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Novel therapeutic nano-particles (lipocores): trapping poorly water soluble compounds

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Abstract

The development of stable spherical lipid-coated drug particles that are termed 'lipocores' is reported here. Unlike conventional lipid-based particles (i.e. liposomes, emulsions, micelles), these particles are comprised solely of a core of a poorly water soluble drug surrounded by polyethyleneglycol conjugated lipid (PEG-lipid) and are formed via a 'kinetic' trapping process. These lipocore particles were made with the acyl chain of 16 carbon length (C16) acyl-chain derivatives of paclitaxel or vinblastine and with the polyene antifungal hamycin. Formation of the particles occurred regardless of the type of PEG-phospholipid used (i.e. acyl chain length, chain saturation, and polymer length) and could also be formed with the negatively charged lipid *N*-glutaryl-dioleoyl-phosphatidylethanolamine (DOPE-GA). Images from both freeze-fracture electron microscopy and electron cryo-microscopy revealed solid spherical structures with no internal lamellae for the PEG-lipid particles made with the C16 derivatives of paclitaxel (BrC16-T) or vinblastine (C16-Vin). From a solute distribution study of lipocores made with BrC16-T and distearoyl-phosphatidylethanolamine-PEG_{2000} (DSPE-PEG_{2000}), the particles were found to have no measurable aqueous captured volume. Fluorescence anisotropy and order parameter measurements revealed the core material of these particles to be highly immobilized. The mole ratio of BrC16-T:lipid in the lipocores was typically $>90:<10$ and as high as 98:2, and the refrigerated lipocores were stable for several months. BrC16-T/DSPE-PEG_{2000} lipocores of 50–100 nm particle size were far less toxic than paclitaxel (Taxol®) after intraperitoneally (i.p.) or intravenously (i.v.) administration in mice and were active against i.p. and subcutaneously (s.c.) planted human (OvCar3) ovarian carcinoma grown in SCID mice. It is believed the high drug:lipid ratio, the stability, and therapeutic efficacy of these novel particles make them a paradigm for delivery of poorly water soluble drugs and/or their hydrophobic derivatives.

Abbreviations: C16, acyl chain of 16 carbon length; DMPE, dimyristoyl-PE; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoyl-PE; DPPE, dipalmitoyl-PE; DSPE, distearoyl-PE; HBS, Hepes buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEG, polyethyleneglycol; SUV, small unilamellar vesicle.

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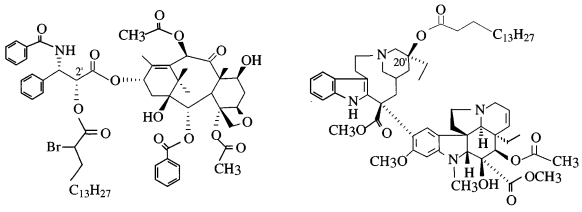
1. Introduction

The poor aqueous solubility of agents such as paclitaxel, vinblastine, camptothecin, etoposide, cyclosporins, and numerous other compounds has made their intravenous use problematic. For aqueous dispersal of these drugs, a biocompatible vehicle is necessary. Numerous carrier systems have been used for intravenous delivery of poorly water soluble drugs and include liposomes, emulsions, micelles (lipid and co-polymer based) (Janoff et al., 1988; Guo et al., 1991; Cohen and Bernstein, 1996; Janoff, 1999; Kwon, 1998; Storm and Crommelin, 1998; Uchegbu and Vyas, 1998). With a limited hydrophobic domain, these carriers can accommodate only a limited amount of hydrophobic drug and the drug/lipid ratio may be especially low for drugs that have a low affinity for the lipid carrier (i.e. poorly lipophilic). Consequently, the administration of a large amount of carrier substance may be required in order to reach a therapeutic concentration of the active agent. In these cases vehicle related toxicity may become limiting to treatment. For example, use of Taxol® (paclitaxel in Cremophor EL®/ethanol) is limited to some extent by the significant, although manageable, vehicle-related side effects (Straubinger, 1995).

Liposome formulations of paclitaxel have been shown to have reduced acute toxicities and maintained therapeutics (Sharma et al., 1993, 1997), but the amount of paclitaxel that can be stably incorporated is quite low (~ 3 mol%). Above this level, paclitaxel gradually dissociates from most bilayers and forms crystals. The inability of the bilayer to accommodate paclitaxel is most likely due to unfavorable packing between lipid and the bulky taxane structure (Balasubramanian and Straubinger, 1994). Recently, Needham and Sarpal (1998) demonstrated that lipid bilayer systems designed to accommodate paclitaxel better could incorporate as much as 12 mol% paclitaxel into liposomes. However, the toxicity of the lysolipid used to achieve this high loading may preclude the clinical use of such a formulation.

While much effort has been spent to make more water soluble derivatives of some of the compounds listed above (Vyas et al., 1995; Li et al., 1996; Iyer and Ratain, 1998; Paradis and Page, 1998), efforts were focused upon making compounds more lipophilic to enhance their association with lipid assemblies. In particular, in a previous study acyl chains were attached to paclitaxel (Mayhew et al., 1996) and later a bromine atom was inserted at the α-carbon of the acyl chain to facilitate hydrolytic cleavage of the prodrug to yield paclitaxel (Mayhew et al., 1997). This derivative, 2′-2-bromohexadecanoyl paclitaxel (BrC16-T) (Fig. 1), was formulated in liposomes and found to be less toxic and more active in an animal model of human ovarian cancer than Taxol® (Ahmad et al., 1996; Mayhew et al., 1997). However, the drug:lipid ratio in these liposomes was lower than desired at about 6 mol% (Ali et al., 1997).

Here a new therapeutic particulate delivery system is described in which high drug:lipid ratios are achieved. These novel particles were produced by the combination of lipophilic derivatives of paclitaxel and vinblastine, as well as the underivatized antibiotic hamycin, with lipids having polyethyleneglycol conjugated to the headgroup. Once formed, these particles are stable for extended periods of time. The studies show that unlike liposomes or emulsion particles, these particles contain no trapped water or oil but rather have a solid core of drug/prodrug, making them



BrC16-T C16-Vin

Fig. 1. Structure of the C16 derivatives of paclitaxel (2′-2-bromohexadecanoyl paclitaxel = BrC16-T) and vinblastine (20′-hexadecanoyl vinblastine = C16-Vin).

highly efficient carriers. In fact, the drug to lipid molar ratio for derivatized paclitaxel/polyethyleneglycol (PEG)-lipid particles was as high as 98:2. Additionally, BrC16-T in these particles exhibited antitumor activity and was found to be much less toxic than Taxol®. These structures have been termed 'lipocores' and it is believed that lipocore formation can be adapted to a broad array of poorly water soluble drugs, their hydrophobic derivatives, and perhaps hydrophobic derivatives of normally water soluble drugs.

