Sterically Stabilized Phospholipid Mixed Micelles: *In Vitro* Evaluation as a Novel Carrier for Water-Insoluble Drugs

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Purpose. Sterically stabilized phospholipid micelles (SSMs) composed of poly(ethylene glycol-2000)-grafted distearoyl phosphatidylethanolamine (PEG(2000)-DSPE) are new and promising lipid-based carriers for water-insoluble drugs. This study investigates and compares sterically stabilized mixed micelles (SSMM), composed of (PEG(2000)-DSPE) plus egg-phosphatidylcholine, with SSM as a novel delivery system for improved solubilization of water-insoluble drugs using paclitaxel as a model.

Methods. Paclitaxel was solubilized in SSM (P-SSM) and SSMM (P-SSMM) by coprecipitation and rehydration with isotonic 0.01M HEPES buffer, pH 7.4. After separation of excess drug by centrifugation, mean particle size and morphology of particles in the supernatant were determined by quasi-elastic light scattering and transmission electron microscopy. The solubilization potentials of SSMM and SSM for paclitaxel were determined by reverse phase high pressure liquid chromatography (RP-HPLC). Cytotoxic activity of paclitaxel in SSMM, SSM, and dimethyl sulfoxide (10% DMSO) was determined against human breast cancer cells (MCF-7).

Results. Mean hydrodynamic diameter of P-SSMM and P-SSM were 13.1 ± 1.1 nm and 15 ± 1 nm (n = 3), respectively. SSMM solubilized 1.5 times more paclitaxel than SSM for the same total lipid concentration. Solubilized paclitaxel amount increased linearly with an increase in lipid concentration. A therapeutically relevant lipid concentration (15 mM) of SSMM solubilized 1321 ± 48μg/ml of paclitaxel. Paclitaxel in the absence of sufficient SSM aggregated to form lipid-coated crystals. P-SSMM, P-SSM, and paclitaxel in DMSO had comparable cytotoxic activities against MCF-7 cells.

Conclusions. SSMM showed increased solubilization potential compared with SSM while retaining all of its own advantages. Therefore, it can be used as an improved lipid-based carrier for water-insoluble drugs.

KEY WORDS: Water-insoluble drugs; paclitaxel; PEGylated lipids; micelles; sterically stabilized mixed micelles; sterically stabilized micelles; sterically stabilized crystals.

INTRODUCTION

Phospholipids and PEGylated phospholipids have been shown to be relatively nontoxic and safe for parenteral administration (1,2), and in recent years, carrier systems using phospholipids have been introduced in the market as liposomes and lipid emulsions. However, these systems are not applicable to all water-insoluble drugs. Solubility in oil may be a problem with some water-insoluble drugs to be formulated as emulsions. Drugs with bulky structures such as paclitaxel may have stability problems in liposomes because of difficulty in packing in the bilayer (3–5). Work in our laboratory has concentrated on the use of phospholipid systems as micelles for parenteral delivery of water-insoluble drugs. We have previously investigated phospholipid-bile salt mixed micelles as a delivery vehicle for water–insoluble drugs such as teniposide and paclitaxel (6,7). Even though these types of mixed micelles are attractive because of their lower toxicity than simple bile salt micelles, slight changes in bile salt/phospholipid ratio result in significant changes in micelle size and structure.

Recently, phospholipid micelles composed of PEGylated phospholipids such as poly(ethylene glycol)-grafted distearoyl phosphatidylethanolamine (PEG-DSPE) have been explored by us and other researchers for the solubilization of water-insoluble drugs (8–10). These phospholipid micellar systems are safe, biocompatible, and nontoxic. The PEG on the surface of the micelles renders them sterically stabilized, preventing opsonization and reticular endothelial system uptake (11). These sterically stabilized micelles (SSM), because of their low critical micellar concentration (CMC) values and also most likely because of the strong interaction between the acyl chains in the core region, are also relatively stable on dilution (9,12). In addition, because of their small size, these carrier systems can provide targeted delivery to cancer tissue or other injured tissues by selective extravasation through leaky vasculature (12). More important, preparation of these SSMs is simple and efficient compared with bile salt/phospholipid mixed micelles or liposomes.

