Ara-C activities either in the free or entrapped form.

REV

The lipid mixture was evaporated to dryness as described for SUV but was then redissolved in 6 ml freshly distilled diethyl ether (PS:PC liposomes) or 3 ml chloroform and 3 ml isopropyl ether (PS:PC:CHOL liposomes). The drug solution (usually 2 ml) was added and the suspension sonicated for 5 min at room temperature. The chloroform and ether were evaporated at 30–37°C and the resulting liposome suspensions were extruded through 0.4 μm polycarbamate membranes. After preparation, SUV and REV liposomes were dialyzed four times against 250 ml PBS for $\frac{1}{2}$ hr followed by overnight dialysis against 500 ml PBS. The amount of entrapped drug was determined from radioisotopic measurements and the capture (% drug entrapped from original solution) was 1–2% for SUV and 20–35% for REV. The liposomes were used for experiments within 24 hr.

Liposome stability

Aliquots of liposome containing entrapped ^{3}H-Ara-C were put in 1 cm dialysis bags and diluted to 1.0 μmol lipid/ml with PBS, 10% or 80% heat inactivated fetal calf serum. The bags were incubated at 37°C in PBS with changes of dialysate at 1, 2, 4, 6, 8, 24, 48 and 72 hr. The radioactivity of the dialysates was determined and from this data the rate of leakage of Ara-C from liposomes were calculated and expressed as the time required for 50% of Ara-C to be lost from the liposomes ($t_{1/2}$) [11].

Chemicals and isotopes

Ara-C (bare) was obtained from Sigma Chemical Co., St. Louis, MO. Ara-CTP (tetralithium salt, trihydrate), 96–98% pure by HPLC was purchased from Calbiochem, La Jolla, Ca. [5-^{3}H]-Ara-CMP (50 ci/mmol), 99% pure by radioactivity, was obtained from Moravek Biochem., City of Industry, CA. [5-^{3}H] Ara-C (22 ci/mmol) and [methyl-^{3}H] TdR (2 ci/mmole) were purchased from Amersham/Searle, Arlington Heights, IL.

Tumor Cells

L1210/0 (wild type) and L1210/Ara-C (resistant to Ara-C) were routinely transplanted into DBA/2J mice and used on day 3 or 4 following an i.p. transplant of 1×10^6 cells. The resistance of L1210 cells to Ara-C is characterized by the lack of *in vivo* sensitivity and by the limited uptake and activation of Ara-C to the Ara-CTP level. Lewis lung carcinoma cells were established in culture by Dr. J. Bertram of this Institute (unpublished results). B16-F10 melanoma cells were kindly supplied by Dr. I. J. Fidler of the Frederick Cancer Research Center, Frederick, MD. Both LLC and B16-F10 melanoma were maintained in culture and checked frequently for tumorigenicity in mice.

HPLC system

Separation of Ara-C metabolites: Ara-C metabolites, namely Ara-CMP, Ara-UMP, Ara-CDP and Ara-CTP, were separated on a Dupont Model 830 equipped with a dual cell detector (254 nm) and a U-shaped ABX column (1 × 2 m). Other conditions were identical to those previously described [16, 17]. Purity of Ara-CTP and ^{3}H-Ara-CMP were

determined as follows: ^{3}H-Ara-CMP was eluted from the column isocratically using 2.5 mm potassium phosphate buffer, pH 3.0. One-minute fractions were collected and counted using a Packard Tri-Carb Model 330 liquid Scintillation Spectrometer with 30% efficiency. This compound was found to be 99% pure. Ara-CTP was eluted with a phosphate buffer gradient 2.5 mM pH 3.0, to 0.5 M pH 4.4) using a concave gradient. Under these conditions, Ara-CTP was eluted as a single peak but separable from CTP and dCTP. In all experiments the Ara-CTP was 96–98% pure. The purity of encapsulated materials was tested prior to each experiment. Free liposomes and liposome entrapped drug were extracted with chloroform–methanol (3:1) and chromatographed on HPLC as described above.

