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Delivery of therapeutic doses of doxorubicin to the mouse lung using lung-accumulating liposomes proves unsuccessful

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Summary. *Addition of solid doxorubicin or solutions to pre-formed liposomes proved to be the optimal method for incorporating the drug into liposomes whilst maintaining their size distribution and hence ability to accumulate in the lung. Liposomes prepared in this way lost doxorubicin only slowly on dialysis but dilution with an equal volume of saline at 37° C resulted in the loss of 80% of the incorporated doxorubicin within 30 min. These liposomes were thus ineffective in altering doxorubicin disposition in vivo and produced no enhanced activity compared with free drug and a non-lung-accumulating carrier liposome in the EMT6 cell-Balb/c mouse model lung tumour.*

Introduction

We have developed a liposome preparation which is capable of accumulating in lung to the extent of 20%–30% of the administered dose [3], presumably as a result of the formation of local microemboli [9]. The lung is an important site for metastatic lodgement during the progression of neoplastic disease [6], and lung-accumulating liposomes are a relatively specific drug delivery vehicle to attack such tumours. After delivery to the lung, liposome contents would be released directly into the microenvironment of the developing tumours, thus achieving high local concentrations of the drug. The inability of liposomes to cross the continuous endothelial lining of the lung vascular bed precludes any more specific targeting of vesicles to extravascular tumour cells in the lung [12].

We have attempted to incorporate the widely used antineoplastic agent doxorubicin [5] into lung-accumulating liposomes and to use these vesicles to treat an experimental murine lung tumour model.

Materials and methods

Chemicals. All lipid materials were purchased and assayed as previously described [2, 3]. Doxorubicin HCl (Adriamycin; ADR) was supplied by Adria Laboratories Inc. (Columbus, OH, USA) and assayed by a fluorometric technique [4]. Radioactive-iodine-labelled *p*-hydroxybenzamidine phosphatidylethanolamine (^{125}I-BPE) was used as a liposomal lipid marker [2].

Liposome preparation. ADR-containing liposomes were prepared in one of four ways.

1) ADR-lipid mixture resuspended as liposomes [10]. A methanol solution of ADR (4 μmol) was mixed with a chloroform solution of 80-μmol lipids (phosphatidylcholine/phosphatidylserine/cholesterol/α-tocopherol = 4 : 1 : 5 : 0.2 molar ratio including 0.4 μCi ^{125}I-BPE) and the mixture evaporated to dryness in vacuo. The lipids were resuspended in 4 ml 0.9-g% sodium chloride solution (isotonic saline) and the whole mechanically shaken for 2 h. The resultant liposomes (from all four protocols) were extruded [3] through a polycarbonate membrane of pore size 8.0 μm and dialyzed at 4° C for 2 days against frequent changes of saline in 1-ml dialysis cells containing membranes with pore size 5.0 μm.

2) ADR solution used to suspend lipids as liposomes [13]. A dry lipid mixture as described above (20 μmol) was resuspended in 2.0 ml saline containing 1 μmol ADR.

3) ADR solution added to pre-formed liposomes. A dry lipid mixture as described above for the first method (20 μmol) was resuspended in 1.7 ml saline and mechanically shaken for 1 h. A further 0.3 ml saline containing 1 μmol ADR was then added and shaking continued for 1 h. In a modification of this protocol (modified protocol 3) the pre-formed liposomes were added to solid ADR and a higher final ADR : total lipid ratio was obtained.

4) Encapsulation of ADR-phosphatidylcholine micelles in liposomes i.e., the method of Forssen and Tokes [7]. Dry phosphatidylcholine (2 μmol) was sonicated for 10 min in the presence of 2 ml saline containing 1 μmol ADR. The resultant suspension was then added to 20 μmol of a dry lipid mixture and liposomes formed as described for protocol-1.

Incubation of ADR-containing liposomes with saline. A batch of modified protocol-3 liposomes (ADR : total lipid ratio 1 : 80) was prepared for incubation by centrifuging twice at 12,800 × *g* and resuspending the pellets in saline. Incubations consisted of 0.5 ml washed liposomes plus 0.5 ml saline. Samples were mixed and incubated at 37° C for 0.5, 1, or 2 h. Time course samples were centrifuged after incubation (controls immediately) to form a liposomal pellet. The ADR concentration of the supernatant was then determined.