However, with all these advantages, SSMs are not devoid of problems. One of them is SSMs' limited solubilization potential, which is controlled by the total number of micelles in the system. In this study, we report an improvement in the solubilization potential of SSM by including a water insoluble phospholipid such as phosphatidylcholine (PC) to form a novel delivery system: sterically stabilized mixed micelles (SSMMs). Our rationale in developing SSMMs to increase the solubilization potential is to increase the hydrophobic core of each SSM by incorporating PC. These SSMMs are the second generation of SSM, and retain all the advantages of SSMs described above while increasing the solubilization capacity of the micelle for a hydrophobic drug. This study investigates SSMM *in vitro* as a novel drug delivery system for water-insoluble drugs, and compares it with SSM, using paclitaxel, which has serious formulation problems (13–15), as a model drug.

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ABBREVIATIONS: PEG(2000)-DSPE, poly(ethylene glycol-2000)-conjugated distearoyl phosphatidylethanolamine; SSM, sterically stabilized micelles; EPC, egg phosphatidylcholine; SSMM, sterically stabilized mixed micelles; P-SSM, paclitaxel solubilized in sterically stabilized micelles; P-SSMM, paclitaxel solubilized in sterically stabilized mixed micelles; HEPES, (N- [2-hydreoxyethyl] piperazine-N′-[2-ethanesulfonic acid]); TEM, transmission electron microscopy; DMSO, dimethyl sulfoxide; SSC, sterically stabilized crystals.

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MATERIALS AND METHODS

Chemicals

Poly(ethylene glycol-2000)-conjugated distearoyl phosphatidylethanolamine was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Paclitaxel (> 99%) and dimethyl sulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Egg-phosphatidylcholine was obtained from LIPOID GmbH (Ludwigshafen, Germany). HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific (Ithasca, Illinois). (N- [2-Hydreoxyethyl] piperazine-N′-[2-ethanesulfonic acid]) (HEPES) (Sigma) and all other reagents were analytical grade.

Preparation of Aqueous Dispersions of Paclitaxel

Paclitaxel solubilized in (PEG(2000)-DSPE) and paclitaxel-mixed micelles were prepared by coprecipitation. Briefly, for simple micelles, paclitaxel and poly(ethylene glycol-2000)-grafted distearoyl phosphatidylethanolamine (PEG(2000)-DSPE), in a molar ratio of 0.16, were dissolved in methanol. The solvent was then removed by vacuum rotary evaporation under a stream of argon to form a dry film. This dry film was further dried under vacuum overnight to remove any traces of remaining solvent. The dried film was rehydrated with isotonic 0.01M HEPES buffer, pH 7.4. The solution was then flushed with argon, sealed, and equilibrated for 12 h at room temperature. The unsolubilized excess paclitaxel was removed by centrifugation at 13,000 g for 5 min to obtain a clear dispersion. The maximum solubility of paclitaxel in the absence of crystal formation was determined in simple micelles of PEG(2000)-DSPE by keeping the phospholipid concentration fixed at 5 mM and systematically reducing the drug concentration (drug:phospholipid, molar ratios, 0.076, 0.078, 0.082, 0.088) until a single homogenous system was determined as confirmed by a single peak by size analysis. To prepare SSMM solubilizing paclitaxel, initially various molar ratios of PEG(2000)-DSPE and egg-phosphatidylcholine (EPC) (90:10, 85:15, 80:20 and 75:25) were coprecipitated along with 500 μg paclitaxel, and the same procedure as described above was followed. The total phospholipid concentration was kept constant at 5 mM. Each formulation was prepared in triplicate. The prepared dispersions were then characterized for their size and morphology and assayed for their drug content. The optimal formulations of SSM or SSMM were then chosen based on their formation of a homogenous system with maximum solubilization potential for paclitaxel. These optimal SSM and SSMM formulations were then tested for bioactivity.

