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# Preparation and stability of liposome encapsulated doxorubicin and mitoxantrone and their internalisation by murine peritoneal macrophages

J.C. Lilley *, L.H. Patterson, M.J. Taylor

*Department of Pharmacy, School of Applied Sciences, De Montfort University, The Gateway, Leicester LE1 9BH, UK*

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**Abstract**

Liposomal encapsulation of the cytotoxic drugs doxorubicin or mitoxantrone was achieved with a lipid combination of 10:1:10 DPPC/DPPG/cholesterol. Both drugs were observed, by confocal fluorescence microscopy, to be almost entirely associated with the liposome bilayers. A method is described for loading of macrophages intraperitoneally with liposome encapsulated doxorubicin or mitoxantrone and the subsequent isolation of viable macrophages ($83.5 \pm 6.6\%$) using a separative technique based on Ficoll and Percoll. Confocal microscopy also revealed that the intact liposomes were internalised in macrophages and that liposomal drug was located in phagosomes. By comparison the free drug was located, in the case of both mitoxantrone and doxorubicin, around the periphery of the nucleus. No intracellular leakage of mitoxantrone from the liposomes after internalisation by the murine peritoneal macrophages was observed whilst some leakage was apparent for doxorubicin.

*Key words:* Liposome; Doxorubicin; Mitoxantrone; Murine peritoneal macrophage; Confocal microscopy

## 1. Introduction

Liposome encapsulated doxorubicin has reached clinical trial stage in humans (Daoud et al., 1989). Advantages to the use of liposomal doxorubicin compared to the free drug appear to be a decrease in symptoms of toxicity including cardiotoxicity (Rahman et al., 1986b; Treat et al., 1990), immunotoxicity (Rahman et al., 1986a), nausea,vomiting and alopecia (Sells et al., 1987) and vesicant properties (Balazsovits et al., 1989). In addition liposome encapsulation of doxorubicin has resulted in increased maximum tolerated dose (Gabizon et al., 1989) which is associated with a similar or increased performance of doxorubicin in human tumours (Balazsovits et al., 1989; Rahman et al., 1990) and rats (Gabizon et al., 1988). Mitoxantrone is a synthetic agent, broadly based on the anthracyclines and is often used in a similar clinical setting to doxorubicin. Liposomal encapsulation of mitoxantrone has also been described (Law et al., 1991; Schwendener et al., 1991) and it is anticipated that encapsulation will achieve a similar reduction in systemic toxicity comparable to that seen with doxorubicin.

* Corresponding author.


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There is speculation regarding the role of macrophages in the distribution of liposomal doxorubicin injected in vivo and the possibility that macrophages may act as a depot slowly releasing free drug (Storm et al., 1989b). In the present work we describe the development of techniques used to isolate macrophages loaded with liposomal encapsulated doxorubicin and mitoxantrone and to investigate the interaction of such liposomal drug with macrophages.