In vitro *studies*

Inhibition of [^{3}H]-TdR incorporation into DNA was determined as follows, L1210 tumor cells ($3–10 \times 10^6$/2 ml) were incubated at 37°C in RPMI 1640 containing 10% fetal calf serum, 2% HEPES/MOPS (complete medium). Free and encapsulated Ara-CTP or Ara-C at the appropriate concentrations were added to the incubation medium and at 60 or 120 min thereafter cells were centrifuged and washed three times with 0.9% NaCl. Cells were then suspended in 4 ml of complete medium containing 5 μCi [^{3}H]-TdR and incubated for an additional 15 min. Cells were then centrifuged, washed twice with 0.9% NaCl, and the cell pellet was extracted three times with 6% PCA. Cell pellets were then taken up in 0.5 ml of 6% PCA, and boiled at 95°C for 15 min. Aliquots were then removed for counting.

[^{3}H]-*Ara-CMP metabolism*

5×10^6 L1210/0 and L1210/Ara-C cells were incubated at 37°C in 4 ml of complete medium containing 1.5 mM of free or encapsulated Ara-CMP solution containing 5×10^6 cpm/ml [^{3}H]-Ara-CMP. At 60 and 120 min, 2 ml of cell suspension was removed, washed three times with 0.9% NaCl and cell pellets were extracted with PCA, centrifuged and the resulting supernatants were neutralized with KOH. Acid-soluble fractions thus prepared were then analyzed for Ara-C metabolites using the HPLC system described above. Incorporation of Ara-C into nucleic acids were determined by washing the acid-insoluble fractions three times with PCA. The pellet was then taken up into counting vials and counted twice each time for 10 min.

Antitumor activity

Female DBA/2J mice weighing 18–20 g were inoculated (day 0) i.p. with 1×10^6 L1210/0 or L1210/Ara-C and 24 hr (day 1) later i.p. treatment with free and encapsulated drug was initiated. Control mice received 0.01 ml of 60 μM lipid per g body weight.

RESULTS

Metabolism of Ara-C by L1210/0 *and* L1210/*Ara-C*

Table 1 summarizes the intracellular activation of [^{3}H]-Ara-C by L1210 sensitive and resistant to Ara-C. The data indicate that although Ara-C was activated up to the tri-

Table 1. In vitro *metabolism of* [^{3}H]-*Ara-C* (20 Ci/mmole) *by* L1210/0 *and* L1210/Ara-C *cells*

Cell line	pmol/10^7 cells ± S. D.			
	Ara-CMP	Ara-UMP	Ara-CDP	Ara-CTP
L1210/0	12.1 ± 2.4	2.2 ± 0.4	20.7 ± 0.4	185.2 ± 35.6
L1210/Ara-C*	1.8 ± 0.6	0.3 ± 0.6	1.7 ± 0.5	3.6 ± 1.5
L1210/0:L1210/Ara-C ratio	6.7	7.3	12.2	51.5

Cells (5.0×10^7) were incubated at 37°C for 15 min in 5 ml of RPMI 1640 media containing 10% fetal calf serum. 2% HEPES/MOPS, and 1 μg/ml Ara-C and 20 μCi/ml[^{3}H]-Ara-C. Cells were then washed three times with 0.9% NaCl, extracted with PCA/KOH and acid-soluble fractions were analyzed by HPLC for Ara-C metabolites. The values reported herein were the average of three separate experiments.
*CdR-Kinase activity in L1210/0 and L1210/Ara-C were 110 and 2.6 pmol/mg protein/min.

phosphate level and incorporated into nucleic acids in both cell lines, the amount of Ara-CTP formed by L1210/Ara-C was only 1–2% of that found in the acid soluble fraction of L1210/0. This suggests that the flow of Ara-C through the metabolic pathway is dramatically reduced in this resistant cell line. For example, while the Ara-CTP/Ara-ADP and Ara-CDP/Ara-CMP in L1210/0 cells were 13.5 and 1.6, respectively, in L1210/Ara-C these ratios were 2.4 and 0.9 respectively. The Ara-CMP/Ara-UMP ratios in the two cell lines, however, were identical. The data in Table 1 also shows that the Ara-CTP pools in L1210/0 were about 52-fold greater than that found in L1210/Ara-C.