Organ distribution of free and incorporated ADR. Groups of three mice (male, ICR from Simonsen Laboratories Inc., Gilroy, CA, USA; mean body weight 20.8 ± 1.4 g) received

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Table 1. The effect of ADR incorporation technique upon liposome physical parameters

ADR incorporation protocol	% Initial ^{125}I-BPE remaining after dialysis	% Initial ADR remaining after dialysis	% Liposome dose present in mouse lung 2 h post-I.V. administration, (mean ± SD) $n = 3$	ADR : total lipid molar ratio
1. ADR-lipid mixture resuspended as liposomes	10	6	0.3 ± 0.1 (79.6 ± 3.6)[a]	1 : 100
2. ADR solution used to suspend lipids as liposomes	6	1.2	5.8 ± 2.4 (61.0)	1 : 75
3. ADR solution added to pre-formed liposomes	13	2.1	12.7 ± 3.9 (76.4)	1 : 83
4. Method of Forssen and Tokes	11	4.0	6.8 ± 1.0 (75.4)	1 : 101
Control (no ADR)	14	–	14.5 ± 6.3 (78.8)	–

[a] Total radioactivity remaining in vivo, estimated by summation of values for whole lungs and remaining carcase

IV injections (tail vein) of either 0.3 ml saline containing 102 μg ADR (4.9 mg/kg body weight) or 0.3 ml saline containing 180 μg ADR (8.7 mg/kg) incorporated in 11.9 μmol total lipid modified protocol-3 liposomes (ADR : total lipid ratio 1 : 38) and were killed at 1, 5, and 24 h after the injection. The organ disposition of the liposome dose was determined [3], plasma and packed red cells prepared from blood samples, and all samples frozen and stored at −20° C. Organs from groups of animals receiving the same dose at a given time point were pooled and extracted, after which the resultant levels of ADR were quantitated by high-pressure liquid chromatography [11].

Treatment of lung tumor model with encapsulated ADR. Balb/C mice, body weight 28.3 ± 1.7 g were given IV injections (tail vein) of 3,904 live and 9.8×10^5 irradiation-killed EMT6 cells in a total volume of 0.24 ml [8]. Twenty-four hours later groups of animals received one of the following: 61.4 μg ADR in 0.03 ml; 61 μg ADR incorporated in 7 μmol total lipid-modified protocol-3 liposomes (ADR : total lipid = 1 : 64 molar ratio) in 0.3 ml; or 60 μg ADR incorporated into 14.3 μmol total lipid French press liposomes (ADR : total lipid = 1 : 130 molar ratio) in 0.3 ml. The small, non-lung-accumulating French press liposomes were prepared from modified protocol-3 liposomes [1]. The ADR dose was 2.2 mg/kg throughout. A group of control animals received no ADR. Fourteen days after the cell dose all animals were killed and any lung tumours quantitated [8].

Fates of liposomes. The in vivo organ distribution pattern of liposomes was followed, using liposomes with ^{125}I-BPE incorporated. All procedures were as previously described [2].

Results

The effect of ADR incorporation protocol upon liposome physical parameters

Our objective was to incorporate as much ADR as possible whilst preserving the large diameter of these liposomes (our empirical assay for size is the ability of a liposome dose to accumulate in the lung in vivo). Whereas all the incorporation protocols gave similar ADR : total lipid ratios, only protocol 3 resulted in a preparation having lung accumulation properties comparable to those of controls, and it was therefore adopted for all subsequent experiments (Table 1). Liposomes from all four protocols had similar in vivo stabilities (as measured by total dose remaining in vivo at 2 h).

Loss of ADR from ADR-containing liposomes

Dialysis at room temperature of protocol-3 liposomes in a 1-ml cell using a membrane with pore-size 0.2 μm and changing the saline dialysate at 6 and 18 h resulted in the loss of 3.6% of the initial total lipid and 40.1% of the initial ADR. The ADR alone had a half-life for diffusion of 24 min under these conditions. Clearly liposome-incorporated ADR is capable of slow diffusion out of these liposomes in a manner not involving lipid loss.

