J. Pharm. Pharmacol. 1997, 49: 16–21
Received March 21, 1996
Accepted April 19, 1996

A Submicron Lipid Emulsion Coated with Amphipathic Polyethylene Glycol for Parenteral Administration of Paclitaxel (Taxol)

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Abstract

Paclitaxel is a promising anticancer agent with poor solubility in water and requires a suitable formulation for intravenous administration. Presently paclitaxel is formulated for clinical use in ethanol and Cremophor EL (Diluent 12), a solvent system associated with severe adverse effects. In this study paclitaxel was entrapped in lipid emulsion droplets with triolein as oil core and dipalmitoyl phosphatidylcholine as the principal emulsifier. The emulsion was further stabilized with polysorbate 80 and polyethylene glycol-dipalmitoyl phosphatidylethanolamine.

The drug-emulsion droplets (diameter about 40 nm) were physically and chemically stable during several months at 4°C. Lyophilized preparations in 5% glucose were completely restored by distilled water. Studies of the integrity of the drug-emulsion showed a release of the drug from emulsion globules and surface transfer was found to be the major mechanism for cellular uptake. The in-vitro antiproliferative activity of paclitaxel against T-47D cells was retained by the drug-emulsion with an ID50 value of 7 nM compared to 10 and 35 nM for paclitaxel in liposomes and Diluent 12, respectively.

Long-circulating submicron lipid emulsions may prove useful, not only for replacement of the more toxic Cremophor EL vehicle, but also by improving the distribution of the drug to the tumour.

Paclitaxel is a diterpenoid originally isolated from the Western yew (*Taxus brevifolia*) and has shown promising antineoplastic activity particularly against drug-refractory ovarian (Runowicz et al 1993) and breast cancer (Holmes et al 1991). Paclitaxel represents a new class of anticancer agents and functions by promoting the assembly and stability of microtubules thereby causing mitotic arrest (Schiff & Horwitz 1980). Since paclitaxel is poorly soluble in aqueous media and is orally inactive, it requires a suitable formulation for intravenous administration. Presently, the vehicle used clinically is Cremophor EL (polyethoxylated castor oil) containing 50% ethanol, designated Diluent 12 by the National Cancer Institute, which is administered as a supersaturated oil-in-water emulsion (Rose 1992). This vehicle has been associated with acute life-threatening anaphylactoid reactions (Weiss et al 1990), is very effective at leaching of plasticizers from PVC infusion bags (Waugh et al 1991) and has been shown to antagonize paclitaxel cytotoxicity in cell survival assays (Liebmann et al 1993). Lately, paclitaxel has been solubilized in a number of vehicles for intravenous administration, including phosphatidylglycerol/phosphatidylcholine liposomes (Sharma & Straubinger 1994), triactin emulsion (Tarr et al 1987) and bile salt/phosphatidylcholine mixed micelles (Alkan-Onyuksel et al 1994).

The purpose of this study was to develop an aqueous vehicle which is better tolerated than Diluent 12. Submicron lipid emulsions have many favourable properties as drug carriers: they are biodegradable, physically stable and easy to produce on a large scale. Coating of the emulsion droplet with a hydrophilic polymer like polyethylene glycol-modified phosphatidylethanolamine (PEG-PE) results in a prolonged circulation lifetime (Lundberg et al 1996). Such long-circulating drug-carriers have been shown to accumulate in tumours and enhance the antitumour activity of the incorporated drug (Mayhew et al 1992). The reformulation of paclitaxel in a long-circulating lipid emulsion could thus improve the therapeutic index, both by decreased toxic effect of the vehicle and increasing the accumulation of drug in the tumour.

Materials and Methods

Materials

Paclitaxel, triacetin, tributyrin, tricaproin, tricaprylin, tricaprin and Cremophor EL were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanolamine were from Avanti Polar-Lipids Inc. (Birmingham, AL, USA). Triolein (TO) and cholesteryloleate were obtained from Nu Chek Prep, Inc. (Elysian, MN, USA) and polysorbate 80 from Fluka Chemie AG (Buchs, Switzerland). The radiolabelled compounds glycerol tri[9,10(n)^{3}H]oleate, cholesteryl [^{14}C]oleate and di[1-^{14}C]palmitoyl phosphatidylcholine were supplied by Amersham International plc (Amersham, UK). [^{14}C]Paclitaxel was a generous gift from Bristol-Myers Squibb Company (Wallingford, CT, USA). PEG-PE was synthesized by the reaction of PEG2000 with carbonyldiimidazole, followed by addition of dipalmitoyl phosphatidylethanolamine (Allen et al 1991). PEG-PE was purified by adding a small amount of water to the reaction residue and the resulting micellar solution was dialysed against water using a Spectra/Por 300 000 MWCO dialysis bag (Spectrum Medical Industries, Inc. Houston, TX, USA) and then lyophilized (Maruyama et al 1992). All tissue culture media were from Gibco Biocult (Paisley, UK).