Particle Size Determination

Particle size distribution and mean diameter of the prepared aqueous dispersions of paclitaxel were determined by quasi-elastic light scattering using a NICOMP 380 Submicron Particle Sizer (Santa Barbara, CA, USA) equipped with a 5 mW helium-neon laser at 632.8 nm and a temperature-controlled cell holder as described previously (7). The mean hydrodynamic particle diameter, $\bar{d}_h$ was obtained from the Stokes-Einstein relation using the measured diffusion of particles in solution ($\eta = 0.933$, T = 23°C, n = 1.33). Data were analyzed in terms of volume and intensity-weighted distributions. Each reported experimental result is the average of at least three $\bar{d}_h$ values obtained from analysis of the autocorrelation function accumulated for at least 20 minutes.

Transmission Electron Microscopy

The morphology of paclitaxel in the presence and absence of PEG(2000)-DSPE was visualized by transmission electron microscopy (TEM) using negative staining. A drop of the prepared paclitaxel dispersion with PEG(2000)-DSPE (molar ratio, 0.16) and a drop without PEG(2000)-DSPE were placed on a carbon-coated copper grid and stained with 1% phosphotungstic acid. After air-drying for 2–3 minutes, the drop was viewed under an electron microscope (JEOL 100CX) and photographed.

Assay of Solubilized Paclitaxel

The amounts of paclitaxel solubilized both in the SSM and the SSMM was determined by RP-HPLC. The clear aqueous dispersion was diluted with methanol. Twenty μl of each sample was injected at least three times into a μBondapak C-18 column, 3.9 mm X 30 cm (Waters, Milford, MA, USA) equipped with a C18 column guard. The column was eluted with acetonitrile/water (60:40) at 1.0 ml/min (Waters 600). Detection was by UV absorption measurement at 227 nm (Waters 490). Peak areas were calculated by interfacing the detector to an electronic integrator (Hewlett Packard, Palo Alto, California). The drug concentration was calculated from standard curves. The assay was linear over the tested concentration range, and there was no interference of the phospholipid with the assay.

In Vitro Cytotoxicity

The cell line used to evaluate the *in vitro* activity of the formulations was MCF-7 (ATCC # HTB-22). This cell line is derived from a human mammary gland adenocarcinoma and is used extensively to determine the effects and mechanisms of drugs against breast cancer, such as paclitaxel (16,17). The cell line was maintained in RPMI 1640 medium containing 10% fetal bovine serum and 1.0% antibiotics (penicillin and streptomycin) in a 5% carbon dioxide humidified atmosphere at 37°C.

Optimum solutions of paclitaxel-SSM and paclitaxel-SSMM chosen from the solubilization studies were used as the test solutions. A 10% dimethyl sulfoxide (DMSO) solution of paclitaxel also was tested as a control. Drug-free SSMs and SSMMs in 0.01M HEPES buffer, pH 7.4, also were prepared at the same concentrations as the test solution and were used as controls. Solvents, 10% DMSO and HEPES buffer, were tested at the highest concentration used in the formulations. All the samples were prepared and tested in triplicate.

The procedure used to test the *in vitro* cytotoxic activity of the formulation is as previously described (18). Briefly, samples were prepared as described earlier and serial dilutions were made to obtain paclitaxel concentrations ranging from 0.0013–4 μg/ml using the respective solvent that is either HEPES buffer or 10% DMSO. Then 190 μl of cell suspension at a density of 6×10^4/ml was plated in a 96-well plate. After that, 10 μl/well of the test solutions and controls were added to the microtiter plates. Control groups with 10 μl of the solvents also were added. Each sample was evaluated in trip-

licate. The plates were then incubated for 3 days in a 5% CO_2 humidified atmosphere at 37°C. After the incubation period, the cells were fixed to the plates by adding 100 μl/well of cold 20% trichloroacetic acid and incubating for 1 hr at 4°C. The plates were then washed, air-dried, and stained with 100 μl/well of 0.4% sulforhodamine B in 1% acetic acid for 30 min. Then, the plates were washed with 1% acetic acid and rinsed, and 10 mM Tris buffer (200 μl/well) was added. The optical density was then read at 515 nm, and the readings obtained for the solvent controls were used to define 100% growth after correcting for the value obtained for the zero day control. These values were then expressed as % survival and ED_{50} values calculated using nonlinear regression analysis (percent survival vs. concentration).