## 2. Materials and methods

### 2.1. Materials and animals

Mitoxantrone dihydrochloride was obtained from Lederle (Gosport, Hampshire, U.K.) and dissolved in methanol (1.33 mM) for incorporation into liposomes. Doxorubicin formulated with lactose and hydroxybenzoate was supplied by Farmitalia Carlo Erba Ltd (St. Albans, Herts, U.K.) and reconstituted with 0.01 N HCl in methanol (1:99 v/v) to produce a solution of 2.5 mg/ml. All drug solutions were stored protected from light at 4°C and used within 48 h. Dipalmitoylphosphatidylglycerol (DPPG) (99.9%), obtained as the ammonium salt, dipalmitoylphosphatidylcholine (DPPC) (99.9%) and cholesterol were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). RPMI 1640 with Hepes buffer (25 mM, pH 7.2) was prepared from powder by sterile filtration and supplemented with L-glutamine (200 mM, 1%), penicillin (5000 IU) and streptomycin (5000 $\mu$g) (Flow laboratories, Irving, U.K.). Hanks' balanced salt solutions, complete (HBSS) and incomplete (HBSSI), and Percoll (Sigma) solutions were prepared using a refractometer as described by Ali (1986). Ficoll-Hypaque 1077 was obtained from Sigma. Trypan blue dye, methyl green, naphthol AS-BI and $\alpha$-naphthyl butyrate (Sigma) were dissolved in PBS. Trypan blue was dissolved in phosphate-buffered saline (PBS), filtered and stored at 4°C protected from light. Methyl green was dissolved in distilled water and the impurities were extracted with chloroform (Ali, 1986). Formal acetone was prepared by mixing formaldehyde with 60% acetone in phosphate buffer (pH 6.6) (1:2 parts). All other chemicals and reagents were obtained from BDH Chemicals Ltd (Enfield, Middlesex, U.K.). Swiss female mice (weight $30 \pm 5$ g) fed on 'diet 41b' (modified) supplied by Pilsburys (Birmingham, U.K.) and given water ad libitum were used throughout.

### 2.2. Multilamellar vesicle liposome preparation

Mitoxantrone (1.33 $\mu$mol) in 1 ml methanol, or doxorubicin (4.31 $\mu$mol) in 1 ml 0.01 N HCl in methanol and DPPG (3.64 $\mu$mol in 1 ml methanol) were mixed thoroughly in a flask followed by the addition of DPPC (36.36 $\mu$mol) and cholesterol (36.36 $\mu$mol) in chloroform (2.0 ml). To produce a lipid/mitoxantrone film the solvents were removed under reduced pressure using a rotary evaporator at 50°C. The remaining procedures were carried out under aseptic conditions. The lipid film was resuspended in sterile PBS (1 ml, pH 7.2). To remove the unentrapped drug the liposomal suspension was passed through a pre-packed Sephadex PD10® column according to the manufacturer's instructions (Pharmacia, LKB Biotechnology, Uppsala, Sweden). The column was primed with sterile PBS (20 ml) prior to the addition of the liposomes. Liposomes were eluted in the void volume through a 19-gauge needle attached to the column, into an autoclaved glass vial which was pre-sealed with a rubber septum and crimped aluminium ring. All liposome suspensions were stored at 4°C, protected from light, and vortexed vigorously prior to use. Liposomal drug content was determined by dissolving the liposomes in methanol and using the extinction coefficient for mitoxantrone (10890 $M^{-1}$ $cm^{-1}$) at 674 nm and doxorubicin (12993 $M^{-1}$ $cm^{-1}$) at 494 nm. No attempt was made to restrict the size distribution of the liposomes.

### 2.3. Stability of liposomes containing mitoxantrone in PBS and supplemented medium

Fresh liposomes (1.5 ml) were prepared containing mitoxantrone and diluted to 8 ml with (a) PBS or (b) supplemented RPMI 1640 medium, placed into sterile, sealed vials and stored at 4 and 37°C, respectively. Three samples (0.5 ml)

were withdrawn from the vials every 24 h and the samples centrifuged at $10000 \times g$ for 10 min (Jouan microcentrifuge). The supernatant was aspirated from the liposome pellet into an Eppendorf tube (1.5 ml) and stored at 4°C. Samples were taken over 120 h, stored at 4°C and the concentration of mitoxantrone determined.