Preparation of drug emulsions

Paclitaxel was incorporated into the submicron lipid emulsion by a method described in details elsewhere (Lundberg 1994). In short, the emulsion components were dispersed from stock solutions into vials, the solvent was evaporated and the samples lyophilized overnight. Thereafter 2 mL of phosphate-buffered saline (PBS) was added, the sample heated to 45°C, vortexed for 20 s and then sonicated (3 × 20 s) with a sonifier equipped with a titanium probe. The same procedures were used for preparation of liposomes. The solubilization capacity of the drug vehicles for paclitaxel was quantitated by adding an excess of [^{14}C]paclitaxel, centrifugation of the sonicated preparations for 10 min at 10 000 *g* and filtration through a 0·4 μm filter. The amount of solubilized paclitaxel, as well as the losses of labelled triolein and dipalmitoyl phosphatidylcholine, were quantitated by liquid scintillation counting.

Characterization of drug-emulsion droplets

The size of the emulsion droplets was measured by quasielastic laser light scattering using a Malvern 4700 submicron particle analyzer (Malvern Instruments, Malvern, UK) and by electron microscopy. Emulsions were stained on formvar-coated grids with 2% phosphotungstic acid and viewed on a Jeol 2000 FX transmission electron microscope.

The integrity of the emulsion droplets was checked by passing samples containing radiolabelled components through a Sephacryl 300 column (30 × 2 cm) eluted with PBS (0·25 mL min^{-1}). The elution profiles were obtained by measuring the radioactivity in the fractions. The release of paclitaxel from the vehicles was studied by the dialysis bag method (Rubinstein et al 1991). The dialysis bag (MW cut-off 50 000) was found to be permeable to paclitaxel.

The colloidal stability of sterile emulsion samples during prolonged storage at 4°C was determined by measurement of droplet size by the laser particle sizer and by quantitation of the recovery of radioactive label after centrifugation for 15 min at 10 000 g.

Cell culture

T-47D breast cancer cells were grown in Dulbecco's modified Eagle's medium. The media were supplemented with 2 mM L-glutamine, 0·08% (w/w) sodium bicarbonate, 10 μg mL^{-1} streptomycin, 10 μg mL^{-1} penicillin and 10% (v/v) foetal calf serum (FCS). Cells were maintained at 37°C and gassed with 5% (v/v) CO_2 in air. The experiments were carried out with cells in mid-logarithmic growth phase.

Cellular uptake and cytotoxicity

The cellular uptake of paclitaxel was determined by incubation of T-47D cells with [^{14}C]paclitaxel. The experiments were performed in 35 mm Petri dishes with fresh growth medium at 37°C. Emulsions containing the non-exchangeable compound cholesteryl [^{14}C]oleate were used as comparison to clarify the relative contribution of cellular uptake of paclitaxel by whole emulsion particles respective surface transfer. After completed incubation, the cells were washed three times with cold PBS and then detached by trypsin treatment. The cells were collected on Whatman GF/C filters and washed twice with cold PBS. The filters were dried for 30 min in an oven at 50°C, transferred to scintillation vials and counted for radioactivity.

The ability of paclitaxel, solubilized in lipid emulsion, liposomes and Diluent 12, to inhibit cell proliferation was determined by measuring [^{3}H]thymidine incorporation. Paclitaxel was dissolved in Diluent 12 at a concentration of 6 mg mL^{-1} and the solution was diluted 100 times with PBS just before use. Control cultures were treated with drug-free vehicles. Three hours before the end of the experiment, [^{3}H]thymidine (1 μCi mL^{-1}) was added to the incubation medium. After completed incubation the cells were harvested on Whatman GF/C filters and measured for radioactivity in the same way as in the uptake experiments.

Analytical procedures

The purity of paclitaxel were checked by HPLC using a 25 cm UltraTechnosphere 5-ODS column (HPLC Technology Ltd) eluted with methanol/water (6:1). The UV absorbance detector was set at 227 nm. The octanol/water partition coefficient was measured by the shaking-flask method. Liquid scintillation counting was performed with a 1216 Rackbeta scintillation counter (Wallac, Finland) with OptiPhase HiSafe II (LKB Scintillation Products) as scintillation fluid. Protein was measured by a modified Lowry method (Markwell et al 1978) using bovine serum albumin as standard.