2. Materials and methods

2.1. Materials

All lipids were obtained from Avanti Polar Lipids (Alasbaster, AL). Paclitaxel ($>99\%$) was obtained from Hauser Chemical Research (Boulder, CO). Taxol® and amphotericin B were obtained from Bristol-Myers Squibb, New Brunswick, NJ. Vinblastine was purchased from Sigma. Hamycin was purchased from Hindustan Antibiotics, India. Paclitaxel derivatives were made where BrC6, BrC8, BrC12, BrC14 and BrC16 acyl chains (bromine on the α-carbon) were attached at the 2′ position of paclitaxel as previously described (Mayhew et al., 1997) (Fig. 1). Vinblastine was derivatized by attaching a 16 carbon acyl chain at the 20′-OH position. Chromiumoxalate (trihydrate) (potassium salt) and 4-oxo-TEMPO (Tempone) were obtained from Aldrich (Milwaukee, WI). All the chemicals used were the highest purity available.

2.2. Lipocore preparation and assay

Two methods were used to make drug/lipid particles. The first involved dissolving both lipid and the derivatized drug in chloroform and adding this to Hepes buffered saline (HBS) (10 mM Hepes, 150 mM NaCl, pH 7.4). The solvent was then removed by vacuum rotary evaporation. Not surprisingly particle size was heterogeneous with a broad size distribution for the resulting particles. As this may be undesirable for an intravenously (i.v.) injectable, a second method was sought by which particle size could be controlled.

The second method was an ethanol injection process. Both lipid and the drug at the desired molar ratio were co-dissolved in ethanol; a typical final concentration of paclitaxel derivative was 200 mg/ml. Typically, this solution was then injected via syringe into HBS heated to $\sim 60°C$ that was being vortexed such that the final concentration of drug was ~ 10 mg/ml (hydrolysis of BrC16-T to paclitaxel was $<1\%$). The final concentration of ethanol as determined by gas chromatography was less than 5% due to evaporation at 60°C. For some of the BrC16-T/distearoyl-phosphatidylethanolamine (DSPE)-PEG_{2000} animal studies, particles were also formed where the ethanol solution was injected into HBS at a point just inside the modified tip of an operating Silverson L4RTA Homogenizer (Silverson Machines, Long Meadow, MA). This process was needed for making larger scale preparations and under the appropriate operating conditions yielded comparable particles to those made by vortexing.

For hamycin/DSPE-PEG_{2000} particles, drug and lipid were dissolved in DMSO and this was injected into HBS (1:9 v/v). DMSO was then removed by dialysis using a membrane with a 10 kDa molecular weight cutoff.

The phospholipid concentration of all samples was measured by a modified version of the procedure to measure phosphate content (Chen et al., 1956). For determination of paclitaxel derivative concentration, samples were dissolved in ethanol and the absorbance at 227 nm was compared to standards. Absorbances were recorded on an UV-2101PC UV-scanning spectrophotometer from Shimadzu Scientific Instruments (Princeton, NJ). Particle size was determined by light scattering using a Nicomp Model 370 Submicron Particle Sizer from Pacific Scientific (Menlo Park, CA) in the solid particle mode. For animal testing, all particle suspensions were made by the ethanol injection and the particles had mean diameters of 50–100 nm.

2.3. Characterization of lipocores

2.3.1. Sucrose density gradient analysis

Sucrose gradients (12 ml of ~10–50% sucrose (w/v)) were established using a Gradient Master Model 106 device (Biocomp Instruments, Fredericton, NB, Canada) where time = 2 min, angle = 81.5°, speed = 19 rpm. Approximately 0.2 ml of sample was placed atop the gradient and the tubes were centrifuged at 208 000 × *g* overnight at 5°C. The gradients were then fractionated manually starting from the top into 1 ml fractions. The fractions were assayed for lipid and for BrC16-T as described above.

2.3.2. Microscopy

All light microscopy was conducted using an Olympus BH-2 microscope (New York/New Jersey Scientific, Middlebush, NJ) equipped with Nomarski optics. Freeze-fracture electron microscopy (EM) was performed as previously described (Perkins et al., 1992). For electron cryo-microscopy, frozen-hydrated samples were prepared and examined as previously described (Li et al., 1998).

2.3.3. Captured volume determination

Captured volumes were measured as previously described (Perkins et al., 1993). The spin label Tempone was either added to the performed BrC16-T/DSPE-PEG_{2000} (85:15) particles or added to the ethanol mixture prior to particle formation. The BrC16-T and Tempone final concentrations were 10 mg/ml and 1 mM, respectively. Samples concentrated 4-fold via centrifugation were also examined (to ~40 mg/ml BrC16-T), as well as DSPC liposomes used as a positive control.

2.3.4. Fluorescence anisotropy and electron spin resonance (ESR) order parameter determinations

BrC16-T/DSPE-PEG_{2000} (initially 85:15 mol/mol) lipocores were made by ethanol injection; the BrC16-T concentration was 10 mg/ml and samples contained either 0.5 mol% 1,6-diphenyl-1,3,5-hexatriene (DPH) or 1 mol% 10-doxyl-nonadecane (10DND) which were added to the ethanol prior to injection. To remove micelles from the lipocores, samples were centrifuged at 10 000 × *g* for 1 h and the supernatant replaced with fresh buffer. This washing procedure was performed again and the final pellet was re-suspended in buffer solution.

For dimyristoyl-phosphatidylcholine (DMPC) liposomes and DSPE-PEG_{2000} micelles, lipid and probes (0.5 mole DPH or 1 mol% 10DND) were dried to thin films from chloroform by rotary evaporation in round bottom flasks and hydrated with HBS to form spontaneously multilamellar vesicles for DMPC (200 mg/ml lipid) or micelles for DSPE-PEG_{2000} (50 mg/ml lipid). For the C18-alkane emulsion, octadecane and DSPE-PEG_{2000}, with either DPH or 10DND, were mixed in ethanol and then injected into HBS heated to 60°C. The final octadecane/DSPE-PEG_{2000} ratio was 1:1 mol/mol, respectively, with either 0.5 mol% DPH or 1 mol% 10DND.

Fluorescence anisotropy measurements were conducted as previously described (Lakowicz, 1983) using a PTI Alpha Scan Spectrofluorometer (PTI, Princeton, NJ). Samples were diluted to a typical DPH concentration of 1 μM and excitation and emission wavelengths were set at 355 and 427 nm, respectively. For ESR measurements, a Bruker 100D instrument equipped with a temperature control device was used (Bruker, Billericia, MA). Order parameters were derived for undiluted samples from the ESR spectrum of 10DND as previously described (Griffith and Jost, 1976).