In vitro *stability of Ara-C entrapped in liposomes*

Table 2 shows that PS/PC liposomes were more leaky than PS/PC/chol liposomes in PBS or in serum. The 10% serum data gives the stability under similar conditions to those used in the *in vitro* experiments. The use of 80% serum was an attempt to mimic more closely the normal high *in vivo* serum concentration. These leakage data were obtained in a cell-free system and it is not known whether *in vivo* liposome interaction with cellular components and membrane induces faster rates of leakage.

Uptake and activation by leukemic cells of free and liposome encapsulated [^{3}H]*-Ara-CMP*

The data in Table 3 compare the *in vitro* ability of L1210 cells sensitive and resistant to Ara-C to take up free and encapsulated Ara-CMP. Although the data show that liposomal [^{3}H]-Ara-CMP was taken up by L1210/0, this amount represented less than 1% of the amount of label found intracellularly when free Ara-C was used. Examination of the stability of Ara-CMP in the incubation mixture revealed only a 1% breakdown of free Ara-CMP to Ara-C during the 2 hr of incubation. During the same period, the amount of Ara-C released into the media from encapsulated Ara-CMP was 0.1–3%. Encapsulation of Ara-CMP in liposomes did not increase the uptake of Ara-CMP by

Table 2. In vitro stability of Ara-C encapsulated in REV and SUV liposomes with different lipid composition

Liposome type and composition	Molar ratio	Incubation media PBS	Incubation media $t_{1/2}$* 80% Serum
PS/PC-REV	1:4	25 hr	1.0 hr
PS/PC/CHOL-REV	1:4:5	58 hr	28 hr
PS/PC-SUV	1:4	15 hr	<1.0 hr
PS/PC/CHOL-SUV	1:4:5	42 hr	21 hr

Liposome entrapping Ara-C were incubated at 37°C with either PBS or 80% fetal calf serum. The identity of Ara-C in liposomes at various times of incubation were identified by HPLC.
*Time required for 50% of entrapped Ara-C to leak out of liposomes.

Table 3. Uptake of free and REV entrapped [^{3}H]-Ara-CMP (5 Ci/mmol) *by* L1210/0 *and* L1210/*Ara-C*

Conditions	Lipid composition	pmol/10^7 cells L1210 60 min	L1210 120 min	L1210/Ara-C 60 min	L1210/Ara-C 120 min
Ara-C-free†	PS/PC*	590.3±32.4	—	3.9±1.2	—
Ara-CMP-free	PS/PC*	26.0±3.1	67.9±18.7	1.26±0.56	0.87±0.5
REV-Ara-CMP	PS/PC	2.21±0.19	5.67±2.0	0.38±0.05	0.14±0.11
REV-Ara-CMP	PS/PC/CHOL	1.7 ±0.22	1.76±0.9	0.38±0.16	0.48±0.10

Leukemic cells (5×10^7 cells) were incubated at 37°C with the drug for 60 and 120 min in RPMI 1640 containing 10% fetal calf serum and 2% HEPES/MOPS. At each time point cells were washed, extracted with PCA/KOH and acid-soluble fractions were counted for radioactivity.
*Free drug mixed with empty liposomes.
†Ara-C-5[^{3}H] (4 μM + 10 μCi) in presence of 5.0×10^7 cells, 1 hr incubation.

