J Membrane Biol (2007) 219:83–91
DOI 10.1007/s00232-007-9064-6

Effect of Fluidizing Agents on Paclitaxel Penetration in Cervical Cancerous Monolayer Membranes

A. Preetha · N. Huilgol · R. Banerjee

Received: 12 October 2006 / Accepted: 26 June 2007 / Published online: 22 August 2007

Abstract The aim of this study was to compare modulation of paclitaxel penetration in cancerous and normal cervical monolayers by four fluidizing agents: PCPG (9:1 DPPC:PG), PCPE (9:1 DPPC:DOPE), ALEC (7:3 DPPC:PG) and Exosurf (13.5:1.5:1.0 DPPC:hexadecanol:tyloxapol). Presence of the fluidizing agents improved drug penetration significantly. PCPG and PCPE were promising penetration enhancers. PCPG 0.1% caused 3.8– and 1.7-fold higher maximum increments in surface pressure due to drug penetration, $(\Delta\pi)_{max}$, than the control in cancerous and normal monolayers, respectively, at 20 mN/m. In cancerous monolayer at 20 mN/m, presence of 0.1%, 0.5%, 1%, 5% and 10% PCPE produced 3.4-, 5.7-, 7.4-, 9.6- and 9.8-fold higher drug penetration compared to the control monolayer without PCPE, respectively. In cancerous monolayer at 20 mN/m, PCPG and PCPE liposomes having 1 mg lipid gave 2.1 and 3.6 times higher $(\Delta\pi)_{max}$ compared to the control, respectively. Further, the liposomal drug penetration was found to be directly proportional to the liposomal lipid content. The effect of the fluidizing agents was confirmed by increased calcein release from model cervical cancer liposomes. These results may have implications in using the above biocompatible lipids and surfactants as penetration enhancers along with anticancer drugs or as carriers for liposomal formulations of anticancer drugs for improved membrane penetration.

Keywords Paclitaxel · Cervical cancer · Langmuir model membrane · Drug penetration · Liposome

A. Preetha · R. Banerjee (✉)
School of Biosciences and Bioengineering, Indian Institute of Technology, Mumbai 400076, India
e-mail: rinti@iitb.ac.in

N. Huilgol
Division of Radiation Oncology, Nanavati Hospital, Mumbai 400 056, India

Introduction

Paclitaxel is a diterpenoid derived from the bark and needles of the pacific yellow tree and has a complex chemical structure. It is a highly hydrophobic molecule with less than 5% solubility in water and has proven antineoplastic activity against a variety of cancers including breast, brain, head and neck, lung, colon, cervical and ovarian tumors (Zhao & Feng, 2004; Crosasso et al., 2000; Ruan & Feng, 2003; Fonseca, Simoes & Graspar, 2002; Brannon-Peppas & Blanchette, 2004; Ruel-Gariepy et al., 2004; Zhang et al., 2004; Koziara et al., 2004). Several clinical trials have placed paclitaxel, either as a single agent or in combination with other drugs, as one of the most active drugs for cervical cancer (Savarrese & Cognetti, 2003; Park et al., 2004). The efficacy of a drug is strongly related to its ability to penetrate the biological membranes and to drug-membrane interactions (Gambinossi et al., 2004). Although representative models of biomembranes, Langmuir monolayers offer a way to evaluate drug-membrane interactions (Lopez-Castellano et al., 2000). The aim of this study was to compare the modulation of paclitaxel penetration by different fluidizing agents using Langmuir monolayer membrane models. We evaluated the effect of four fluidizing agents: PCPG (DPPC:PG in the ratio 9:1), PCPE (DPPC:DOPE in the ratio 9:1), ALEC (7:3 DPPC:PG) and Exosurf (13.5:1.5:1.0 DPPC:hexadecanol:tyloxapol). All