Results and Discussion

Drug formulation

The formulation of paclitaxel (Fig. 1) for intravenous administration is a very difficult problem. The bulky taxane skeleton and the peripheral aromatic rings of paclitaxel, combined with a propensity to self-aggregate (Balasubramanian et al 1994), make the compound poorly soluble in water. A value of 12·8 μM for the aqueous solubility at 20°C was obtained in this study while the values obtained by other authors range from 0·77 (Mathew et al 1992) to 35 μM (Ringel & Horwitz 1991). At the same time the scattered hydroxyl groups at the 7, 15 and 2′ positions make the compound too polar to be readily soluble in oil. The solubility in triolein at 20 and 37°C were found to be as low as 3·1 and 4·6 μg mg^{-1}, respectively. The octanol/water partition coefficient of 311 obtained for paclitaxel at 20°C can be compared to those of 1165 and 10 755 obtained for cholesterol and cholesteryl oleate, respectively. Although paclitaxel exhibits both polar and hydrophobic structural elements, characteristic of amphipathic molecules, the polar hydroxyl groups are too scattered and the hydrophobic diterpenoid ring system too bulky for the molecule to be readily incorporated into phospholipid bilayer structures and a value of

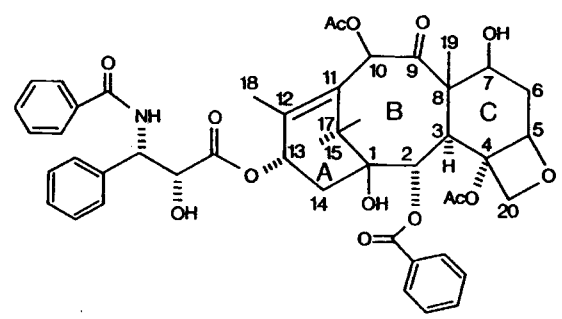

FIG. 1. Chemical structure of paclitaxel.

about 3 mol % has been reported (Balasubramanian & Straubinger 1994). It can thus be concluded that the structural and physicochemical features of paclitaxel render it difficult to solubilize in both aqueous and oily excipients as well as in phospholipid bilayers.

Design and characterization of drug-emulsion

A previous study (Lundberg 1994) showed that the combination of phosphatidylcholine with the non-ionic surfactant polysorbate 80 enables the preparation of stable lipid emulsions with a particle diameter of about 50 nm. Coating the surface of such emulsion particles with PEG-PE results in a prolonged circulation-time (Lundberg et al 1996). In this work a series of short-chain triglycerides were first tested as oil core components, since the bulk-phase solubility of paclitaxel in these are greater than that in triolein (Tarr et al 1987). However, the triglycerides with the shortest acyl chains, triacetin and tributyrin, gave coarse, unstable emulsions. Tricaproin, tricaprylin and tricaproin gave more stable emulsions, but the solubilization capacities were not substantially better than that of the triolein emulsion. The conclusion was that triolein is the preferable oil core component. The solubilization capacities of lipid emulsions and liposomes were measured in parallel experiments and both amounted to 5 mol % when calculated on phospholipid content. This result indicates that most of the paclitaxel is situated in the surface layer of the emulsion droplet. To avoid potential problems with over-saturated preparations a loading of 3 mol % paclitaxel was chosen for both emulsion and liposomes. The composition chosen for the preparations used in the further experiments was for lipid emulsion – triolein : dipalmityl phosphatidylcholine : polysorbate 80 : PEG-PE : paclitaxel at mass ratios of 1 : 1 : 0·4 : 0·1 : 0·03 and for the liposomes – dipalmitoyl phosphatidylcholine : PEG-PE with a mass ratio of 1 : 0·1.

The particle size of the emulsion droplet is an important feature since both a good physical stability (Lundberg 1994) and a prolonged clearance time (Lundberg et al 1996) are dependent on a small particle diameter. The laser particle sizer gave a mean particle diameter of 40 nm for the lipid emulsions (Table 1) and 60 nm for liposomes, while electron microscopy revealed emulsion particles ranging in size between 20 and 40 nm (Fig. 2).