### *2.4. Preparation of murine peritoneal macrophages loaded with liposomes containing doxorubicin or mitoxantrone in vivo*

Drug-containing liposomes or an equivalent concentration of free drug were injected intraperitoneally (i.p.) into Swiss female mice. 20 h after injection peritoneal exudate cells were removed from mice by a peritoneal lavage procedure (adapted from Hunt, 1987). In order to isolate a population of macrophages containing internalised liposomes from contaminating neutrophils and red blood cells a purification procedure on Ficoll-Hypaque 1077 was developed as follows: a suspension of unwashed peritoneal exudate was carefully layered onto Ficoll-Hypaque 1077 (1.5 ml) in a sterile conical tube (10 ml, Sterilin) and centrifuged at $500 \times g$ for 30 min at 20°C. The upper 'plasma cell' layer was discarded and the middle layer of cells, within the Ficoll, pooled with similar layers from other tubes, diluted with HBSSI (to 10 ml), and centrifuged at $325 \times g$ for 20 min at 20°C. Cells were subsequently washed and resuspended in HBSSI (5 ml) twice to remove traces of Ficoll.

Macrophage internalised liposomes were separated from uninternalised liposomes using a Percoll solution (28% SIP in PBS, 5 ml). The loaded macrophages pelleted through the Percoll and the uninternalised liposomes remained at the Percoll/buffer interface.

Macrophage viability was determined using the Trypan blue exclusion dye assay (Hunt, 1987) and cell type was determined by non-specific esterase staining (adapted from Shibata et al., 1985).

### *2.5. Examination of liposomes and macrophages containing liposomes using confocal laser microscopy*

Liposomes containing drug were spun onto polylysine treated microscope slides using a Shandon Elliott cytospin and then mounted in a high density anti-fade mountant prior to examination under the confocal laser microscope. Cells were examined in suspension under a glass coverslip. The confocal imaging system used was a BioRad MRC-600 (BioRad Microscience Ltd, Hemel Hempstead, U.K.) scanning assembly incorporating a 25 mW argon ion laser and coupled to a Nikon Optiphot II fluorescence microscope. Excitation was at 514 nm wavelength (red fluorescence monitored) and the scanning beam intensity was used at the maximum with no neutral density filtration. The objective lens was a $60 \times$ oil immersion and the digitised images were accumulated under Kalman filtration to reduce the signal-to-noise ratio. The system was operated close to the minimum confocal aperture, collecting fluorescence from approx. 1 $\mu$m deep sections of each cell.

## 3. Results

The method of liposome preparation described produced multilamellar vesicles (MLV) and the concentration of drug in the liposomes was mitoxantrone 390 $\mu$M or doxorubicin 203 $\mu$M. Fig. 1 depicts liposomes containing (a) mitoxantrone or (b) doxorubicin examined by confocal laser microscopy under fluorescent laser light of wavelength 514 nm. This reveals that liposome entrapped mitoxantrone or doxorubicin were contained within the bilayer of the liposomes. Fig. 2 shows that there was effectively no loss of mitoxantrone from liposomes suspended in PBS as measured by no change in mitoxantrone concentration in the supernatant over a period of 4 days at 4°C. However, in RPMI 1640 medium at 37°C an increase of approx. 2 $\mu$M mitoxantrone (representing 10% loss) in the supernatant over the first 24 h was evident, followed by no further change up to 120 h. This suggests that the liposomes are stable at 4°C over 4 days but at 37°C do give rise to an initial loss of drug. As the temperature approached the transition temperature of the lipid it is possible that some of the drug molecules were freed from the lipid bilayer. This would cause an alteration of the free drug to lipid drug ratio.