fluidizing agents mentioned above are biocompatible phospholipid-based, known to be surface-active and approved for human use. ALEC and Exosurf are commercially available protein-free synthetic surfactants widely used in exogenous surfactant therapy (Takahashi, Nemoto & Fujiwara, 1994). The role of PG in ALEC and hexadecanol in Exosurf is to improve the adsorption and respreading of DPPC, indicating their fluidizing nature (Bangham et al., 1984; Yu, Harding & Possmayer, 1984). The surfactant properties of mixtures of PC with PE and PG have already been established with reference to those of ALEC and Exosurf by Banerjee & Bellare (2001). However, the effect of these surfactants in connection with drug-membrane interactions has not yet been explored. This study has been carried out to evaluate the effect of the above-mentioned four surfactant systems on drug penetration through membranes. The concentration of fluidizing agent used was 0.1% of the model membrane. PCPE was used as a representative fluidizer to evaluate the effect of concentration of fluidizer on the penetration of paclitaxel to cervical cancerous model membranes. Varying amounts of PCPE used were 0.1%, 0.5%, 1%, 5% and 10% of the cancerous model membrane. The promising fluidizing agents were selected to prepare liposomal formulations of paclitaxel, and their effects on paclitaxel penetration were also evaluated. The effect of liposomal paclitaxel with varying amounts of lipids on penetration to cancerous model membranes was also evaluated using PCPE liposomal paclitaxel, with lipid contents ranging 0.5–10 mg. Atomic force microscopy (AFM) and calcein release from liposomes was used to confirm our Langmuir monolayer penetration results.

Materials and Methods

Laboratory Chemicals

High-performance liquid chromatography-grade methanol and chloroform for tissue extraction were purchased from Loba Chime (Mumbai, India). Analytical reagent-grade (AR) methanol and acetone for cleaning of the Langmuir trough were purchased from SRL (Mumbai, India). Dipalmitoylphosphatidylcholine (DPPC), phosphatidylglycerol (PG), Triton X-100 and dioleoylphosphatidylethanolamine (DOPE) were obtained from Sigma-Aldrich (St. Louis, MO). Sterile freeze-dried powder of artificial lung expanding compound (ALEC, Pumactant) was bought from Britannia Pharmaceutical (Redhill, UK). Exosurf (freeze-dried powder) was obtained from Wellcome Foundation (London, UK). High-purity water purified by a Milli Q Plus water purifier system (Millipore, Billerica, MA), with a resistivity of 18.2 MΩcm, was used in all experiments.

Preparation of Monolayer Materials

Human biopsy specimens of cervical cancerous tissues (n = 8) and normal cervical tissues (n = 8) were obtained from the Radiation Oncology Division of Nanavati Hospital (Mumbai, India). All cancerous samples were of stage III squamous cell carcinoma of the cervix. Normal controls of cervical tissues were obtained from hysterectomy patients having noncervical disorders. All normal cases were reported to be free from malignancy by histopathological analysis. All tissue samples were washed thoroughly with normal saline, dried on a tissue paper and weighed. The weighed samples were processed using liquid nitrogen and dissolved in measured volumes of normal saline to obtain a tissue homogenate of known concentration. The organic phases containing the lipophilic components of the tissue homogenates were separated using the Bligh-Dyer extraction procedure. The organic phases of the cancerous tissues were pooled to obtain the pooled cancerous organic phase. Similarly, organic phases of normal cervical tissues were separately pooled to obtain the pooled normal organic phase. These pooled organic phases corresponding to 1 mg of tissue were used to form the monolayer membrane models for the drug penetration study. The surface activities of the normal and cancerous cervical tissue extracts have been previously standardized (Preetha, Huilgol & Banerjee, 2005a; Preetha, Banerjee & Huilgol, 2005b).

Drug Penetration Studies

Drug penetration studies were performed by evaluating the change in surface pressure of organic phase monolayers due to injection of drug using a computer-controlled Langmuir-Blodgett film balance (KSV Mini trough model; KSV Instruments, Helsinki, Finland) at 37°C. The lipid monolayer was formed at the air-liquid interface; a 30-min wait time was given for the evaporation of organic solvents, and then the monolayers were compressed to a surface pressure of 20–30 mN/m. These surface pressures were relevant to the packing in the biological membranes (Fidelio, Maggio & Cumar, 1986). Paclitaxel solution in methanol (1 mg/ml) was injected into the subphase to achieve a drug concentration of 600 nM in the subphase. The subphase was stirred using a Teflon-coated magnetic stirrer to ensure uniform drug distribution. Surface pressure was then recorded as a function of time until 3,000 s.