2.4. Erosion of BrC16-T/DSPE-PEG_{2000} lipocores in rat plasma

The BrC16-T/DSPE-PEG_{2000} (85:15 mol/mol) lipocore suspension (10.3 mg/ml BrC16-T in saline) was added to freshly obtained plasma from female Sprague–Dawley rats to give a concentration of 250 μg BrC16-T/ml plasma. The plasma was then split into 0.6 ml aliquots, 3 per time point, which were incubated at 37°C for 0, 4, 24, or 72 h. After incubation, the samples were centrifuged at 50 000 × *g* for 30 min. A 0.2 ml aliquot of the resulting supernatant was removed and frozen (−80°C). The remaining material was resuspended by vortexing and transferred to a separate tube. A 0.2 ml aliquot of saline was used to

wash residual material from the centrifuge tube which was added to the tube containing the resuspended fraction. This was also frozen and stored at −80°C until HPLC analysis was performed. The extraction and HPLC procedures were a modification of those developed by Dr George Thomas (personal communication). All plasma fractions were thawed and 0.2 ml was taken and mixed 1:1 with acetonitrile containing 250 μg/ml C12-T as an internal standard. An additional 0.6 ml of acetonitrile was then added to give a final plasma:acetonitrile ratio of 1:4, v/v. The plasma/acetonitrile mixtures were held for 15 min on ice and then centrifuged at 16 000 × *g* for 10 min. The supernatants were collected and analyzed using a Waters HPLC system (Waters 600 Multi Solvent Delivery System, Waters 712 WISP Autosampler) from Waters Corporation (Milford, MA) with a Spectroflow 757 UV detector (227 nm) from ABI Applied Biosystems (Ramsey, NJ). The column used was an Inertsil ODS2 (150 × 4.6 mm, particle size 5 μm) from Keystone Scientific (Bellefonte, PA). A solvent gradient of water/acetonitrile (3:1 v/v) and acetonitrile was used with a flow rate of 2 ml/min. The efficiency of extraction was approximately 90%.

2.5. In vivo toxicity studies

BrC16-T/DSPE-PEG_{2000} (85:15) and Taxol® toxicities were evaluated both i.v. (tail vein) and intraperitoneally (i.p.) in 5 day dosing studies (one administration per day for 5 days) in CDF1 female mice (6–8 weeks old, 5–20 mice per group). The daily doses of BrC16-T and paclitaxel are indicated in Tables 2 and 3. Drug suspensions were diluted in PBS (Dulbecco's® phosphate buffered saline 1 ×), which served as vehicle control (dose volume 15 ml/kg i.v., 25 ml/kg i.p.). Mortality was recorded daily and body weights taken at least twice weekly for a period of at least 30 days.

2.6. In vivo efficacy studies

For in vivo efficacy, an ovarian cancer animal model was used where CB-17 SCID female mice (6–7 weeks old, 6–10 per group) were inoculated i.p. on day 0 with 5×10^6 human ovarian cancer (OvCar3) cells (obtained from DCT Tumor Repository NCI Cancer Research Facility, Frederick, MD) and treated with either BrC16-T/DSPE-PEG_{2000} (85:15) or Taxol®. Treatment was given either i.p. in a delayed treatment study where dosing was on days 20, 22, 24, 26 and 28 or given i.v. in an early treatment study where dosing was on days 1, 3, 5, 7, and 9. The samples were diluted in PBS, which served as vehicle control (dose volume 15 ml/kg i.v. or 25 ml/kg i.p.). Survival was monitored over a period of 300 days.

The efficacy of the BrC16-T/DSPE-PEH_{2000} formulation against s.c. OvCar3 human ovarian cancer was evaluated as follows: CB-17 SCID female mice (6–7 weeks old, 5 per group) were inoculated (at day 0) with 2×10^6 OvCar3 cells s.c. Mice were administered i.v. with either BrC16-T/DSPE-PEG_{2000} (85:15) lipocores, Taxol®, or PBS (control) on days 1, 3, 5, 7 and 9. Tumor measurements were recorded twice weekly and mortality was recorded daily until the mean tumor volume of each group exceeded 1500 mm^3. Tumor volume was calculated from measurements of width and length as: tumor volume = $(\text{width}/2)^2 \times \text{length} \times \pi$.

3. Results and discussion

An amphipathic carrier (e.g. micelle, emulsion, liposome) is usually required for the dispersal of poorly water soluble drugs in aqueous solution for i.v. administration. Lipid and co-polymer micro- and nano-assemblies have allowed poorly water soluble drugs to be dispersed as particulates for intravenous use. Not always optimal, many systems have been improved by reformulation from one type carrier system to another. Such has been the case for the antifungal drug amphotericin B, whose toxicity has been reduced significantly by reformulation from deoxycholate micelles (Fungizone®) into lipid based complexes (Abelcet® and Amphotec®) and liposomes (Ambisome®) (Janoff et al., 1988; Guo et al., 1991; Singh and Perdue, 1998).

If the compound also has a low or limited affinity for lipid assemblies (low lipophilicity), the

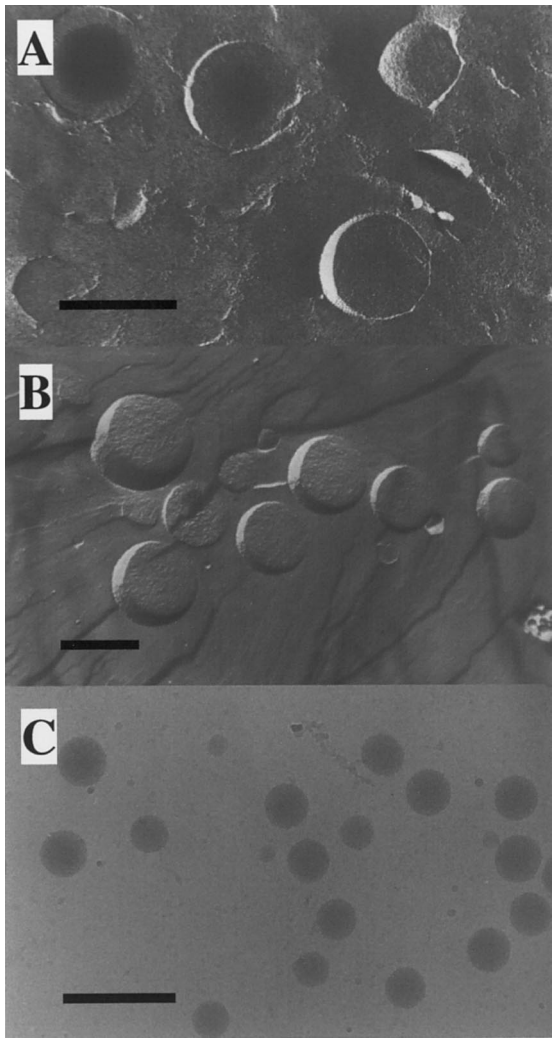


Fig. 2. Electron micrographs of lipocore particles. Images were obtained by freeze-fracture electron microscopy (EM) (A and B) and by electron cryo-microscopy techniques (C). For A and B, particles were made by solvent removal using either (A) C16-Vinblastine/distearoyl-phosphatidylethanolamine (DSPE)-polyethyleneglycol $(PEG)_{2000}$ mixed at 60:40 mol ratio or (B) 2′-2-bromohexadecanoyl paclitaxel/dioleoylphosphatidylcholine (DOPC)/dioleoyl-phosphatidylethanolamine (DOPE)-PEG_{2000} mixed at 20:30:50 mol ratio; the mol% BrC16-T in the resulting particles was found to be $\sim$94% when isolated on a sucrose density gradient. (C) Particles were made by ethanol injection using BrC16-T/DSPE-PEG_{2000} mixed at a 85:15 mol ratio. Bars represent 500 nm (A,B) and 200 nm (C).

design of suitable carrier is even more difficult. In order to increase the lipophilicity of two such agents, paclitaxel and vinblastine, they were modified by covalently attaching acyl chains (Fig. 1). In the case of the paclitaxel derivatives, a prodrug with a cleavable acyl chain attached at the 2′ position of paclitaxel was designed; a Br atom at the α-carbon was included to expedite hydrolysis (Ali et al., 1996; Mayhew et al., 1997).