Data and Statistical Analyses

All the data are expressed as means ± SD. Solubilization potential of SSMM for paclitaxel is represented as the amount of paclitaxel solubilized per ml of dispersion. The increase in solubilization with increase in total lipid amount for SSMM was determined to be linear by regression analysis and R-square value and equation to the line determined. Cytotoxic activity was expressed as percentage survival of the cells and compared with baseline using repeated measures analysis of variance with Neuman-Keuls post hoc test. ED_{50} values were calculated for each formulation and compared statistically using one-way analysis of variance. A p value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Particle Size

The aqueous dispersions of PEG(2000)-DSPE containing paclitaxel indicated the presence of two populations by quasi-elastic light scattering analysis (χ^2 values > 1000 for Gaussian analysis). One species had a mean diameter of ~16 nm, which is consistent with the size of PEG(2000)-DSPE micelles (9,10). It has been demonstrated in our laboratories and by other investigators that PEG(2000)-DSPE above 0.8–1 μM forms micelles with an average diameter of about 16 nm (9,10). The other observed population consisted of particles with a mean diameter of ~200 nm (Fig. 1a.). The second population was hypothesized to be an aggregate of paclitaxel with a PEG(2000)-DSPE monolayer coating its surface. These so-called sterically stabilized crystals (SSCs), being small and lipid-coated, remained in the supernatant after centrifugation and formed a clear solution. Paclitaxel, being hydrophobic, may favor this structure in the absence of sufficient PEG(2000)-DSPE micelles. Paclitaxel has been shown to undergo concentration-dependent self-aggregation, possibly due to hydrogen-bonded stacking (5). Quasi-elastic light scattering (NICOMP) analysis helped in studying the homogeneity of the system. The second, larger species in the system indicated the presence of paclitaxel crystals. However, no conclusion about the shape of the particle can be made since NICOMP size analysis assumes spherical shape of particles for calculating $\bar{d}_h$. We therefore studied the larger species by further using TEM. (See below.) The formation of this second species of lipid-coated crystals, SSC, is being explored as a potential delivery system, though that is not reported in this