Modification of Drug Penetration

We performed penetration studies in the presence of some membrane fluidizing agents in the monolayers and with

liposomal formulations of paclitaxel to improve the paclitaxel penetration into cervical tissue lipid monolayers. We evaluated four fluidizing agents: PCPG (DPPC:PG in the ratio 9:1), PCPE (DPPC:DOPE in the ratio 9:1), ALEC (7:3 DPPC:PG) and Exosurf (13.5:1.5:1.0 DPPC:hexadecanol:tyloxapol). To evaluate the effect of the fluidizing agent, the agent was added to the monolayer and drug penetration in the presence of fluidizing agent was evaluated as explained earlier and compared with that in the absence of fluidizing agent.

Liposomal formulation of paclitaxel was prepared via the thin film hydration method using PCPG and PCPE fluidizing agents as the lipid. Briefly, appropriate volumes of chloroform solutions of lipids and paclitaxel were mixed in a suitable round-bottomed flask. The flask was then connected to a rotary evaporator. Vacuum was applied to the flask for half an hour to evaporate the chloroform and to form a homogeneous film on the flask wall. The dry film was then hydrated with normal saline solution by rotating the flask at about 200 rpm at 45°C until the lipid film was completely hydrated and a homogeneous dispersion was formed (approximately 1 h). The liposome dispersion was then extruded (11 times) through 100-nm polycarbonate filters (Whatman, Clifton, NJ) using a Mini-Extruder (Avanti Polar Lipids, Alabastar, AL) at 40–42°C. The mean particle size of liposomes after extrusion was determined using photon correlation spectroscopy based on the principle of dynamic light scattering (BI MAS, multisizing option on Zetaplus; Brookhaven Instrument, NY, USA) at 25°C. The intensity of the scattered light was detected at 90° to an incident beam. Liposomal paclitaxel corresponding to 600 nM drug was added to the subphase, and drug penetration was evaluated as above. PCPE and PCPG liposomes containing 1 mg lipid were used to evaluate the effect of liposomal paclitaxel on penetration to cancerous cervical model membranes. PCPE liposomal paclitaxel containing varying amounts of lipid (0.5, 1, 5 and 10 mg) was also studied to evaluate the effect of liposomal fluidizer content on paclitaxel penetration in cancerous cervical model membranes.

Calculation of Parameters

To cancel out the effects of film desorption and to obtain a measure of drug penetration alone, we calculated the difference between the surface pressure of the monolayer in the presence and absence of drug and denoted this as $\Delta\pi$. $\Delta\pi$ was then plotted against time, and the $\Delta\pi$ *vs.* time curves were used for the interpretation of our results. Figure 1 is a schematic diagram showing the possible profiles of $\Delta\pi$ values with respect to time. The increase of $\Delta\pi$ values with respect to time indicates the penetration of drug to the monolayer. Achievement of a steady surface pressure represented by a horizontal plateau indicates establishment of penetration equilibrium within the monolayer system. The decrease of $\Delta\pi$ values after reaching a maximum is correlated with the desorption of the drug from the monolayer. A zero value for $\Delta\pi$ is indicative of complete desorption of the drug, while negative values may be due to destabilization and dragging of some of the monolayer material to the subphase. We formulated a parameter, namely $(\Delta\pi)_{max}$ – the maximum increment in surface pressure due to drug penetration to quantify the drug penetration.

Calcein Release from Liposomes

In order to confirm the fluidizing effect revealed by the Langmuir monolayer model membrane study, calcein release from liposomes was evaluated. Model cancerous cervical membrane liposomes containing cholesterol, PC and sphingomyelin (SM) were used as model systems in this part of the study. The cholesterol:PC:SM ratio was 10:0.6:0.3, similar to that in the cancerous cervical tissues. PCPE was used as a representative fluidizing agent. Calcein-entrapped liposomes with and without varying amounts of PCPE fluidizing agent were prepared by thin film hydration as explained above. The hydrating medium was 63 mM calcein solution in phosphate-buffered saline (PBS), and the hydration temperature was 60°C. The