3.1. Particle formation

Using solvent removal or ethanol injection methods, the C16 derivatives of paclitaxel (BrC16-T) and vinblastine (C16-Vin) were combined with lipids having a PEG headgroup to produce stable spherical particles as observed by light microscopy (data not shown) and electron microscopy (see Fig. 2). When the underivatized compounds were combined with the PEG-lipid only crystals and globular aggregates were observed. Stable spherical particles could also be formed when the paclitaxel derivative BrC16-T was combined from solvent with DOPE-PEG_{1000} or DOPE-PEG_{5000} or with PEG_{2000}- or PEG_{5000}-modified lipids of various chain compositions (DMPE-, DPPE-, and DSPE-). Although dioleoylphosphatidylcholine (DOPC) could be incorporated along with DOPE-PEG_{2000} and BrC16-T to form particles, combination of BrC16-T with DOPC alone resulted in the formation of large irregularly shaped globular aggregates, indicating that the polymer headgroup is essential to the maintenance of stable particle structure.

3.2. Particle characterization

As shown in Fig. 2, the resulting BrC16-T and C16-Vin particles yielded only cross-fractures by freeze-fracture EM. Images obtained by electron cryo-microscopy indicated that the particles were comprised of a continuous material with no distinguishable lamellae (Fig. 2C). Two populations could be resolved by sucrose gradient analysis: one contained primarily lipid and the other contained primarily BrC16-T, respectively (see Fig. 3). Upon examination of the two separated populations by light microscopy it was found that the spherical particles occupied the higher density population that settled to the bottom of the gradi-

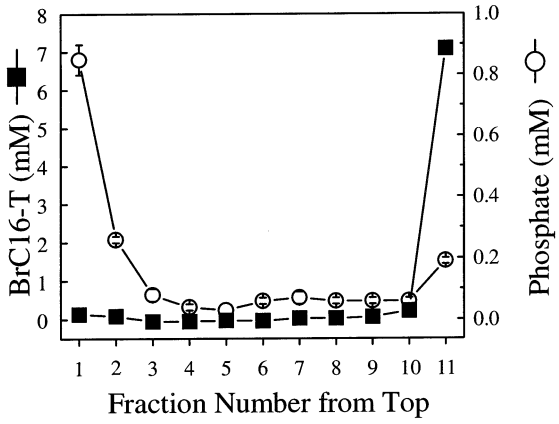


Fig. 3. Sucrose density gradient of 2′-2-bromohexadecanoyl paclitaxel (BrC16-T)/distearoyl-phosphatidylethanolamine (DSPE)-polyethyleneglycol $(PEG)_{2000}$ mixture (85:15 mol ratio). Fractions were taken from the top and represent lower to higher density. The percents of the total BrC16-T (■) and phospholipid (○) are indicated.

ent; the top band was clear and most likely micellar. Fraction 11 contained 95% of the total BrC16-T and the drug to lipid ratio in that fraction was 97:3. In subsequent gradient studies, the high density band appeared to be isodense with ~55–60% sucrose (w/v) and the typical drug/lipid molar ratio in the high density population was found to be ~98:2. Interestingly, stable particles could not be directly made at this mole ratio. To avoid aggregate formation the drug/lipid ratio needed was found to be at or below 90:10 even though the isolated particles contained a higher drug/lipid ratio.

Considering their morphology and the very high drug/lipid ratio, it is believed these structures to be cores of the derivatives surrounded by an outer coating of PEG-lipid. For this scenario, little or no internalized water would be expected to be present. To confirm the absence of an entrapped aqueous phase, the distribution of an aqueous ESR spin probe used previously to establish captured volumes in liposomes was measured (Perkins et al., 1993). Whether the probe was added to the ethanol solution prior to particle formation or added afterward to the already formed particles, the results were the same — no measurable aqueous captured volume. From measurement of the signal of incorporated probe molecules by fluorescence anisotropy and ESR techniques, motions within the particles were found to be highly restricted (Table 1). Examination of the particles by X-ray diffraction revealed no crystal formation. Taken together, these data suggest that the interiors consist of amorphous drug solid. Additionally, by modeling the particles as monolayer coated spheres with an area per lipid of 2–5 nm^2 (Torchilin, 1996), a monolayer thickness of 4–5 nm and a BrC16-T core density of ~1.2 g/ml (estimated from sucrose gradient profiles), reasonable agreement was obtained between the mol% BrC16-T and the observed particle size distributions that were measured by laser light scattering. Presumably during sample injection or solvent removal the PEG-lipid orients itself at the aqueous phase boundary. Perhaps the polymer provides an interfacial steric barrier to particle coalescence which would explain why ag-

Table 1
Fluorescence anisotropy and electron spin resonance (ESR) order parameter measurements

	Fluorescence anisotropy[a]		Order parameter[b]	
	15°C	37°C	15°C	37°C
BrC16-T/DSPE-PEG_{2000} (lipocores)	0.30	0.30	>0.9	>0.9
DSPE-PEG_{2000} (micelles)	0.27	0.12	0.24	0.09
DMPC (liposomes)	0.32	0.09	0.63	0.12
Octadecane/DSPE-PEG_{2000} (emulsion)	0.27	0.04	0.25	0.11

[a] Fluorescence anisotropy was measured using diphenylhexatriene (DPH).
[b] ESR order parameters were measured using 10-doxyl-nonadecane (10DND). For reference, the values for probes incorporated into various other particles are included. See Section 2 for formation procedures.

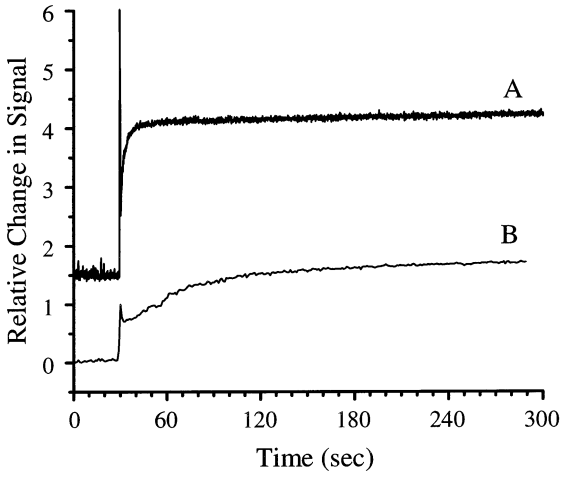


Fig. 4. Change in solution turbidity (A) or fluorescence intensity (B) upon injection of 2′-2-bromohexadecanoyl paclitaxel (BrC16-T)/distearoyl-phosphatidylethanolamine (DSPE)-polyethyleneglycol $(PEG)_{2000}$ ethanol mixture (85:15 mol ratio) into HBS (pH 7.4, 60°C). The arrow denotes the time of injection. For fluorescence, λ_{ex} and λ_{em} were 230 and 325 nm, respectively.

gregates arose when DOPC alone was combined with BrC16-T. The hydrophobic derivatives then form the core of the resulting particles.