L1210/0 or L1210/Ara-C. The amount of drug taken up by leukemic cells in the case of free and encapsulated Ara-CMP could be accounted for on the basis of leakage of Ara-CMP from liposomes and the subsequent breakdown to Ara-C.

The extent of metabolic activation of free and liposome encapsulated Ara-CMP in L1210/0 and L1210/Ara-C was investigated and the results are shown in Fig. 1. The data

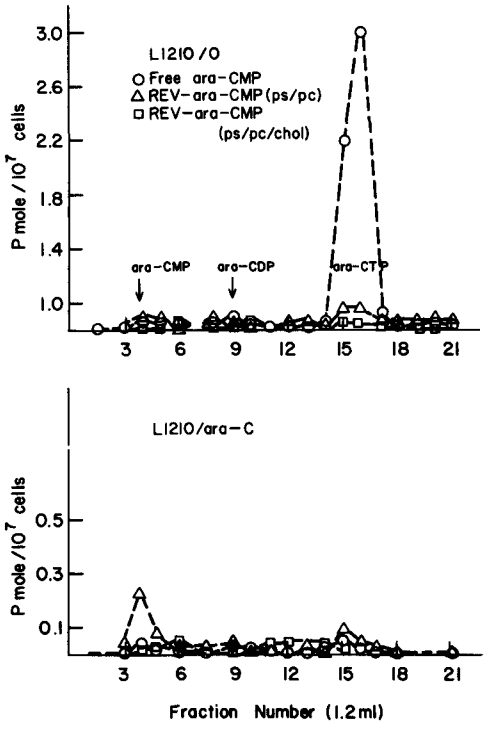

Fig. 1. *Metabolism of free and liposome encapsulated Ara-CMP by* L1210/0 *and L1210/Ara-C. Two hours following* in vitro *incubation of cells with labeled drugs cells were centrifuged, washed and cell pellets were extracted with PCA and neutralized to* pH 7.0 *with KOH. Ten to* 50 *microliters of the cell extract (acid-soluble fraction) was applied on to an HPLC column and eluted as described under Materials and Methods. Fractions were collected every* 1.5 min *and counted for radioactivity.*

indicate that only in L1210/0 were free and encapsulated Ara-CMP metabolized to the level of Ara-CTP. The amount of Ara-CTP formed seems to correlate with the rate of leakage and breakdown of Ara-CMP and Ara-CTP into Ara-C with its subsequent uptake of and phosphorylation to the triphosphate level in L1210/0. In contrast, in L1210/Ara-C, where resistance is due to a relatively lower level of deoxycytidine kinase (see Table 1), there was no appreciable formation of Ara-CTP. The relatively higher level of Ara-CMP found when the drug was encapsulated in liposomes (PS/PC) could be due to residual association of liposome entrapped drug with the cell membrane.

Inhibition of [^{3}H]-*TdR incorporation into DNA by Ara-CTP*

The effects of free and liposome encapsulated Ara-CTP on the incorporation of TdR into DNA of L1210/0 and L1210/Ara-C were investigated. The data which are outlined in Table 4 indicate that in L1210/0 both free Ara-C and Ara-CTP inhibited TdR incorporation by over 90%. The extent of inhibition by free Ara-CTP was correlated with the degree of Ara-CTP breakdown to Ara-C in the incubation medium, and this was determined to be 1–5% in a period of 120 min. Encapsulation of Ara-CTP in liposomes did not enhance the inhibition of TdR incorporation into DNA at lower doses. In fact, data in Table 4 indicate that the inhibition which occurred at 10–20 μg/ml of encapsulated Ara-CTP can be accounted for by the leakage of Ara-CTP from liposomes and the subsequent breakdown to Ara-C in the medium, followed by its activation to Ara-CTP in L1210/0 but not in L1210/Ara-C. In L1210/Ara-C there was no significant inhibition of TdR incorporation at any concentration used. Similarly, the effects of Ara-CTP encapsulated in lipid vesicles on the incorporation of TdR into DNA was evaluated in two other cell lines with marginal *in vitro* sensitivity to Ara-C, namely Lewis Lung carcinoma (LLC) and B16-F10 malignant melanoma cells. The data which are outlined in Table 5 indicate that encapsulated Ara-CTP did not inhibit the incorporation of TdR into DNA in Lewis Lung carcinoma. In B16-F10 cells inhibition by free drug was greater than that found when Ara-CTP was encapsulated in liposomes.