The physicochemical stability of the drug-emulsion was found to be excellent, in agreement with previous findings (Lundberg 1994). No aggregation or change in particle size were noted during several months of storage. However, the most practical method for storage and distribution of paclitaxel emulsions would apparently be in the form of a lyophilized powder. Emulsions lyophilized in 5% glucose and rehydrated with distilled water showed the same physicochemical properties (size and stability) as the parent vehicles.

Table 1. Particle size distribution of representative lipid emulsion preparations with the standard composition (mass ratios) triolein : dipalmitoyl phosphatidylcholine : polysorbate 80 : PEG-PE : paclitaxel (1 : 1 : 0·4 : 0·1 : 0·03).

Size class (nm)	Intensity
20–30	0·22
30–40	0·87
40–50	0·72
50–60	0·58
60–70	0·32
70–80	0·10

Intensity is as measured by quasielastic laser light scattering.

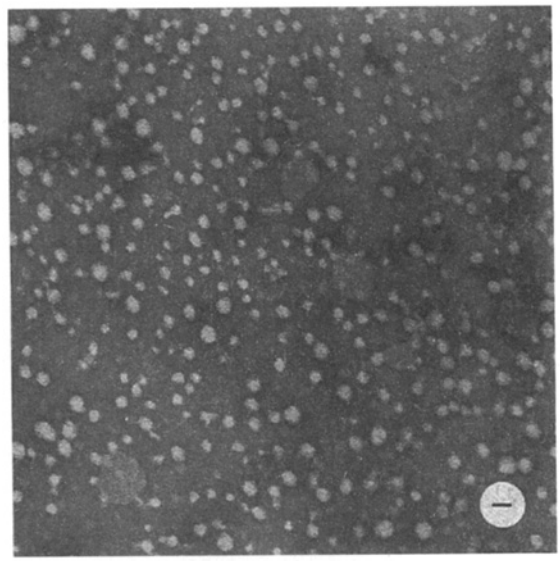

FIG. 2. Negative-staining electron microscope appearance of representative lipid emulsion preparations with the standard composition (mass ratios) triolein : dipalmitoyl phosphatidylcholine : polysorbate 80 : PEG-PE : paclitaxel (1 : 1 : 0·4 : 0·1 : 0·03). The scale bar represents 50 nm.

The lipid components of the emulsion eluted as a single peak from a Sephacryl 300 column (Fig. 3A). About 15% of the paclitaxel co-eluted with the lipids, while the rest eluted as a broad peak at larger volumes. This behaviour indicated that paclitaxel leaked out of the emulsion globules. The release of paclitaxel from emulsion globules compared with that from liposomes and Diluent 12 was further studied by the diffusion of the free agent trough a dialysis membrane (Fig. 3B). The results show the fastest release from emulsions, while the values for liposomes and Diluent 12 are quite similar. From these experiments it can be concluded that the paclitaxel emulsion can be characterized more like a sustained-release formulation than a stable drug-carrier system.

Paclitaxel uptake and cytotoxicity

The concentration-dependent uptake of [^{14}C]paclitaxel, solubilized in lipid emulsions, liposomes and Diluent 12, by T-47D cells during 1 h is presented in Fig. 4A. The uptake of the non-exchangeable lipid cholesteryl [^{14}C]oleate from emulsion is shown as comparison. At near saturated conditions the data show an only marginal difference in uptake between the three vehicles. Considerably less uptake was noted for the cholesteryl ester and when compared with the accumulation of paclitaxel it was estimated that surface transfer accounts for about 65% of the drug accumulation from emulsion globules. Such a transfer process is thought to involve desorption into, and diffusion through, the water interphase of the exchanging molecules (Phillips et al 1987). The time-course curves for paclitaxel uptake (Fig. 4B) show a fast uptake of the drug and steady state is reached after about 1 h for emulsions and