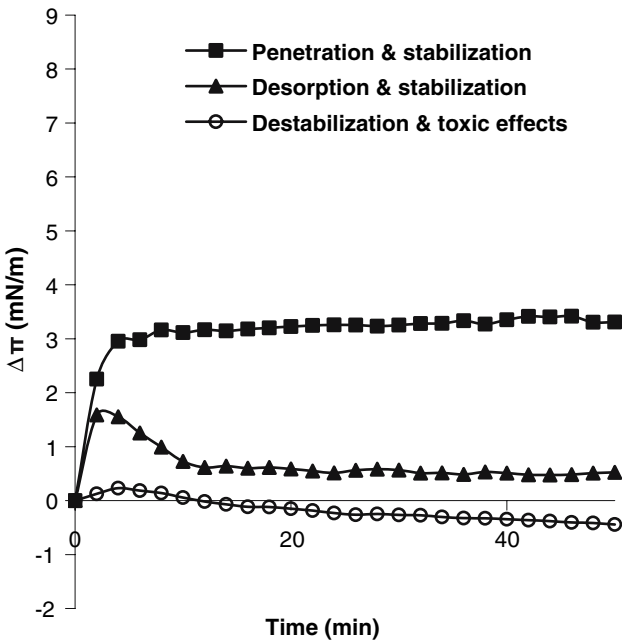

Fig. 1 Schematic representation showing three possible penetration profiles

multilamellar liposomal vesicles were then sonicated for half an hour to form unilamellar calcein-entrapped liposomal vesicles. The unencapsulated calcein was separated by centrifugation. For evaluation of calcein release, 60 µl of calcein liposomes (10 mg/ml of lipid) were added to 600 µl of PBS in an Eppendorf tube. The samples were kept at 37°C in a water bath up to 15 min. Initially and after 15 min, 20 µl of samples were added to 2 ml of PBS and mixed well, and fluorescence intensity was measured in a spectrofluorimeter (Hitachi, Tokyo, Japan) using 485 excitation and 520 emission filters. For 100% release of calcein from liposomes, 60 µl of calcein liposomes were added to 600 µl of 1% Triton X-100, vortexed for 1 min and kept at 55–60°C for 30 min; then, 20 µl of mixture was diluted with 2 ml PBS, and fluorescence intensity was measured as described above. Percentage of calcein release at time (t) = 15 min was calculated using the formula

$$\%\text{Release} = (\mathrm{F^t} - \mathrm{F^i})/(\mathrm{F^f} - \mathrm{F^i}) \times 100$$

Where F^i is the initial fluorescence intensity of the liposomal suspension and F^t is that at t = 15 min. F^f is the fluorescence intensity of the liposomal suspension after the addition of 1% Triton X-100.

Langmuir-Blodgett Film Deposition and AFM

To confirm the fluidizing effect on the cervical cancerous lipid model membranes, AFM images of the monolayers with and without a representative fluidizing agent (PCPE) were taken and a surface roughness of the images was analyzed.

The monolayer at the air-water interface was transferred onto a clean mica sheet (30 x 10 x 1.5 mm) using the Langmuir-Blodgett deposition technique. The monolayer was first compressed to a predetermined target surface pressure value (20 mN/m) with the help of a servo mechanism, and a clean mica sheet, immersed into the subphase prior to monolayer spreading, was withdrawn from the monolayer at a speed of 5 mm/min. The transfer ratio was monitored throughout the dipping process, and films having transfer ratios close to or equal to 1 were taken for AFM measurements after drying in air for 2 h. Monolayer with and without 1% PCPE fluidizing agent were deposited onto mica for AFM studies. AFM measurements were made at room temperature (25°C) using an optical lever microscope (Nanoscope IV; Digital Instruments, Santa Barbara, CA) and contact mode imaging. Topographic images were taken using an oxide-sharpened silicon nitride cantilever at a scan rate of 1 Hz.

Statistical Analysis

The data are expressed as mean ± standard deviation (SD). Statistical comparison for penetration enhancement by fluidizing agents/liposomal formulation was done using one-way analysis of variance (ANOVA) and Newman-Keuls test ($p < 0.05$ was taken as the cut-off for significance).