The data on the inhibition of TdR incorporation into the DNA of L1210/0 and L1210/Ara-C are summarized in Fig. 2. As indicated in Tables 4 and 5, only in L1210/0 was there significant inhibition of TdR incorporation into DNA. In studies not reported here, results indicated that incubation of L1210/Ara-C cells with Ara-CTP encapsulated in liposomes for up to 4 hr did not increase the extent of inhibition of TdR incorporation into DNA.

Antitumor activity of free and encapsulated Ara-CTP

The activities of free Ara-C, free Ara-CTP and Ara-CTP encapsulated in lipid vesicles against mouse leukemia L1210/0 and

Table 4. Inhibition of [methyl-^{3}H]TdR incorporation into DNA by free and liposome encapsulated Ara-C and Ara-CTP

Condition†	Concentration (μg/ml)	% Inhibition* L1210/0 60 min	L1210/0 120 min	L1210/Ara-C 60 min	L1210/Ara-C 120 min
Ara-C-free	0.1	92.0	88.2	N.D.	8.6
	1.0	98.2	98.3	7.8	12.2
	10.0	99.6	99.8	14.5	32.1
	20.0	99.7	99.8	31.5	34.3
Ara-CTP-free	1.0	79.4	94.3	0	0
	10.0	95.1	94.6	4.9	0
	20.0	95.0	99.2	4.2	13.6
REV-Ara-CTP	1.0	44.5	49.1	0	12.9
	10.0	79.6	82.6	0	12.1
	20.0	61.9	77.6	3.6	10.9
SUV-Ara-CTP	1.0	80.3	N.D.	0	0
	10.0	95.3	N.D.	0	0
	20.0	91.1	95.1	4.8	2.2

L1210/0 and L1210/Ara-C (1.0×10^7cells/ml) were incubated with various concentrations of drug at 37°C for 60 and 120 min in presence of RPMI 1640 containing 10% fetal calf serum and 2% HEPES/MOPS. Ara-CTP was encapsulated in REV and SUV with a lipid composition of PS/PC/CHOL in the ratio of 1:4:5. Free drug was mixed with empty liposome just prior to initiation of experiment.

*Tdr incorporation in the 60 min control samples were 53.6 ± 15.8 and 19.7 ± 6.5 pmol/10^7 cell for L1210/0 and L1210/Ara-C, respectively. These values represent an average of two experiments.

† The lipid concentration in each condition was 7.5 mM.

Table 5. Inhibition of [^{3}H]-TdR incorporation into the DNA of Lewis Lung Carcinoma (LLC), and F-10 melanoma cells by free and liposome encapsulated Ara-C and Ara-CTP

Conditions	Drug concentration (μg/ml)	% Inhibition† F-10	LLC
Ara-C-free	0.1	74.6	22.0
Ara-CTP-free	20.0	78.1	61.2
REV-Ara-CTP	1.0	2.6	0
	5.0	51.4	N.D.
	10.0	42.7	N.D.
	20.0	53.4	0

3.0×10^6 tumor cells were each incubated with free or encapsulated Ara-C or Ara-CTP at 37°C for 120 min.* Other conditions are similar to those outlined in Table 3.

*Average of 2 experiments with 15–20% variation.

†TdR incorporation into DNA of control samples (without drug) was $156.3 + 22.8$ and 134.9 ± 18.8 pmol/10^7 for F10 and LLC, respectively.