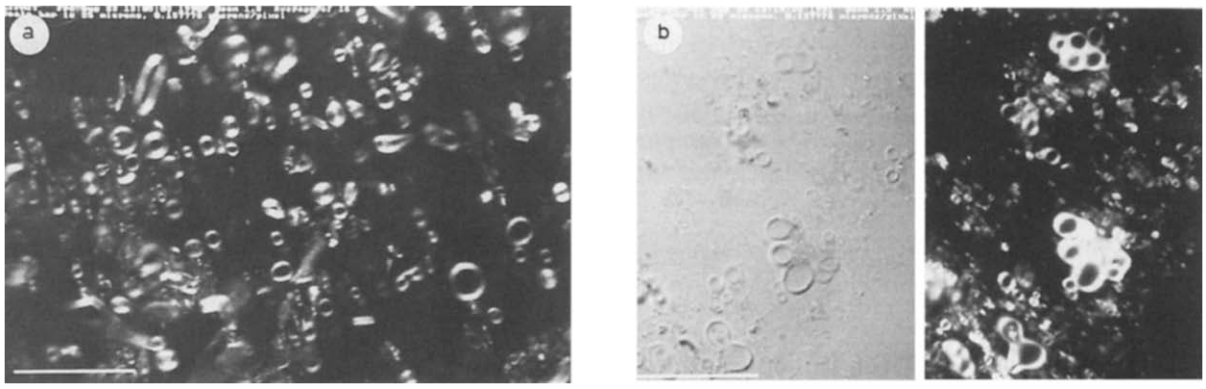


Fig. 1. Liposomes containing: (a) mitoxantrone and (b) doxorubicin photographed using confocal laser scanning microscopy as described in section 2 under laser light (excitation $\lambda$ 514 nm) (a) or white light (LHS) followed by laser light (RHS) (b). Magnification $\times$ 60 (oil immersion). Scale bars represent 25 $\mu$m.

### 3.1. Uptake of liposomes by murine peritoneal exudate cells in vivo

Direct injection of the liposomes into the peritoneal cavity of mice resulted in an extensive level of phagocytosis of the liposomes by peritoneal macrophages. The macrophages were viable (83.5 $\pm$ 6.6%) after separation on Ficoll and Percoll and appeared 'gorged' with liposomes (refer to Fig. 4b, i and ii). Viability of the cells containing ingested mitoxantrone liposomes, as determined by the trypan blue dye assay, was maintained throughout the subsequent PEC purification and separation procedure and compared favourably with results obtained previously with the separation of untreated PEC. The mean viability of macrophages containing liposomes was 83.5 $\pm$6.6% after separation on Ficoll and Percoll compared to 90.7 $\pm$4.8% for macrophage preparations unexposed to liposomes and separated on Ficoll alone. The non-specific esterase

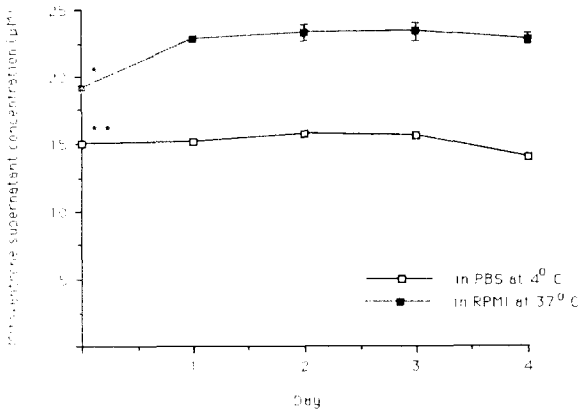


Fig. 2. The leakage of mitoxantrone from dipalmitoylphosphatidylcholine/cholesterol/dipalmitoylphosphatidylglycerol liposomes incubated in PBS at 4°C or supplemented RPMI 1640 at 37°C. Values are the mean $\pm$ SD of three samples except * two samples and ** one sample and include the mitoxantrone supernatant concentration.

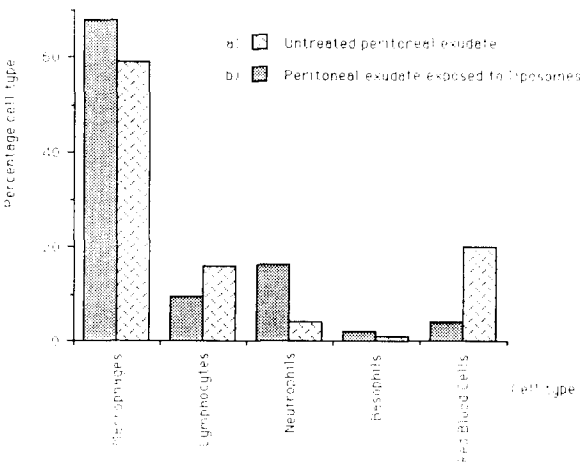


Fig. 3. Percentage recovery of cells from murine peritoneal exudate: (a) untreated and (b) exposed to mitoxantrone containing liposomes in vivo.