3.3. Formation mechanism

3.3.1. Kinetics of formation

To assess the kinetics of lipocore formation, 10 μl of ethanol containing 0.2 mg of BrC16-T and 0.083 mg DSPE-PEG_{2000} was injected into a cuvette containing 2 ml of HBS which was being stirred. Turbidity changes were measured by monitoring the absorbance at 500 nm. In a separate experiment, changes in the inherent fluorescence of BrC16-T as a function of time were also measured. From the time course of the formation process monitored by turbidity and fluorescence measurements, it appeared that particle formation proceeded quite rapidly (see Fig. 4). The change in turbidity was nearly complete within 10 s from the time of ethanol injection into the buffered saline. The increase in fluorescence intensity of BrC16-T reflects exclusion of the solvents ethanol and water from the environment of the fluorophore. The difference in time course between the turbidity and fluorescence profiles may simply have reflected different aspects of the process. That is, ethanol from the micro-droplets entering solution should diffuse away rapidly thus leaving the insoluble hydrophobic drug to self associate. The rapid change in turbidity would correspond to the initial particle formation phase. The fluorescence change then could simply have reflected the slower condensation event of BrC16-T, as ethanol diffused into the bulk solution, to form an essentially solid core. In this proposed scheme, the amphipathic lipid would have oriented at the interface with its hydrophilic portion extended toward the aqueous and thus provided the surface coating. This proposed process leads to the trapping of drug within each particle. By adjusting injection rate and mixing (solution flow) rate the ultimate particle size could be controlled, a likely consequence of controlling the micro-droplet size entering solution.

3.3.2. Role of lipid during particle formation

Because replacing the PEG-lipid with DOPC resulted in the production of aggregates, it is believed the steric barrier provided by the PEG polymer coating was essential to preventing particle growth/aggregation (Needham et al., 1992). Since electrostatic repulsion should work equally well to prevent coalescence, we tried making particles with BrC16-T and the doubly negatively charged lipid *N*-glutaryl-dioleoyl-phosphatidyl-ethanolamine (DOPE-GA). Because the cross-sectional area per lipid is significantly lower than that for PEG-lipids, the molar ratio was increased to 1:1. Injection of an ethanol solution of BrC16-T and DOPE-GA (48 mg/33 mg) into HBS resulted in a suspension of stable particles that exhibited a Gaussian size distribution with a mean diameter of 106 nm as measured by laser light scattering (intensity weighting). The formation of these particles confirmed the belief that the surface lipid is essential to stability once the particles form. However, for i.v. delivery, highly charged carrier systems such as these may experience strong complement opsonization which shortens circulation lifetime (Ahl et al., 1997). Work was therefore continued with just the PEG-lipid systems.

3.3.3. Acyl chain length dependence of pro-drugs upon particle formation

Because BrC16-T and C16-Vin, but not their underivatized parents, could form particles with PEG-lipids, the extent to which the length of attached acyl chain influenced particle formation was examined. Several paclitaxel derivatives were combined with PEG-lipid as per the ethanol injection procedure. The resulting suspensions were assessed by light microscopy (Table 2). For BrC16-T and BrC14-T mixtures with PEG-lipid, no crystals were observed but small particle formation was confirmed by laser light scattering. For BrC12-T an occasional aggregate was noted by light microscopy as well as some large spherical particles; small particles were again confirmed by laser light scattering. For BrC8-T, and BrC6-T, crystals were apparent alongside particles with the greatest crystal content for the BrC6-T formulation. As stated above for underivatized paclitaxel, combination with even excess lipid resulted in only crystals.

The preference for paclitaxel derivatives of > 12C acyl chain length to form particles over crystals may reflect an inherent increased stability that their resulting particles have as compared to those made with shorter chain derivatives; perhaps this involves a chain length dependent interaction with the acyl chains of the PEG-lipid. Alternatively, the avoidance of crystals may simply reflect the lower aqueous solubility of the BrC14-, BrC16-T compounds as compared to the derivatives with shorter chains. That is, when solvent and aqueous phases are mixed some portion of the shorter chain paclitaxel derivatives may diffuse along with solvent into the bulk aqueous phase and it is from this material that crystals are seeded. This latter solubility explanation might also explain why C16-Vin but not vinblastine formed stable particles.

3.4. Formation of particles with hamycin

Because vinblastine is similarly bulky in structure and may have similar solubility properties to paclitaxel, it was not known if lipocore formation with C16 derivatives of these compounds was coincidental and thus specific to just these derivatized compounds. Therefore, two poorly water soluble polyene antibiotics, amphotericin B and hamycin, were examined to see whether they also could form lipocores. Hamycin is a heptaene macrolide with a poly-hydroxyl side like amphotericin but is less soluble in some solvents (Zameer et al., 1996). Interestingly, mixtures of DSPE-PEG_{2000} with hamycin but not amphotericin B resulted in stable spherical particles; amphotericin B/PEG-lipid yielded aggregates. The reason for this difference is unclear but it may involve differences in aqueous solubilities, self interaction within the core, or interaction of drug with the PEG-lipid.

Table 2
Acyl chain length dependence of particle formation[a]

Agent	-PEG_{2000} lipid	Drug/lipid	Particles	Aggregates	Crystals
BrC6-	DOPE-	80:20	+		+++
BrC8-	DOPE-	80:20	+	−	++
BrC12-	DOPE-	80:20	++	+	−
BrC14-	DOPE-	80:20	+++	−	−
BrC16-	DOPE-	80:20	+++	−	−
BrC16-	DSPE-	90:10	+++	−	−
Paclitaxel	DSPE-	20:80	−	−	+++

[a] Derivatives and paclitaxel not incorporated in the particles formed crystal structures whose presence was monitored by light microscopy. Particles were made using the ethanol injection technique (see Section 2) using the indicated molar ratios of drug to lipid. For BrC14-T and BrC16-T, which were crystal and aggregate-free, a few large spherical particles could be seen; the majority of the particles were too small to be resolved — a small size distribution was confirmed by quasi-elastic light scattering.