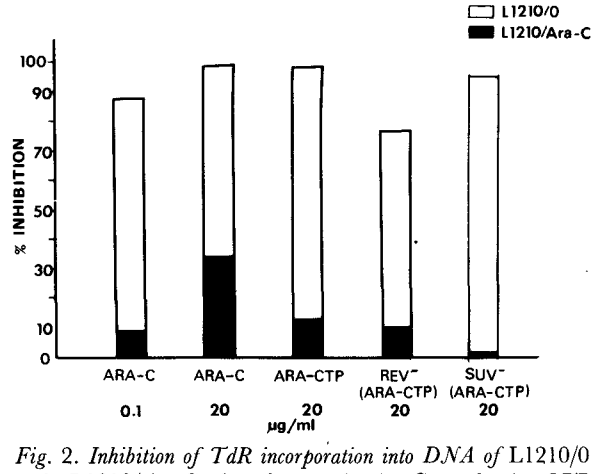

Fig. 2. Inhibition of TdR incorporation into DNA of L1210/0 *and* L1210/*Ara-C by free and Ara-C and Ara-CTP encapsulated in liposome. Cells were incubated with the drug* (0.1–20 μg/ml) *for* 2 hr *and* 15 min *with labeled TdR (see Materials and Methods), REV (PS/PC/CHOL) with a molar ratio of* 1:4:5.

L1210/Ara-C were examined and the results are summarized in Table 6. The data indicate that while encapsulated Ara-CTP exhibited antitumor activity against L1210/0, there was no significant effect against L1210/Ara-C. Furthermore, the data in Table 6 indicate

Table 6. Antitumor activity of free and REV-encapsulated Ara-CTP against DBA/2J mice

Treatment	Dose (mg/kg)	L1210/0 Average survival time (days)	L1210/0 30-day survivors	L1210/Ara-C Average survival time (days)	L1210/Ara-C 30-day survivors
0.9% NaCl		7.3±0.5	0/20	7.5±0.6	0/20
Free Ara-C	10	8.1±1.5	0/10	7.8±0.5	0/10
Ara-C in REV		21.6± 4.9	3/8	7.4±0.5	0/8
REV+Ara-CTP-free	5	7.8±1.2	0/5	8.1±0.9	0/5
Ara-CTP-free	50	8.1±1.7	0/20	8.6±1.5	0/20
Ara-CTP in REV	50	15.7± 3.7	0/10	9.3±0.8	0/7
	25	12.2± 5.8	1/6	7.2±1.0	0/6
	10	14.7± 5.3	1/10	8.8±1.3	0/10

Treatments (i.p.) were initiated 24 hr after an i.p. inoculation of 1×10^6 tumor cells. REV (PS/PC/CHOL) with a molar ratio of 1:4:5 was used and 7.5 μmol/mouse was injected in each treatment.

that while free Ara-C possessed no antitumor activity against either cell line, Ara-C encapsulated in the lipid vesicle produced a significant increase in life span; 3/8 mice 30 day survivors in L1210/0, but no significant increase in life span was observed against L1210/Ara-C.

DISCUSSION

The rationale for the experiments using Ara-CMP described in this report was as follows: if liposomes containing Ara-CMP were delivered to L1210/0 and L1210/Ara-C cells intact and released their contents intracellularly, Ara-CMP would then be metabolized to Ara-CDP and Ara-CTP in both cell types and inhibit DNA synthesis. If, on the other hand, Ara-CMP was released from liposomes extracellularly, the monophosphate would be transported into both cell types poorly compared to Ara-C [1], but that which did enter could be phosphorylated as described above. However, if the extracellular Ara-CMP was hydrolyzed to Ara-C in the extracellular environment, the drug would then be taken up and phosphorylated by L1210/0 and not by L1210/Ara-C cells.