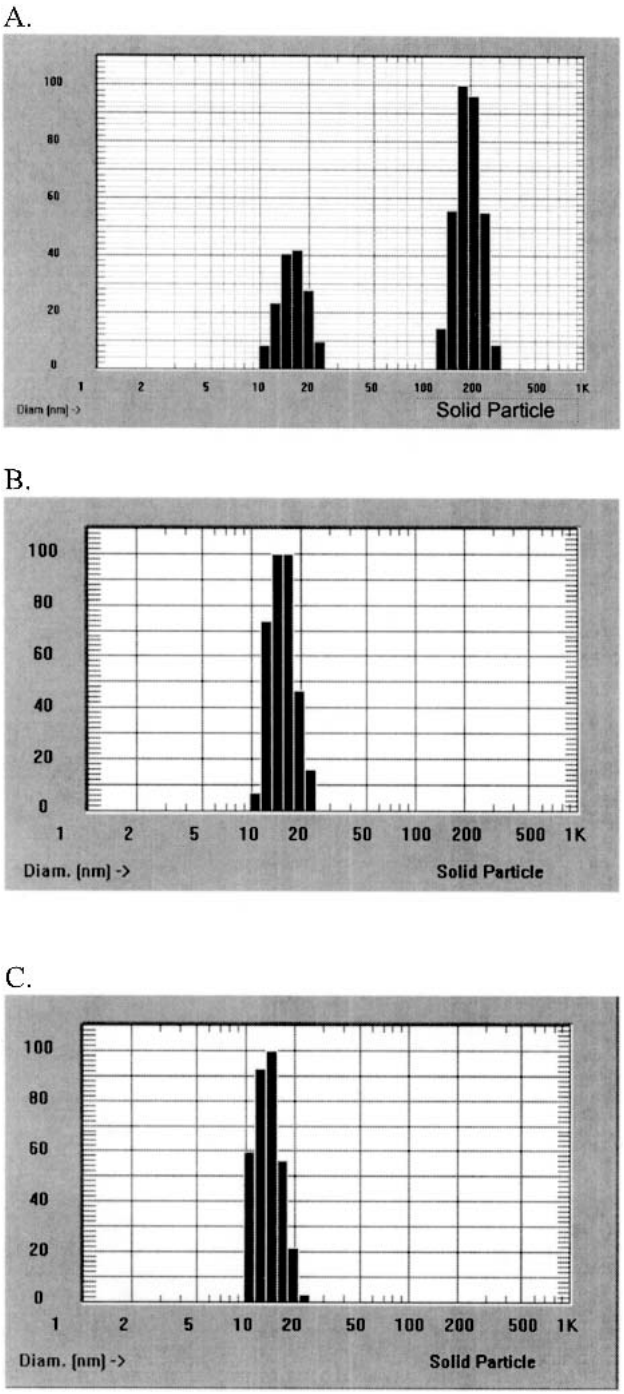

Fig. 1. Representative NICOMP size distribution analysis of paclitaxel and PEG(2000)-DSPE dispersions: (A) SSC of paclitaxel coexisting with paclitaxel-SSM; (B) Paclitaxel-SSM; (C) Paclitaxel-SSMM.

article. This current study focuses on only sterically stabilized mixed and simple micelles as improved lipid-based delivery systems. Therefore, the amount of paclitaxel added to a constant amount of PEG(2000)-DSPE during preparation was reduced systematically until only a single micellar species was observed by light-scattering particle size analysis with a mean diameter of 15 ± 1.0 nm. A representative NICOMP size distribution plot of SSM is shown in Fig. 1b. The presence of a single species as SSM also was confirmed by low χ^2 (< 3) for Gaussian fit.

Size analysis of the SSMM dispersion of paclitaxel containing PEG(2000)-DSPE and EPC at a molar ratio of 90:10 indicated the formation of a homogenous system containing only one peak corresponding to micellar size, with a mean hydrodynamic diameter of 13.1 ± 1.1nm ($\chi^2 < 3$ for Gaussian fit) (Fig. 1c). The slight reduction in the mean size of the micelles indicates the insertion of the EPC into the PEG(2000)-DSPE micelles to form mixed micelles, with a possible transition of the PEG chains from a brushed to mushroom conformation as suggested previously (19). Size analysis of the other mixed micellar samples prepared with higher EPC concentrations indicated the formation of heterogeneous systems with multiple peaks. (Data not shown.) Previously, it has been shown that incorporation of PEG(2000)-DSPE into PCs causes a transition from lamellar to micellar phase (20). We are studying the transition of this system for more details by small angle neutron scattering and small angle X-ray scattering techniques, which will be the subject of another report. However, we are confident that in the mixed micellar system, when the EPC content is increased, a possible transition from spherical micelles to lamellar phase can occur, and this process can be facilitated in the presence of another hydrophobic entity, paclitaxel, resulting in the formation of a heterogeneous system. Since only the molar ratio of 90:10 PEG(2000)-DSPE:EPC under the studied conditions gave a homogenous system and solubilized paclitaxel at higher concentration than simple micelles (see below), this ratio was chosen as an optimal mixed micellar state and was studied further.