test was necessary to distinguish the presence of macrophages and monocytes from other cell types, in particular, large lymphocytes which are of a similar size and can possess a similarly shaped nucleus. In this study, positive but different staining patterns were observed for mononuclear phagocytes, which revealed a diffuse staining pattern throughout the cytoplasm, and lymphocytes which revealed staining of one or two localised granules near the cell membrane (results not shown). No esterase activity in neutrophils was observed. The proportions of cell types determined by non-specific esterase present in unpurified samples of murine peritoneal exudate are shown in Fig. 3. The results show increased proportions of macrophages, neutrophils and basophils in the exudate treated intraperitoneally with liposomes containing mitoxantrone compared to the untreated exudate. Nearly half the population of macrophages (42%) in this preparation phagocytosed the mitoxantrone liposomes.

### *3.2. Examination of peritoneal exudate, exposed intraperitoneally to liposomes using confocal microscopy*

Confocal microscopy utilises the fluorescent properties of mitoxantrone when excited with laser light (514 nm) enabling this technique to uniquely detect the presence of mitoxantrone within living cells (Smith et al., 1992). The present study has extended this approach to mitoxantrone entrapped within liposomes. Confocal microscopy has previously been applied to the

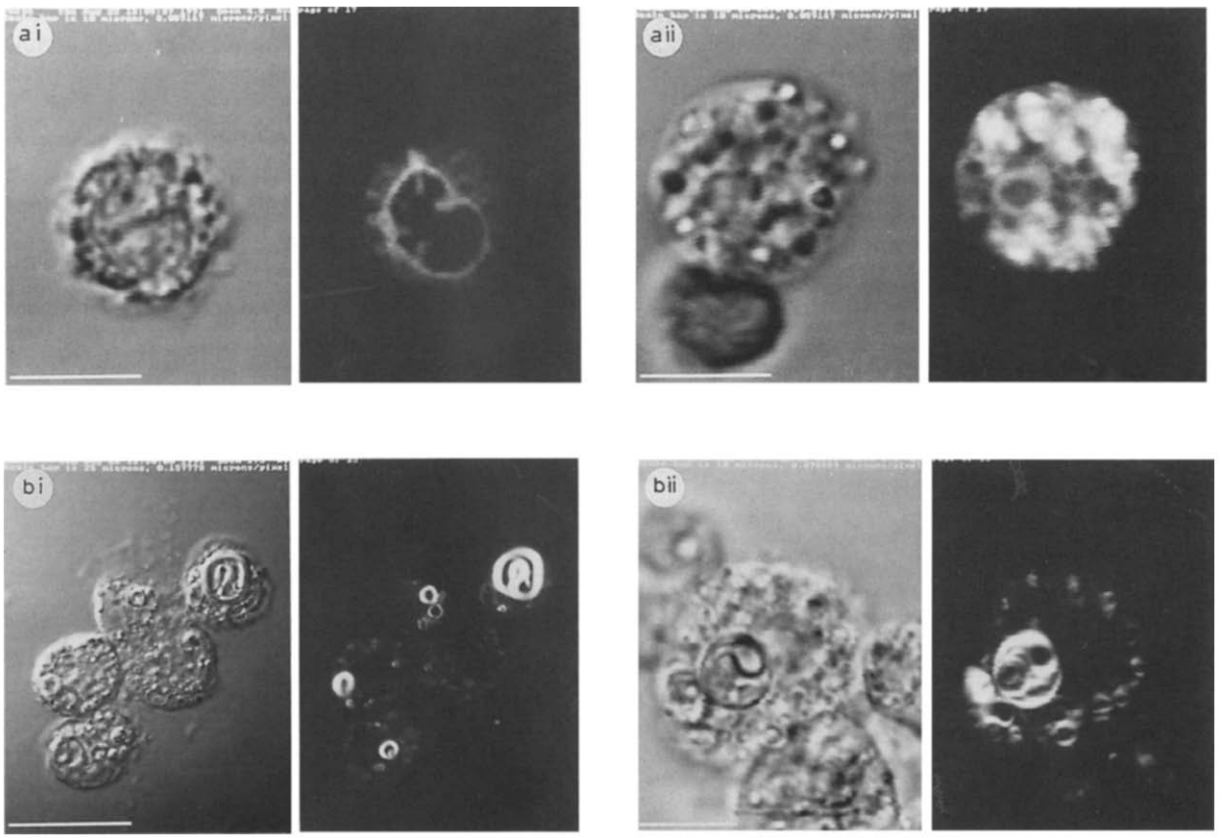


Fig. 4. Murine peritoneal macrophages exposed in vivo to: (a,i) and (a,ii) free mitoxantrone and (b,i) and (b,ii) liposome encapsulated mitoxantrone. The cells and liposomes were examined either under laser light (excitation $\lambda$ 514 nm) or white light using a confocal microscope as described in section 2. Each photograph contains two pictures of the same field examined under white light (LHS) and then laser light (RHS). Magnification: $\times 600$ (oil immersion). Scale bars represent 25 $\mu$m.