The results show clearly that entrapment of Ara-CMP in liposomes resulted in *decreased* uptake and incorporation in both L1210/0 and L1210/Ara-C cells compared to uptake of free nucleoside. Liposomes composed of PS/PC, a lipid composition thought most likely to 'fuse' with membranes [18], did not markedly differ in their delivery capabilities compared with PS/PC/chol liposomes even though their stabilities in serum differed. Also the results showed that both liposome entrapped Ara-CTP and 'free' Ara-CTP inhibited DNA synthesis to the same extent, in L1210/0 cells but not in L1210/Ara-C cells. Thus, the results indicate that liposome entrapment of Ara-CMP and Ara-CTP did not result in increased delivery of the metabolites into L1210 cells. These results are more consistent with those reported by Kaye *et al.* [19] and Kedar *et al.* [20] rather than Poste and Papahadjopoulos [21] regarding the inability of liposome-entrapped drugs to overcome drug resistance *in vivo* or *in vitro*, respectively.

The data presented in the present report can be taken as evidence further supporting the idea that liposomes, where they show chemotherapeutic activity, are probably acting as a slow release system [6, 22] as previously suggested. The data shown in Table 6 demonstrated that encapsulation of the presumed active moiety of Ara-C, namely Ara-CTP, did not lead to inhibition of growth of the resistant cell line while free Ara-CTP did not cause a significant increase in life span in mice bearing sensitive L1210 cells. Encapsulated drug produced a significant effect with long-term survivors, possibly resulting from slow release of Ara-CTP from the liposomes either while still in the circulation or from stably cell or tissue bound liposomes [6, 23]. We propose that the slowly released Ara-CTP would first be hydrolyzed to Ara-C before being taken up and activated by L1210/0 cells. If slow release of drug external to the target cells appears to be the prevailing mechanism *in vivo*, then encapsulation of antimetabolites in *presently available* liposomes to

overcome intracellular resistance (where resistance is due to defect in transport and/or intracellular activating enzyme(s)) may not be useful in cancer chemotherapy. On the other hand, if the cell membrane is the target site of a drug, then encapsulation of drugs in liposomes might be useful as a drug delivery system. Hence, in order to make rational use of drugs encapsulated in liposomes in therapy of drug resistant cancers, mechanism(s) of tumor resistance and drug action must be clearly understood.

Whatever the mechanism of the inhibitory effects of encapsulated Ara-CTP against L1210/0 tumor, the results support the suggestion that liposomes may be of use as carriers of potential drugs which are normally too rapidly metabolized in the free state for practical use [6, 22]. As it is relatively easy to trap many types of substances with high efficiency in presently available liposomes this type of carrier should be considered as a method of screening potentially useful but normally unstable drugs.

Acknowledgements—We thank Dr. D. Papahadjopoulos for useful discussions and R. Lazo, T. Isac, J. Goranson and D. Milholland for technical assistance.