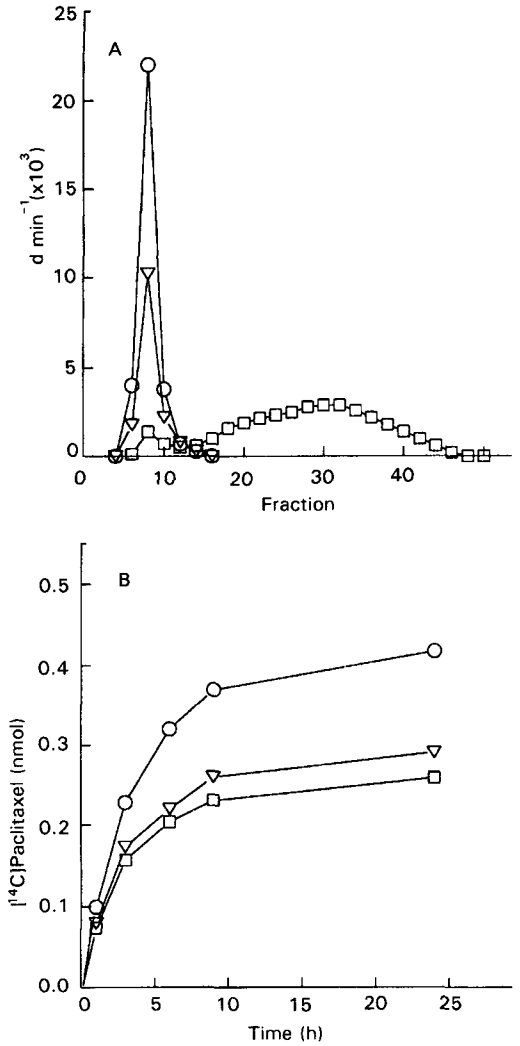

FIG. 3. In-vitro release of paclitaxel from lipid emulsions. (A) Integrity of a lipid emulsion as determined by elution on a Sephacryl 300 column [^{3}H]triolein (○), [^{14}C]dipalmitoyl phosphatidylcholine (▽), [^{14}C]paclitaxel (□). (B) Release of [^{14}C]paclitaxel measured by the dialysis bag method from lipid emulsions (○), liposomes (▽), and Diluent 12 (□).

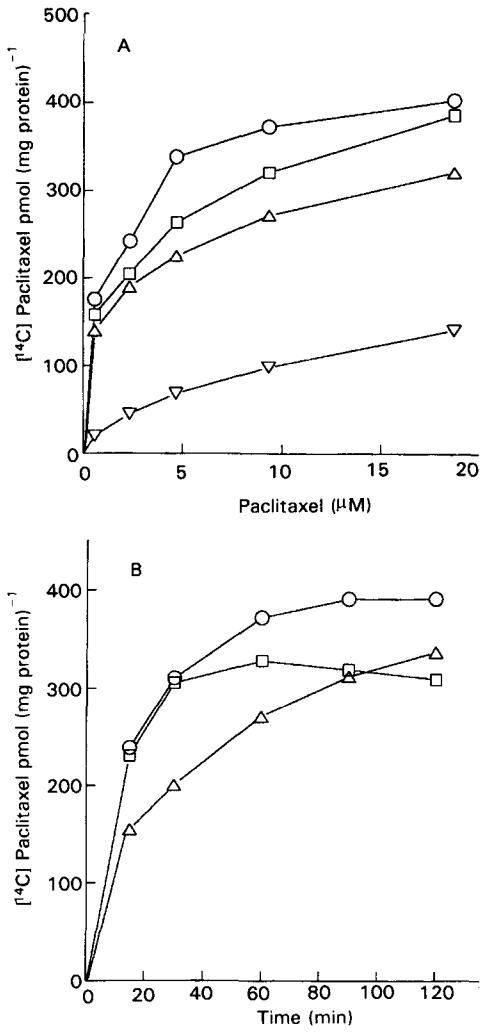

FIG. 4. Uptake of [^{14}C]paclitaxel into T-47D cells from lipid emulsions (○), liposomes (△), and Diluent 12 (□). The cellular uptake of cholesteryl [^{14}C]oleate (▽) is shown as comparison. (A) Concentration-dependent uptake during a 1 h incubation at 37°C. (B) Time-course curves obtained with a drug concentration of 10 μM. Data are the mean of three separate experiments.

Diluent 12, while the process is somewhat slower for the liposomes. The experiments demonstrated that the cellular accumulation of paclitaxel from the lipid emulsion, liposomes and Diluent 12 is essentially similar and, at least from the emulsion, mainly due to physicochemical transfer.

The in-vitro antiproliferative activity of paclitaxel, administered in lipid emulsions, liposomes and Diluent 12, was evaluated on the basis of [^{3}H]thymidine incorporation. The dose-response curves for exponentially growing T-47D cells exposed to paclitaxel for 48 h are biphasic (Fig. 5). The curves are initially steep at low concentrations, but with cancer cell survival plateauing at higher drug concentrations. This effect is especially discernible when paclitaxel is solubilized in Diluent 12. The IC50 values (reducing proliferation by 50%) obtained were 7, 10 and 35 nM for paclitaxel administrated in emulsion, liposomes and Diluent 12, respectively. The cytotoxic effects of the vehicles without paclitaxel were found to be considerable at higher concentrations (Fig. 5) and interfered obviously with the noted antiproliferative action of paclitaxel. Most dramatic was this effect with Diluent 12, which gave an almost total kill at the highest concentration tested. The cytotoxic effect of the vehicles became discernible at concentrations corresponding to 100 nM paclitaxel for both emulsions and liposomes, while the value obtained for Diluent 12 was about 40 nM. From these numbers it can be concluded that the cytotoxic effects of the vehicles should not interfere with the ID50 values.