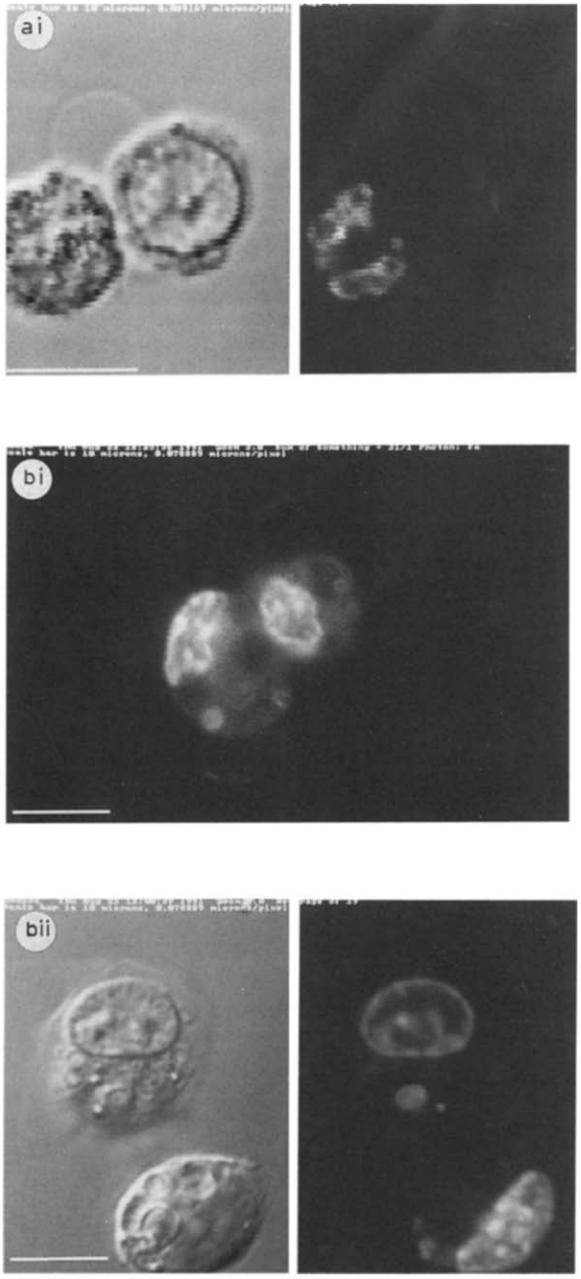


Fig. 5. Murine peritoneal macrophages exposed in vivo to: (a) free doxorubicin and (b,i) and (b,ii) liposome encapsulated doxorubicin. The cells and liposomes were examined either under laser light (excitation $\lambda$ 514 nm) (b,i) or white light using a confocal microscope as described in section 2. Photographs (a) and (b,ii) contain two pictures of the same field examined under white light (LHS) and then laser light (RHS). Magnification: $\times 600$ (oil immersion). Scale bars represent 25 $\mu$m.

visualisation of fluorescein-conjugated microspheres phagocytosed by adherent murine macrophages (Hook and Odeyale, 1989; Odeyale and Hook, 1990). Fig. 4a and b shows photographs of murine peritoneal macrophages containing free or liposome-encapsulated mitoxantrone whilst Fig. 5a and b depicts murine peritoneal macrophages containing free or liposome-encapsulated doxorubicin. Examination using confocal microscopy revealed that the liposomes were located inside the macrophage and not attached to the plasma cell membrane. Interestingly, although fluorescence was associated with most of the liposomes within the macrophages some liposomes were not fluorescent suggesting that there was an uneven distribution of doxorubicin and mitoxantrone amongst the liposome preparations. Contaminating cells in the macrophage preparations did not show the presence of internalised liposomes. It was observed that most of the internalised liposome encapsulated mitoxantrone remained within the liposomes (Fig. 4b) whereas comparatively more doxorubicin was released from the internalised liposome encapsulated doxorubicin (Fig. 5b). The released doxorubicin could clearly be seen associated with the macrophage nuclear membrane (Fig. 5b). The lack of diffused fluorescence within the cells suggests that detectable amounts of mitoxantrone had not leaked from macrophage internalised liposomes. In contrast, macrophages incubated with free mitoxantrone displayed a marked nuclear membrane fluorescence (Fig. 4a) similar to that also observed with free doxorubicin (Fig. 5a).

## 4. Discussion

This study shows that Sephadex PD-10 columns can be used for a rapid (15 min) efficient separation of liposome encapsulated doxorubicin or mitoxantrone with little preparation required. The liposomes can be obtained in concentrated form (in 1 ml) from a commercial PD-10 column. The use of prepacked hydrated Sephadex between two scintered polyethylene frits (pore size 50–159 $\mu$m) is advantageous over other separation methods because the frits remove very large drug-lipid