REFERENCES

1. Gregoriadis G. The carrier potential of liposomes in biology and medicine. *N Engl J Med* 1976; **295:** 704.
2. Gregoriadis G. The carrier potential of liposomes in biology and medicine. *N Engl J Med* 1976; **295:** 765.
3. Gregoriadis G, Neerunjun ED. Homing of liposomes to target cells. *Biochem Biophys Res Commun* 1975; **55:** 537.
4. Gregoriadis G, Ryman BE. Fate of protein-containing liposomes injected into rats. An approach to the treatment of storage diseases. *Eur J Biochem* 1972; **24:** 485.
5. Juliano RL, Stamp D. Lectin-mediated attachment of glycoprotein bearing liposomes to cells. *Nature (Lond)* 1976; **261:** 235.
6. Kimelberg H, Mayhew E. Properties and biological effects of liposomes and their uses in pharmacology and toxicology. *Crit Rev Toxicol* 1978; **6:** 25.
7. Mayhew E, Papahadjopoulos D, Rustum YM, Dave C. Inhibition of tumor cell growth *in vitro* and *in vivo* by 1-β-D-arabinofuranosylcytosine entrapped within phospholipid vesicles. *Cancer Res.* 1976; **36:** 4406.
8. Mayhew E, Papahadjopoulos D, Rustum YM, Dave C. Use of liposomes for the enhancement of the cytotoxic effects of cytosine arabinoside. *Ann NY Acad Sci* 1978; **308:** 371.
9. Rustum YM, Dave C, Mayhew E, Papahadjopoulos D. Role of liposome type and route of administration in the antitumor activity of liposome-entrapped 1-β-D-arabinofuranosylcytosine against mouse L1210 leukemia. *Cancer Res* 1979; **39:** 1390.
10. Kobayashi T, Tsukagoshi S, Sakurai Y. Enhancement of the cancer chemotherapeutic effect of cytosine arabinoside entrapped in liposomes on mouse leukemia L1210. *Gann* 1975; **66:** 719.
11. Mayhew E, Rustum YM, Szoka F, Papahadjopoulos D. Role of cholesterol in enhancing the activity of 1-β-D-arabinofuranosylcytosine, entrapped in reverse phase evaporation vesicles. *Cancer Treatment Rep* (in press).
12. Robles EC, van den Berg D. Synthesis of lecithins by acylation of *O*-(SN-glycero-3-phosphoryl) choline with fatty acid anhydrides. *Biochem Biophys Acta* 1969; **187:** 520.
13. Papahadjopoulos D, Jacobson K, Nir S, Isac T. Phase transition in phospholipid vesicles and permeability measurements concerning the effects of temperatures and cholesterol. Fluorescence polarization. *Biochem Biophys Acta* 1973; **311:** 330.
14. Papahadjopoulos D. Phospholipid model membranes III. Antagonistic effects of Ca^{2+} and local anesthetics in the permeability of phosphatidylserine vesicles. *Biochem Biophys Acta* 1970; **211:** 467.
15. Szoka F Jr., Papahadjopoulos D. A new procedure for preparation of liposomes with large volume and high capture by reverse evaporation (REV). *Proc Natl Acad Sci USA* 1978; **75:** 4196.
16. Rustum YM. Metabolism and intracellular retention of 1-β-D-arabinofuranosylcytosine as predictors of response of animal tumors. *Cancer Res* 1978; **38:** 543.

17. RUSTUM YM, PREISLER HD. Correlation between leukemic cell retention of 1-β-D-arabinofuranosylcytosine 5′-triphosphate and response to therapy. *Cancer Res* 1979; **39:** 42.
18. POSTE G, PAPAHADJOPOULOS D. Lipid vesicles as carrier for introducing materials into cultured cells: influence of vesicle lipid composition on mechanism of vesicle incorporation into cells. *Proc Natl Acad Sci USA* 1976; **73:** 1603.
19. KAYE SB, BODEN JA, RYMAN BE. The application of liposome entrapped cytotoxic drugs to the treatment *in vivo* of drug resistant solid murine tumours. *Proc ASCO/AACR* 1980; **20:** 254.
20. KEDAR A, MAYHEW E, MURPHY GP, MOORE RH. Failure of actinomycin D entrapped in liposomes to prolong survival in renal cell adenocarcinoma bearing mice. *Oncology* (in press).
21. POSTE G, PAPAHADJOPOULOS D. Drug containing lipid vesicles render drug-resistant tumor cells sensitive to actinomycin D. *Nature* (*Lond*) 1976; **26:** 699.
22. MAYHEW E, PAPAHADJOPOULOS D, RUSTUM YM, DAVE C. Use of liposomes for the enhancement of the cytotoxic effects of cytosine arabinoside. *Ann NY Acad Sci* 1978; **308:** 371.
23. MAYHEW E, GOTFREDSEN C, SCHNEIDER Y-J, TROUET A. Interaction of liposomes with culture cells: Effect of serum. *Biochem Pharmacol* 1980; **29:** 877.