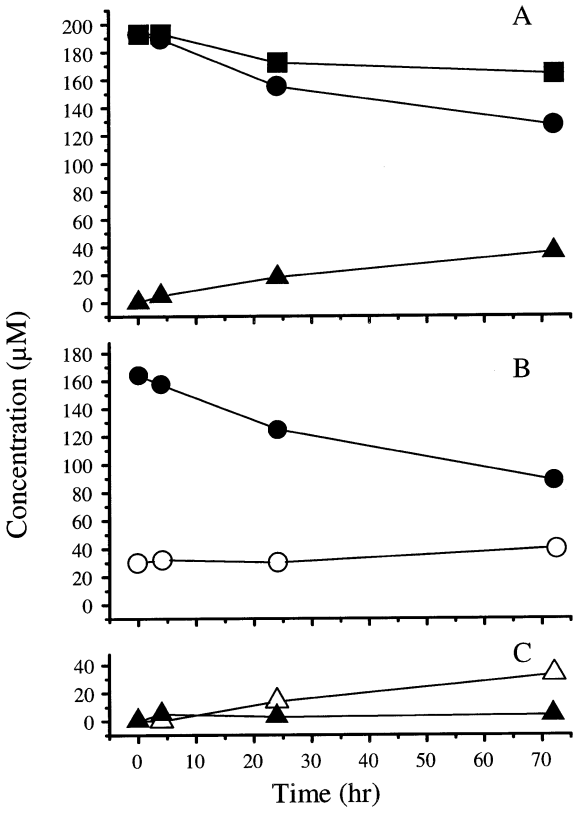


Fig. 5. Conversion in rat plasma (37°C) of 2′-2-bromohexadecanoyl-paclitaxel (BrC16-T) to paclitaxel for particles made with distearoyl-phosphatidylethanolamine (DSPE)-polyethyleneglycol $(PEG)_{2000}$ (85:15 mol/mol). Particles were made by the ethanol injection method and had number weighted mean diameters of 34–54 nm. (A) Total BrC16-T (●), paclitaxel (▲), and BrC16-T + paclitaxel (■) concentrations. (B) BrC16-T concentrations in pelleted fraction (●) and in supernatant (○). (C) Paclitaxel concentrations in pelleted fraction (▲) and in supernatant (△).

3.5. Particle stability

3.5.1. Storage stability

The utility of lipocores as therapeutic delivery vehicles was next examined using BrC16-T. First the stability of BrC16-T particles made with ≥ 85 mol% drug was examined combined with various PEG-lipids (different chain compositions with either -PEG_{2000} or -PEG_{5000} headgroup and having the following chain compositions: dioleoyl-, dimyristoyl-, dipalmitoyl-, and distearoyl). The most stable formulation appeared to be that with DSPE-PEG_{2000} in which crystals were noted only after 2 months of storage at room temperature. When maintained at 4°C, this DSPE-PEG_{2000} formulation was found to be structurally stable for over 1 year with $< 3\%$ hydrolysis of BrC16-T to paclitaxel.

3.5.2. Particle erosion/disassembly in plasma

Hydrolytic cleavage of the acyl chain from the BrC16-T prodrug is believed to be important to its activity (Ali et al., 1996). To correlate this process with particle erosion/disassembly a centrifugation assay was employed to examine BrC16-T/DSPE-PEG_{2000} samples that had been incubated in rat plasma at 37°C for various time intervals (see Fig. 5). Following incubation, samples were centrifuged at $50\,000 \times g$ to sediment intact lipocore particles. The content of BrC16-T and paclitaxel distributed between the pelleted fraction and the supernatant allowed one to quantitate both particle erosion (pellet/supernatant distribution) and conversion (BrC16-T/paclitaxel ratio). Shown in Fig. 5A are the total amounts of paclitaxel and BrC16-T in the entire sample. After 72 h, there was about a 30% loss of BrC16-T, of which half was paclitaxel and the rest was unidentified metabolites. The distribution of BrC16-T between the pelleted fraction (intact lipocore particles) and the supernatant is shown in Fig. 5B. After 72 h, nearly half of the BrC16-T was no longer retrievable in the form of intact particles. Interestingly, the amount of BrC16-T found in the supernatant remained relatively constant. For paclitaxel, the supernatant fraction accounted for essentially all that was present — no appreciable amount was found in the pelleted fraction (Fig. 5C). This supports the concept that hydrolysis of the prodrug occurs outside of the lipocore particle. Using a slightly different rat plasma assay without centrifugation, it was found that for BrC16-T particles made using other -PEG lipids, there was about 25–35% conversion after 72 h for various -PEG_{2000} and -PEG_{5000} lipids (DMPE-, DPPE-, DSPE-, DOPE-) (data not shown).

3.6. Animal studies

3.6.1. In vivo toxicity

The acute toxicity of BrC16-T/DSPE-PEG_{2000} (85:15) particles in vivo was next evaluated from survival of CDF1 mice following injection of these particles either intraperitoneally or intravenously (Table 3). (For all animal testing particles were produced by the ethanol injection method and had mean diameters between 50 and 100 nm.) In both cases, mice were dosed daily for 5 days. For comparison, mice were also injected with Taxol® at the same schedule. For both routes of administration, BrC16-T/DSPE-PEG_{2000} particles were less acutely toxic than Taxol®. The estimated maximum tolerated dose (MTD) values for i.p. and i.v. administration of BrC16-T were approximately ten times the values for paclitaxel (Taxol®).

Table 3
Multiple dose toxicity of BrC16-T/distearoyl-phosphatidyl-ethanolamine (DSPE)-polyethyleneglycol ($PEG)_{2000}$ particles versus Taxol® administered intraperitoneally or intravenously to CDF1 mice

Agent	MTD[a] (mg/kg)	
	i.p.	i.v.
BrC16-T	~200	~200
Taxol®	12.5–25	18.75–25

[a] Maximum tolerated dose where the numbers shown represent the daily dose in mg/kg; mice were injected daily for 5 days. Survival was monitored for 30 days. The cumulative intraperitoneally (i.p.) and intravenously (i.v.) doses corresponding to the maximum tolerated dose (MTD) concentrations were 1000 mg/kg for BrC16-T and 63–125 and 94–125 mg/kg for paclitaxel, respectively. The BrC16-T/DSPE-PEG_{2000} ratio was 85:15 mol/mol.

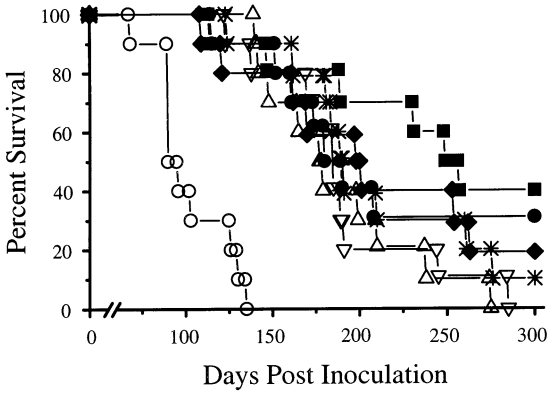


Fig. 6. Intraperitoneal treatment of CB-17 SCID female mice (6–7 weeks old, 10 per group) that were inoculated intraperitoneally (i.p.) on day 0 with 5×10^6 human ovarian cancer (OvCar3) cells. Mice were treated i.p. at days 20, 22, 24, 26 and 28 with either BrC16-T/DSPE-PEG_{2000} (85:15) or Taxol®. For BrC16-T/DSPE-PEG_{2000}, the BrC16-T daily doses were 12.5 mg/kg (*), 25 mg/kg (◆), 50 mg/kg (●), and 100 mg/kg (■). For Taxol®, the paclitaxel daily doses were 12.5 mg/kg (△) and 25 mg/kg (▽). As controls, one group of mice were injected with PBS alone (○).