Transmission Electron Microscopy

To confirm the existence of different populations in paclitaxel aqueous dispersions, the paclitaxel crystals were visualized in buffer in the presence (drug lipid molar ratio 0.16) and absence of PEG(2000)-DSPE before and after centrifugation. As can be seen in Fig. 2A, a large size distribution of particles was observed in the presence of PEG(2000)-DSPE compared with the sample without lipid and without centrifugation (Fig. 2B). Another interesting observation was that when paclitaxel was dried as a film from an organic solvent and reconstituted with buffer, large angular crystals were observed (Fig. 2B) instead of the needle-like crystals seen with native paclitaxel. Also, the particles in the presence of lipid have rounded edges and are not angular, as seen in the case of paclitaxel crystals formed in the absence of PEG(2000)-DSPE (Fig. 2B). Upon centrifugation of this system, which did not contain lipid, paclitaxel totally precipitated, and no detection of drug using HPLC and particles by TEM was seen in the supernatant. (Data not shown.) However, when paclitaxel with PEG(2000)-DSPE was centrifuged and studied by TEM, some dispersed particles were observed in the supernatant. These particles had rounded edges and were much smaller, with an average size of ~200 nm and a narrow distribution (Fig. 2C). We explain the results as follows: Low lipid/drug ratios result in insufficient PEG(2000)-DSPE micelles to disperse the existing paclitaxel molecules in a molecular state in micelles. Therefore, drug molecules aggregate to form crystals. PEG(2000)-DSPE molecules, being amphiphilic, accumulate at the surface of these hydrophobic paclitaxel particles and reduce the surface tension between the drug and buffer interface. This coating not only prevents further growth of the crystals but also prevents particle-particle interaction by providing a steric barrier. On centrifugation, larger particles are removed from the system to give a clear solution. However,

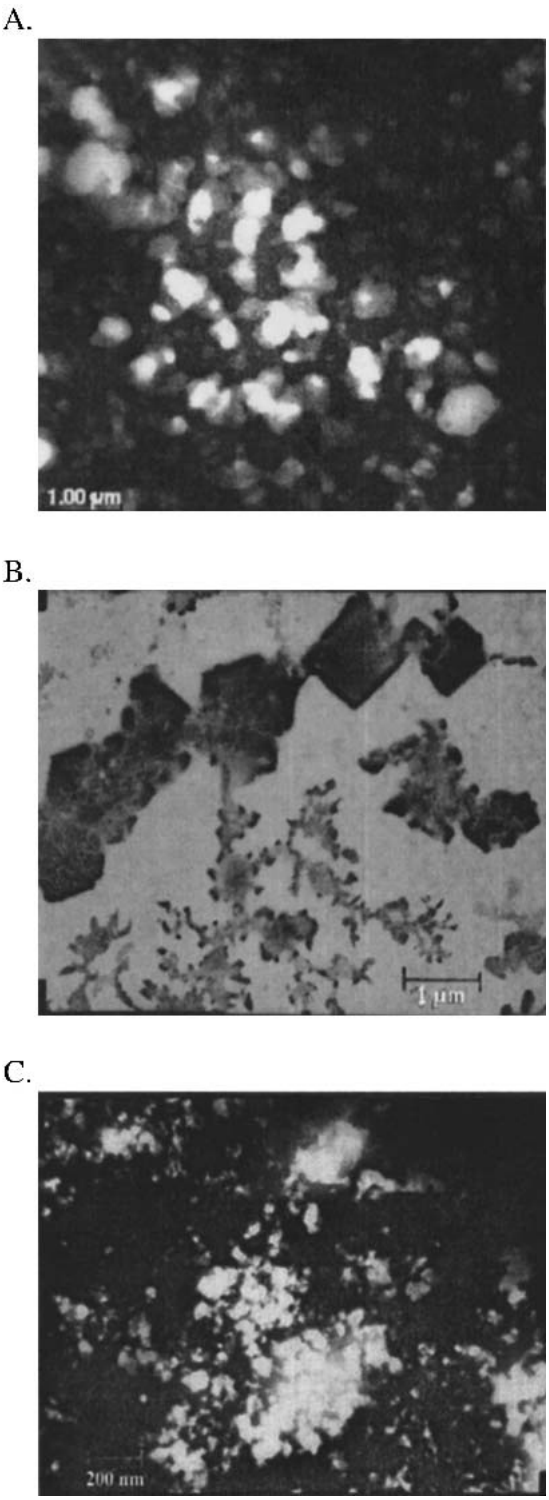

Fig. 2. Transmission electron micrograph of paclitaxel crystals: (A) Sterically stabilized crystals of paclitaxel in the presence of PEG(2000)-DSPE (drug/lipid molar ratio 0.16) before centrifugation (Bar = 1 μm); (B) Paclitaxel crystals in the absence of PEG(2000)-DSPE before centrifugation (Bar = 1 μm); (C) Sterically stabilized crystals of paclitaxel in the presence of PEG(2000)-DSPE (drug/lipid molar ratio 0.16) after centrifugation (Bar = 200 nm).

the smaller phospholipid-coated crystals, SSCs, remain suspended in solution. This situation is not observed with dispersions in the absence of phospholipid, probably because of the growth of paclitaxel particles to a size that precipitates on centrifugation. SSCs, if prepared in a controlled and reproducible fashion, are very attractive as another novel aqueous delivery system for water-insoluble drugs since they can provide a high dose per given volume and are safe for injection because of their small size.