The biphasic character of the dose-response curves has also been noted by other authors (Hill et al 1994) and Liebmann et al (1993) even found an enhanced cell survival at high paclitaxel concentrations. This finding was, however, not verified in our study. The results obtained by other authors regarding the in-vitro cytotoxic effect of Diluent 12 are contradictory. Liebmann et al (1993) found that high concentrations of Diluent 12 antagonised paclitaxel, while Nygren et al (1995) noted a substantial cytotoxic effect of the Cremophor EL component of Diluent 12. Cremophor EL is a non-ionic surfactant, and given the well-known disruption of membrane

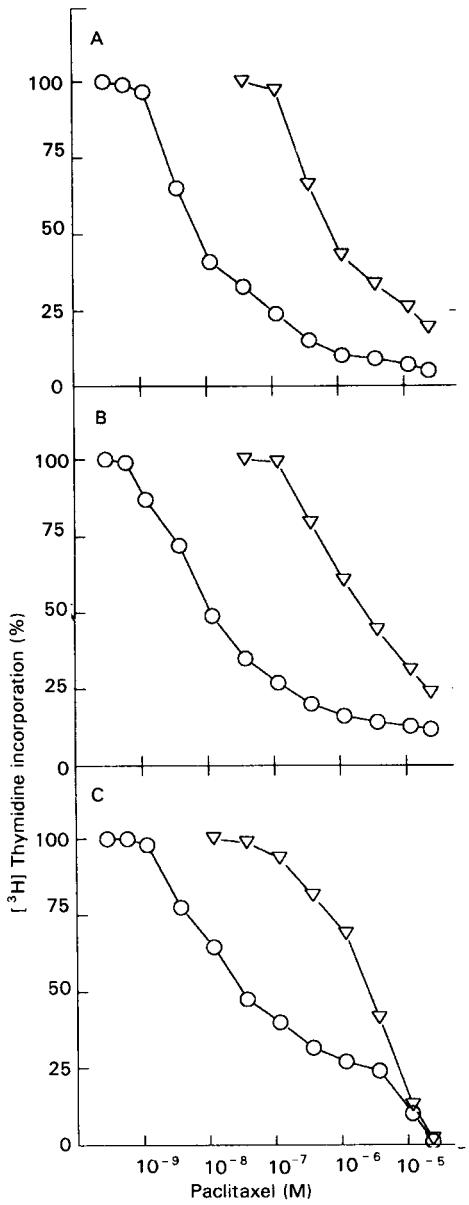

FIG. 5. Inhibition of T-47D cell proliferation by paclitaxel (○), as measured by [^{3}H]thymidine incorporation, in lipid emulsions (A), liposomes (B), and Diluent 12 (C). The antiproliferative effect of corresponding vehicles without paclitaxel (▽) is shown as comparison. Data are the mean of four separate experiments.

integrity and function by this group of compounds, a toxic effect can be expected. More surprising was the antiproliferative action of the lipid emulsion and liposomes. Lipid emulsions have long been used in parenteral nutrition with few adverse reactions (Pelham 1981) and well over 10 g of phospholipid may be administered safely to humans in the form of liposomes (Sculier et al 1986). There is thus reason to expect that the adverse effects of the lipid-based vehicles would be less pronounced when used in-vivo. The optimism regarding lipid-based vehicles is also supported by the findings that liposomal paclitaxel demonstrated greater inhibition of tumour growth compared to standard paclitaxel in human tumours grafted in athymic mice (Riondel et al 1992) and that liposome encapsulation of paclitaxel appeared to reverse multidrug resistance (Rafaeloff et al 1992; Sharma et al 1993). Small, long-circulating drug carriers similar to the submicron lipid emulsion have also been shown to accumulate in areas of inflammation and neoangiogenesis (tumours) (Jain 1989; Mayhew et al 1992). The lipid emulsion also offer good opportunities for specific drug targeting by attaching ligands for cellular receptors like apolipoproteins to the surface of the globules (Lundberg 1987; Rensen et al 1995).

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