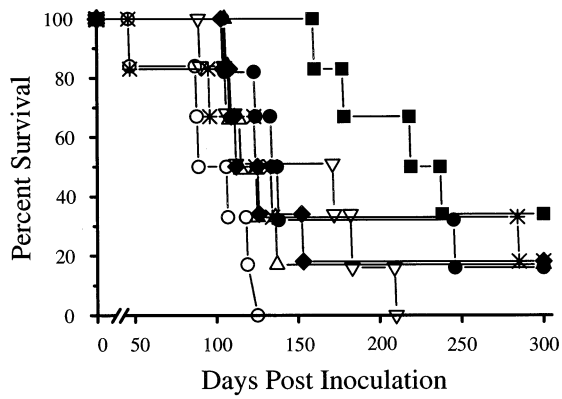


Fig. 7. Intravenous treatment of CB-17 SCID female mice (6–7 weeks old, 6 per group) that were inoculated intraperitoneally (i.p.) on day 0 with 5×10^6 human ovarian cancer (OvCar3) cells. Mice were treated i.v. at days 1, 3, 5, 7 and 9 with either BrC16-T/DSPE-PEG_{2000} (85:15) or Taxol®. For BrC16-T/DSPE-PEG_{2000}, the BrC16-T daily doses were 12.5 mg/kg (*), 25 mg/kg (◆), 50 mg/kg (●), and 100 mg/kg (■). For Taxol®, the paclitaxel daily doses were 12.5 mg/kg (△) and 25 mg/kg (▽). As controls, one group of mice were injected with PBS alone (○).

3.6.2. In vivo efficacy

The BrC16-T/DSPE-PEG_{2000} formulation was at least as active as Taxol® in human ovarian cancer grown in SCID mice by the i.p. (Fig. 6) or i.v. (Figs. 7 and 8) routes. At equivalent doses BrC16-T and paclitaxel demonstrated similar activities. However Fig. 6 shows that the highest dose of 100 mg/kg BrC16-T used, 40% of the animals were still alive after day 300 while all of the mice treated with Taxol® at its highest dose of 25 mg/kg paclitaxel were dead. All BrC16-T treatments produced some long-term survivors (> 300 days) in a dose related fashion.

Using the same i.p. model of ovarian cancer, mice were treated intravenously with either BrC16-T particles or Taxol® (Fig. 7). Again at equivalent doses

of the BrC16-T the activity was similar to that of Taxol®, and again there were long term survivors at all doses of BrC16-T versus Taxol®, which showed none.

The activities of the two formulations against OvCar3 cells grown s.c. in SCID mice was examined next (Fig. 8). Treatment was given i.v. and the volumes of the tumors determined over time. While tumor growth in mice treated with both Taxol® and BrC16-T lipocores at 25 mg/kg was delayed to the same extent ($\sim$ 100 days to a tumor volume of 2000 mm^3), the tumors in animals treated with Taxol® exhibited an initial regression in size. This difference may reflect the fact that fewer paclitaxel equivalents were given since dosing was made based upon weight. That is, because the molecular weights for paclitaxel and BrC16-T are 853 and 1169, respectively, the equivalent paclitaxel dose of BrC16-T was 18 mg/kg as compared to 25 mg/kg for Taxol®. For the mice

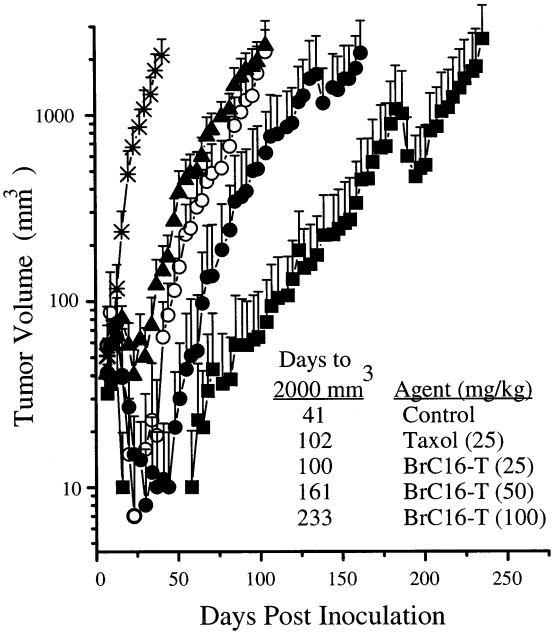


Fig. 8. Intravenous treatment of subcutaneous human ovarian cancer (OvCar3) tumors. CB-17 SCID female mice (6–7 weeks old, 5 per group) were inoculated subcutaneously (s.c.) on day 0 with 2×10^6 OvCar3 cells. Mice were treated intravenously (i.v.) at days 1, 3, 5, 7 and 9 with either BrC16-T/DSPE-PEG_{2000} (85:15) or Taxol®. For BrC16-T/DSPE-PEG_{2000}, the BrC16-T daily doses were 25 mg/kg (▲), 50 mg/kg (●), and 100 mg/kg (■). For Taxol®, the paclitaxel daily dose was 25 mg/kg (○). As controls, one group of mice was injected with PBS alone (*).

treated with BrC16-T particles at 50 and 100 mg/kg, tumor volume initially regressed to near the limit of detection (< 10 mm^3) by day 20 and remained undetectable until days 40 and 60, respectively.

It must be pointed out that the dose scheduling for BrC16-T and Taxol® has not been optimized pending pharmacokinetic studies. However, higher doses of BrC16-T formulated in the particles (beyond the maximum tolerated dose for paclitaxel) produced an increased response as compared to paclitaxel at its highest dose. Although a slow conversion of BrC16-T to paclitaxel and an altered distribution may account for the lower toxicity, the mechanistic details of drug distribution and conversion in vivo remain to be determined.

4. Conclusion

Particles with a high drug:lipid ratio can be formed when the acyl chain derivatives of poorly water soluble compounds, such as paclitaxel or vinblastine, are combined with PEG-lipids or with the negatively charged lipid DOPE-GA. Electron cryo-microscopy and freeze-fracture EM results indicated that the particles appeared to be solid spheres. The low lipid/drug ratio, lack of an aqueous captured volume, and the extent of probe immobilization suggested that these particles are a core of amorphous derivatized molecules surrounded by a lipid coating, unlike an oil-in-water emulsion, which is a liquid within a liquid. Presumably the hydrophilic polymer of the PEG-lipid provides interfacial stability as well as steric protection from particle self association. Because of the high drug-to-lipid ratio these two-component particles are very efficient delivery systems. BrC16-T formulated in these particles was well tolerated and exhibited activity against a human ovarian tumor implanted in SCID mice. The stability and usefulness of the derivatized paclitaxel lipocore system indicates that formulation into lipocores may be useful for a wide variety of compounds and their derivatives.