Results

Free Drug Penetration

Figure 2 depicts the paclitaxel penetration into cancerous and normal cervical lipid monolayers at 20 and 30 mN/m. Paclitaxel penetration in the absence of fluidizing agents leads to $(\Delta\pi)_{max}$ of only 0.89 ± 0.01 and 0.70 ± 0.01 mN/m at initial surface pressures of 20 and 30 mN/m, respectively, in cancerous cervical model membranes. Likewise, in the case of normal cervical model membranes, the $(\Delta\pi)_{max}$ due to free paclitaxel penetration at initial surface pressures of 20 and 30 mN/m were 1.72 ± 0.04 and 0.87 ± 0.02 mN/m, respectively. The free paclitaxel penetration in cervical model membranes was found to be much less compared to that in conventional DPPC model membranes at initial surface pressures of 20 and 30 mN/m. Further, reorganization due to the penetrated drug, as evidenced by a decrease in $\Delta\pi$ values with time, was also observed in cervical model membranes as opposed to the asymptotic penetration equilibrium in conventional DPPC model membranes (Preetha, Huilgol & Banerjee, 2006).

Effect of Fluidizing Agents on Cancerous and Normal Cervical Monolayers

Significantly different drug penetration kinetics was observed in the presence of fluidizing agents for the two types of monolayers in our study. In cancerous lipid monolayer at 20 mN/m, PCPG and ALEC curves showed horizontal regions with respect to the time axis after ~15 min, whereas the other two fluidizing agents showed increased $\Delta\pi$ values with respect to time in the presence of drug. This indicates the establishment of equilibrium drug penetration in PCPG and ALEC curves, whereas drug penetration goes on increasing in the other two cases (Fig. 3). In normal cervical lipid monolayer at 20 mN/m, there was increased drug penetration until 50 min in the presence of PCPE and Exosurf, whereas in the presence of PCPG and ALEC equilibrium drug penetration was attained after ~25 min (Fig. 4). Similarly, all fluidizing agents overcame the complete exclusion of the penetrated

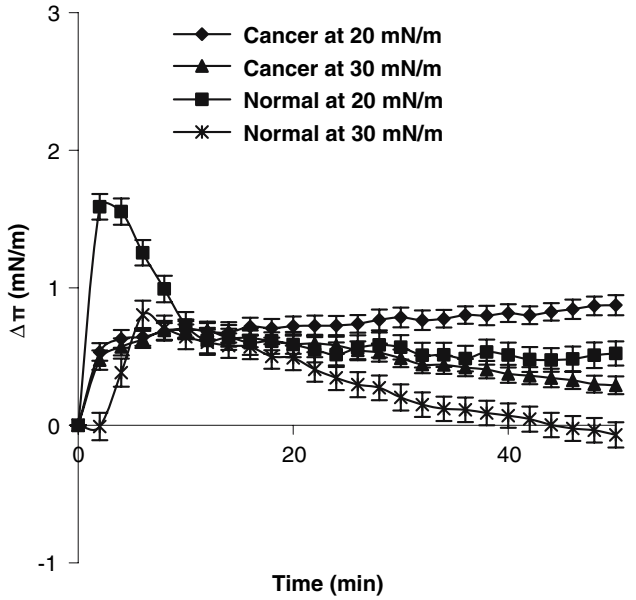

Fig. 2 Paclitaxel penetration into cancerous and normal model membranes in the absence of fluidizing agents. Each curve is a mean of three different trials