complexes whilst the small complexes are retained by the column and are eluted after the higher molecular weight liposomes. Separation of unentrapped drug from liposomal drug by gel filtration using Sephadex has been achieved routinely by others for measurement of encapsulation efficiency. However, it has generally been discarded as a preparative method for liposomes because gel filtration can lead to dilute liposomal samples (Amselem et al., 1990; Law et al., 1991). This problem does not arise using PD-10 columns which contain a comparatively small volume of gel, thus allowing for a significant reduction in the sample volume. Using this method, liposomes containing mitoxantrone were found to be stable in PBS at 4°C for up to 4 days. However, there was a slight leakage, initially, of mitoxantrone from liposomes stored in supplemented RPMI medium at 37°C. This could be due to a temperature shock phenomenon described by Allen and Cleland (1980) which can be reduced by equilibration of the liposomes to the required temperature over a period of 5 min. In common with previous work investigating liposomal doxorubicin and mitoxantrone the liposomes produced were multilamellar vesicles. The combination of DPPC/DPPG/cholesterol in a ratio of 10:1:10 was used to encapsulate doxorubicin and mitoxantrone since it was considered to produce liposomes which were more stable with respect to the physical entrapment of doxorubicin compared to other combinations (Storm et al. 1989a). Those lipids with long chains which are more saturated lipids preferentially resist attack by serum components (Comiskey and Heath, 1990). Specifically, these long chain lipids minimise the extent of lipid peroxidation (Cullis et al., 1987; Weiner et al., 1989). The negatively charged phospholipid DPPG also facilitated entrapment of doxorubicin and mitoxantrone which are both cationic amphiphiles at physiological pH (Mayer et al., 1986). The ionic binding capacity of the acidic phospholipids for the doxorubicin and mitoxantrone is stronger than the hydrophobic reaction of these agents with neutral, zwitterionic phospholipids such as DPPC (De Wolf et al., 1991). DPPG is structurally similar to cardiolipin (1′,3′-di-*O*-(3-*sn*-phosphatidyl-*sn*-glycerol) which is thought to bind doxorubicin at two sites: one is situated deeply buried in the membrane, accessible to the drug at temperatures which confer liquid-crystalline status to the lipids, and second site relatively exposed, thought to be a drug-phospholipid head interaction (Karczmar and Tritton, 1979).