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References

Ahl, P.L., Bhatia, S.K., Meers, P., Roberts, P., Stevens, R., Dause, R., Perkins, W.R., Janoff, A.S., 1997. Enhancement of the in vivo circulation lifetime of L-α-distearoylphosphatidylcholine liposomes: importance of liposomal aggregation versus complement opsonization. Biochim. Biophys. Acta 1329, 370–382.

Ahmad, I., Ali, S., Dank, E., Filep, J., Franklin, C., Janoff, A.S., Minchey, S., Peters, A., Prior, L., Mayhew, E., 1996. Preclinical anticancer effects of 2′,α-bromo-hexanoyl paclitaxel (2′, Br-HTD) liposomes. Proc. Am. Assoc. Can. Res. 37, 1229a.

Ali, S., Mayhew, E., Peters, A., Minchey, S., Janoff, A., 1996. Hydrolysable hydrophobic taxol derivatives. Biophys. J. 70, A119.

Ali, S., Mayhew, E., Minchey, S., Janoff, A., 1997. Calorimetric investigation of binary mixtures of bromoacyl taxanes and phosphocholines. Biophys. J. 72, A74.

Balasubramanian, S.V., Straubinger, R.M., 1994. Taxol-lipid interactions: taxol-dependent effects on the physical properties of model membranes. Biochemistry 33, 8941–8947.

Chen, P.S., Toribara, T.Y., Warner, H., 1956. Microdetermination of phosphorus. Anal. Chem. 28, 1756–1758.

Cohen, S., Bernstein, H. (Eds.), 1996. Microparticulate Systems for the Delivery of Proteins and Vaccines, Marcel Dekker, New York.

Griffith, O.H., Jost, P.C., 1976. Lipid spin labels in biological membranes. In: Berliner, L.J. (Ed.), Spin Labeling Theory and Applications. Academic Press, New York, pp. 453–523.

Guo, L.S.S., Fielding, R.M., Lasic, D.D., Hamilton, R.L., Mufson, D., 1991. Novel antifungal drug delivery: stable amphotericin B-cholesteryl sulfate disks. Int. J. Pharm. 75, 45–54.

Iyer, L., Ratain, M.J., 1998. Clinical pharmacology of camptothecins. Cancer Chemother. Pharmacol. 42, S31–S43.

Janoff, A.S. (Ed.), 1999. Liposomes: Rational Design, Marcel Dekker, New York.

Janoff, A.S., Boni, L.T., Popescu, M.C., Minchey, S.R., Cullis, P.R., Madden, T.D., Taraschi, T., Gruner, S.M., Shyamsunder, E., Tate, M.W., Mendelsohn, R., Bonner, D., 1988. Unusual lipid structures selectively reduce the toxicity of amphotericin B. Proc. Natl. Acad. Sci. USA 85, 6122–6126.

Kwon, G.S., 1998. Diblock copolymer nanoparticles for drug delivery. Crit. Rev. Ther. Drug Carrier Syst. 15, 481–512.

Lakowicz, J.R., 1983. Principles of Fluorescence Spectroscopy. Plenum Press, New York.

Li, C., Yu, D., Inoue, T., Yang, D.J., Milas, L., Hunter, N.R., Kim, E.E., Wallace, S., 1996. Synthesis and evaluation of water-soluble polyethylene glycol-paclitaxel conjugate as a paclitaxel prodrug. Anti-Cancer Drugs 7, 642–648.

Li, X., Hirsh, D.J., Cabral-Lilly, D., Zirkel, A., Gruner, S.M., Janoff, A.S., Perkins, W.R., 1998. Doxorubicin physical state in solution and inside liposomes loaded via a pH gradient. Biochim. Biophys. Acta 1415, 23–40.

Mayhew, E., Franklin, J.C., Bhatia, S., Harmon, P.A., Janoff, A.S., 1996. Hydrophobic taxane derivatives. US Patent 5,580,899, 3 Dec.

Mayhew, E., Ali, S., Janoff, A.S., 1997. Hydrolysis-promoting hydrophobic taxane derivatives. US Patent 5,703,117.

Needham, D., Sarpal, R.S., 1998. Binding of taxol to lipid interfaces: correlations with interface compliance. J. Liposome Res. 8, 147–164.

Needham, D., McIntosh, T.J., Lasic, D.D., 1992. Repulsive interactions and mechanical stability of polymer-grafted lipid membranes. Biochim. Biophys. Acta 1108, 40–48.

Paradis, R., Page, M., 1998. New active paclitaxel amino acids derivatives with improved water solubility. Anticancer Res. 18, 2711–2716.

Perkins, W.R., Minchey, S.R., Boni, L.T., Swenson, C.E., Popescu, M.C., Pasternack, R.F., Janoff, A.S., 1992. Amphotericin B-phospholipid interactions responsible for reduced mammalian cell toxicity. Biochim. Biophys. Acta 1107, 217–282.

Perkins, W.R., Minchey, S.R., Ahl, P.L., Janoff, A.S., 1993. The determination of liposome captured volume. Chem. Phys. Lipids 64, 197–217.

Sharma, A., Mayhew, E., Straubinger, R.M., 1993. Antitumor effect of taxol-containing liposomes in a taxol-resistant murine tumor model. Cancer Res. 53, 5877–5881.

Sharma, A., Mayhew, E., Bolcsak, L., Cavanaugh, C., Harmon, P., Janoff, A., Bernacki, R.J., 1997. Activity of paclitaxel liposome formulations against human ovarian tumor xenografts. Int. J. Cancer 71, 103–107.

Singh, R.M., Perdue, B.E., 1998. Amphotericin B: a class review. Formulary 33, 424–447.

Storm, G., Crommelin, D.J.A., 1998. Liposomes: quo vadis? Pharm. Sci. Technol. Today 1, 19–31.

Straubinger, R.M., 1995. Biopharmaceutics of paclitaxel (Taxol): formulation, activity, and pharmacokinetics. In: Suffness, M. (Ed.), Taxol® Science and Applications. CRC Press, New York, pp. 237–258.

Torchilin, V., 1996. Effect of polymers attached to lipid headgroups on properties of liposomes. In: Lasic, D.D., Barenholz, Y. (Eds.), Handbook of Nonmedical Applications of Liposomes, vol. 1. CRC Press, New York, pp. 263–284.

Uchegbu, I.F., Vyas, S.P., 1998. Non-ionic surfactant based vesicles (niosomes) in drug delivery. Int. J. Pharm. 172, 33–70.

Vyas, D.M., Ueda, Y., Wong, H., Matiskella, J.D., Hauck, S., Mikkilineni, A.B., Farina, V., Rose, W.C., Casazza, A.M., 1995. Phosphatase-activated prodrugs of paclitaxel. In: Georg, G.I., Chen, T.T., Ojima, I., Vyas, D.M. (Eds.), Taxane Anticancer Agents Basic Science and Current Status. ACS Symposium Series 583, USA, pp. 124–137.

Zameer, S., Hideki, E., Keijiro, T., Tadayoshi, Y., Hirofumi, O., 1996. Aggregation of polyene antibiotics as studied by electronic absorption and circular dichroism spectroscopies. Colloids Surf. 7, 31–38.