Incubation of ADR-containing liposomes with saline

A simple 100% dilution of ADR-containing liposomes with saline (controls) resulted in the loss of 42.8% of their ADR to the supernatant. Incubation for 0.5 h increased this loss to 82.3%, while prolonged incubation (up to 2 h) did not release any further ADR. Under these conditions an apparent partition coefficient for ADR between liposomal lipid and saline can be calculated as 0.4. The effect of saline in these experiments is dramatic, and the presence of additional interactive factors (proteins, lipoproteins) is likely to be even more damaging. Protocol 3 does not yield encapsulated ADR, but rather liposome–associated ADR, and interaction with saline accelerates the release of ADR from these liposomes. Nevertheless, sufficient ADR might have remained liposome-associated to produce a therapeutic effect.

Organ distribution of free and incorporated ADR (Table 2)

It was apparent that ADR liposomes could not deliver ADR to the lungs over and above the levels expected for free drug, despite the fact that at 1.5 h, 11.4% of the lipid dose was

Table 2. Disposition of ADR in vivo after IV administration of free or liposome-encapsulated ADR. ICR mice received either 102 μg free ADR or 180 μg ADR incorporated into 11.9 μmol total lipid, lung-accumulating liposomes

Time (h) post drug administration		Organ levels of ADR (% administered dose)				
		Plasma (per ml)	Red blood cells (per ml)	Liver	Lungs	Heart
Free ADR	1.5	0.52	0.27	4.47	0.64	0.55
	5.0	0.06	0.07	3.66	0.6	0.38
	24.0	0.07	0.07	1.39	0.54	0.21
Liposome ADR	1.5	0.73	0.32	7.18 (30.6 ± 2.0)[a]	0.86 (11.4 ± 3.0)[a]	0.68
	5.0	0.02	0.05	5.66 (33.2 ± 3.1)	0.48 (7.9 ± 2.0)	0.29
	24.0	0.06	0.05	3.18 (31.1 ± 2.4)	0.6 (6.9 ± 3.8)	0.21

[a] Figures in parentheses represent mean ± SD or range of values for the disposition of ^{125}I-BPE label incoporated into ADR-liposomes in the liver and lungs of individuals prior to pooling of organs for ADR analysis

present in the lungs. There appeared to be a slight liposome effect upon levels of ADR present in liver at all times, versus ADR controls. Liposome incorporation did not prevent heart muscle from taking up ADR. The drug did not partition extensively into red blood cells. The recovery of ADR (free and liposome-incorporated) in vivo was indicative of extensive removal of ADR from the circulation and major organs.

Treatment of lung tumour model with incorporated ADR

There was no significant difference between the effects of free ADR, lung-accumulating, and non-lung-accumulating ADR liposomes upon lung tumours.

Discussion

The amphipathic nature of ADR poses problems when attempts are made to incorporate it into liposomes whilst maintaining their size distribution. Our results demonstrate that large (lung-accumulating) liposomes may be preserved by adding pre-formed liposomes to either solid or aqueous ADR. The drug binds to, partitions into, and equilibrates with, the liposomal membranes. The affinities of the ADR molecule are such that it resides preferentially in the hydrophobic environment of the vesicle's membrane, and as a result ADR is released only slowly during extensive dialysis. Following dilution of the preparation with saline, a rapid loss of up to 80% of such ADR can result, and this property vitiates the usefulness of these particular liposomes as either in vivo drug carriers or sustained release vehicles. These functions, however, may be preserved for encapsulated ADR when vesicle size is unimportant [10] or the preferred size is smaller than that required for lung accumulation.

The in vivo results clearly show that these ADR liposomes do not enhance accumulation of ADR in the mouse lung, and are ineffective in treating the EMT6-Balb/C mouse lung tumour model, even though the carrier distributes successfully to the lung. These liposomes must release the majority of their ADR within minutes of entering the vascular system, the drug then redistributing as expected but not so fast as to preclude some increase in ADR liver levels. If ADR had stayed associated with the liposomes, then ADR and ^{125}I-BPE levels, on a percent dose basis, would be similar. It should be noted that the free ADR disposition in mice found here differs from reported values in the rat [11], being qualitatively similar to that seen in mice after IP administration of free ADR [13].

Amphipathic compounds such as ADR, because of the relative physicochemical properties of the drug and its carrier, can be poor candidates for use in some liposomes. In such cases alternative carriers or approaches are needed to obtain improved therapeutic availability of the drug.

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