Solubilization of Paclitaxel

Use of mixed micelles at a molar ratio of 90:10 was studied further as this was the only system found to be homogenous and containing only spherical mixed micelles at the conditions studied. Five mM PEG(2000)-DSPE was found to solubilize 319 ± 1.6 μg/ml paclitaxel as simple micelles alone without the formation of SSC. However, a significantly higher amount of paclitaxel (475.6 ± 8.96 μg/ml) was solubilized in 5 mM (90:10) SSMM of PEG(2000)-DSPE and EPC. EPC is a hydrophobic phospholipid. Insertion of a small percentage of this lipid into a micellar system composed of an amphiphilic phospholipid should increase the volume of the hydrophobic region of the micelle. This would therefore provide a larger space for the hydrophobic drug to be solubilized. This is confirmed by the results of this study in which the presence of EPC in the mixed micellar system increased the solubilization potential of paclitaxel by one and a half times when compared with SSM. We believe that this solubilization improvement can vary according to the drug molecule, and we are studying the case.

Since SSMM for the same total lipid concentration of 5 mM showed a higher solubilization potential for the drug than simple micelles (SSM), in our following experiments we tested the improvement of paclitaxel solubilization with increased total phospholipid concentrations of SSMM at the same ratio. As anticipated, we observed a linear increase in solubilized concentration of paclitaxel with increase in total mixed micelle concentration (R^2 = 0.999, y = 84.523x + 50.907) (Fig. 3). It is seen that for a feasible total lipid concentration of 15 mM, paclitaxel can be solubilized at a concentration well above 1 mg/ml. Hence, this approach results in an improvement of about 2000-fold in aqueous solubility of paclitaxel (21). In Fig. 4, we compared our previous data for

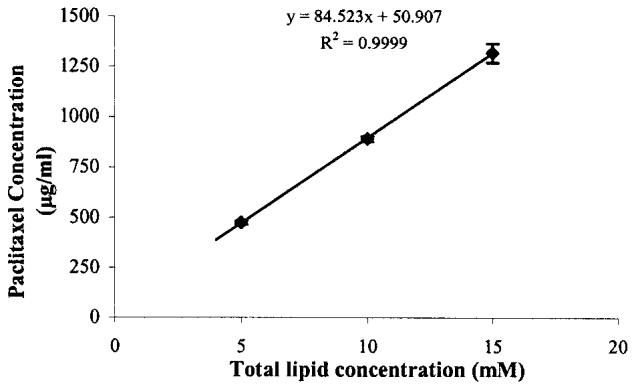

Fig. 3. Effect of total phospholipid concentration on solubilization potential of paclitaxel in sterically stabilized mixed micelles (PEG(2000)-DSPE:EPC, molar ratio 90:10) at room temperature (n = 3 experiments); R = 0.999, R^2 = 0.999; y = 84.523+50.97.

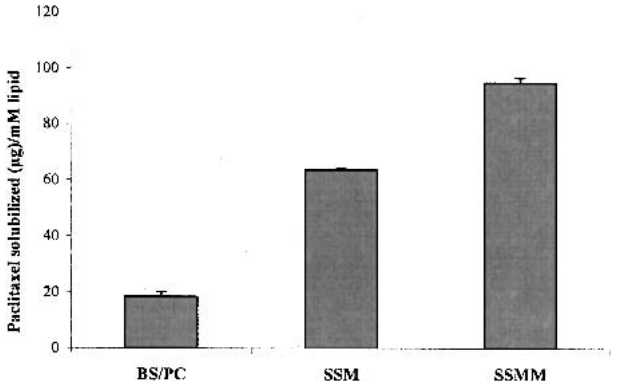

Fig. 4. Solubilization of paclitaxel in: (a) bile salt/phospholipid mixed micelles; (b) SSM; (c) SSMM.