A fundamental objective of cancer therapy is to destroy cancer cells whilst minimising systemic toxicity. The use of liposomal doxorubicin for this purpose is relatively advanced and trials in humans have shown a decreased toxicity compared to injection of the free drug. This effect has been partly attributed to the uptake of liposomal doxorubicin by macrophages of the reticuloendothelial system (RES) in the liver, spleen and elsewhere which then act as a depot for the drug. Storm et al. (1988, 1989b) have shown that doxorubicin can be released intact from macrophages but the intracellular fate of doxorubicin-containing liposomes has not previously been observed. Doxorubicin and mitoxantrone were shown, by confocal microscopy (refer to Fig. 1) to partition preferentially into the lipophilic liposome bilayer. Mitoxantrone fluorescence is normally too weak to be detected by conventional fluorimetry (Bell, 1988). However, use of laser light to maximise the quantum yield of mitoxantrone fluorescence in combination with the excellent detection and image enhancement properties of confocal microscopy has enabled this technique to visualise the distribution of mitoxantrone (and doxorubicin) in liposomes and viable macrophages. The structural similarities of mitoxantrone to doxorubicin, with respect to the hydrophobic chromophore and cationic functionality make it likely that both agents would associate with anionic liposomes in the same manner. Schwendener et al. (1991) have confirmed that mitoxantrone does require the presence of an anionic phospholipid for encapsulation.

Confocal laser microscopy also identified the location of doxorubicin and mitoxantrone within living viable macrophages. The mitoxantrone remained associated with the liposomal bilayer once internalised by the macrophages which contrasts with mitoxantrone localisation at the nuclear periphery when internalised as the free drug (refer to Fig. 4). The results for free doxorubicin

demonstrate that, in contrast to mitoxantrone, this agent was released from the liposomal bilayer upon internalisation of the liposomes by the macrophages since both liposomal and free doxorubicin were observed to associate at the nuclear periphery (refer to Fig. 5). Nuclear association of free doxorubicin has also been demonstrated by Martin et al. (1982) and is consistent with the uptake and release of free doxorubicin in cell lines (Haskill, 1981) and Kupffer cells (Storm et al., 1989b). With respect to liposome encapsulated doxorubicin it is known that the liposome lipid composition influences the amount of doxorubicin released inside phagocytic cells due to resistance to degradation by lysosomal esterases (Storm et al., 1989c). The present study indicates that a lower concentration of the mitoxantrone was leaked from the macrophage ingested liposomes, compared to that of doxorubicin. This implies that the mitoxantrone/liposomal interaction is more stable and more resistant to hydrolytic degradation. The potential for using macrophage ingested liposome encapsulated drugs for delivery to the vicinity of tumour cells is recognised (Taylor, 1991). In this respect lipid encapsulated mitoxantrone should be relatively non-toxic to macrophages, since free drug is not leaked from internalised liposomes a property that is the subject of futher investigations.

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