the solubilization of paclitaxel in bile salt/phospholipid micelles with SSM and SSMM. These data clearly demonstrate the superiority of the solubilization potential of SSMM compared with other systems. Furthermore, PEGylated phospholipid micellar formulations lack organic solvents or detergents and hence are more compatible for human use.

In Vitro Cytotoxicity

As a next step, we investigated whether the interaction between the drug and the lipids in the formulation affected the drug's bioactivity. To determine the cytotoxic activity of paclitaxel in SSM and SSMM, the formulations were tested against MCF-7, a human breast cancer cell line which has been previously used in studying the anticancer activity of paclitaxel (16,17).

As shown in Fig. 5, the phospholipid micellar formulations had cytotoxic activities toward cultured MCF-7 cells similar to those of paclitaxel solution in 10% DMSO. ED_{50} values are summarized in Table I. The results indicate that paclitaxel in simple and mixed micelle is readily available to interact with the cancer cells and retains its antimitotic potency. Moreover, paclitaxel activity is not altered by the presence of lipids. The solutions containing PEGylated lipid alone or in combination with EPC at concentrations similar to those present in the paclitaxel formulations had no cytotoxic ef-

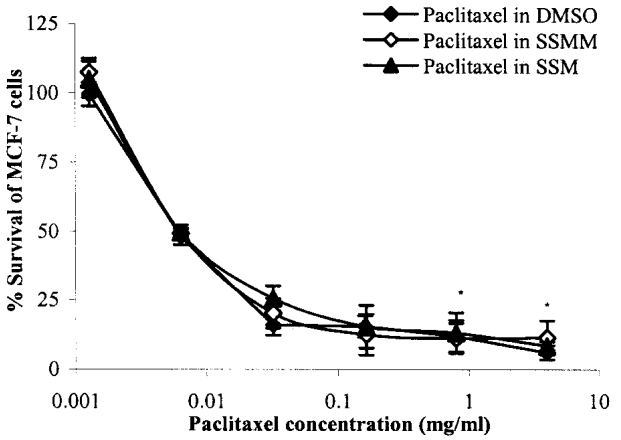

Fig. 5. Cytotoxicity of paclitaxel in 10% DMSO, SSM, and SSMM against MCF-7 cells (n = 3 experiments); * $p < 0.05$ in comparison with baseline.

Table I. Cytotoxic Activity, ED_{50} (Mean ± S.D.) of Various Formulations of Paclitaxel against MCF-7 Cells

Formulation	ED_{50} (μg/ml)*
Paclitaxel solution in 10% DMSO	0.0067 ± 0.0003
Paclitaxel-SSMM	0.0064 ± 0.0002
Paclitaxel-SSM	0.0066 ± 0.0005
SSMM	>104
SSM	>175

Note: (n = 3 experiments) *p > 0.05 for all comparisons

fects. This indicates that the phospholipids at the concentrations used are not toxic to the cells. These results suggest that SSM and SSMM containing paclitaxel could provide an effective chemotherapeutic activity *in vivo* despite the steric layer of the micelles.

CONCLUSIONS

In this study, we have demonstrated by *in vitro* research the suitability of a novel lipid-based drug delivery system, SSMM, for the solubilization of water-insoluble drugs such as paclitaxel. SSMMs solubilized higher concentrations of paclitaxel than simple SSMs, and both formulations showed significant cytotoxic activity against cultured MCF-7 cells. SSMM therefore retain the attributes of SSM while increasing the solubilization potential for water-insoluble drugs. Our current studies are investigating paclitaxel solubilized in SSMM and SSM in animal models to determine the *in vivo* cancer targetability, anticancer activity, and toxicity. We are also evaluating similar mixed micelles with different PEGylated lipids for the solubilization of other water-insoluble drugs.

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