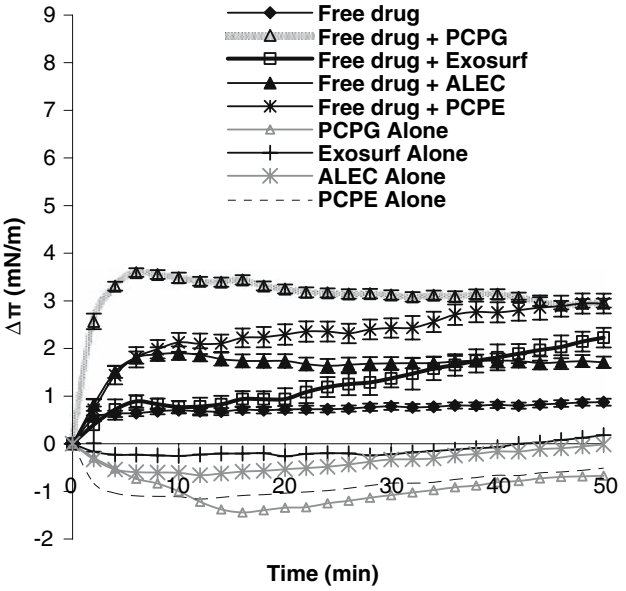

Fig. 3 Effect of fluidizing agents on cervical cancerous lipid monolayers at 20 mN/m. Each curve is a mean of three different trials

drug and destabilization due to free drug in the normal cervical monolayer at 30 mN/m. All four fluidizing agents gave negative $\Delta\pi$ values when present alone in both cancerous and normal monolayers. This negative $\Delta\pi$ value implies membrane destabilization and reorientation. In cancerous lipid monolayers at 20 mN/m, the membrane destabilizing ability was found to be in the order PCPG > PCPE > ALEC > Exosurf (Fig. 3). Similarly, from Figure 4 it is clear that the membrane destabilization in normal cervical monolayers at 20 mN/m is in the order PCPE > PCPG > Exosurf = ALEC.

The effects of different fluidizing agents on $(\Delta\pi)_{max}$ values are summarized in Table 1. Presence of fluidizing agents significantly improved drug penetration in both cervical monolayer systems. At 20 mN/m all fluidizing agents showed significant increase in $(\Delta\pi)_{max}$ values for cancerous monolayers. The $(\Delta\pi)_{max}$ values in the presence of PCPG, PCPE, Exosurf and ALEC were 4.1, 3.3, 2.5 and 2.2 times higher than the control values, respectively. However, at 30 mN/m PCPG and PCPE showed significant increases (2.1 and 1.7 times higher, respectively), whereas ALEC and Exosurf gave similar $(\Delta\pi)_{max}$ values compared to free drug. In normal cervical lipid model membranes, all four fluidizing agents studied increased paclitaxel penetration at 20 and 30 mN/m. At 20 mN/m PCPE gave maximum drug penetration (3.8 times higher than control) in comparison with other fluidizing agents.

The effect of increasing amount of fluidizer (PCPE) on paclitaxel penetration to cancerous cervical model membranes at initial surface pressure of 20 mN/m was

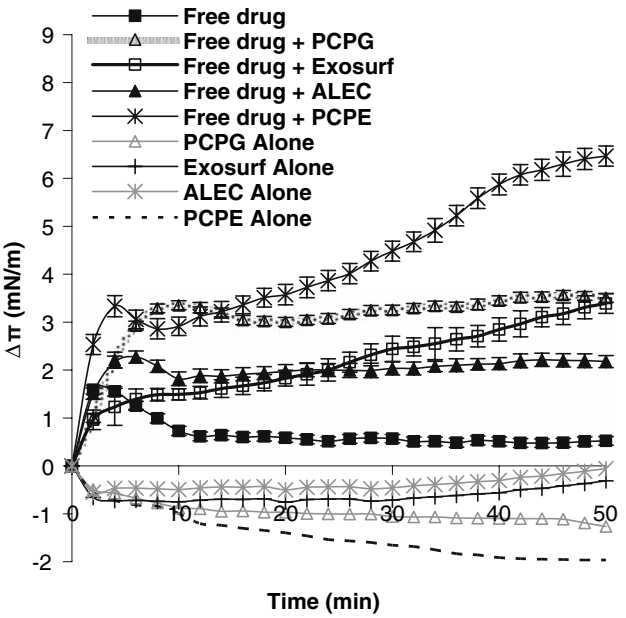

Fig. 4 Effect of fluidizing agents on normal cervical lipid monolayers at 20 mN/m. Each curve is a mean of three different trials

evaluated, and the results are depicted in Figure 5. It can be noted that the drug penetration increases with increase in fluidizer concentration until 5%. The $(\Delta\pi)_{max}$ values in the presence of 0.1%, 0.5%, 1%, 5% and 10% of PCPE were found to be 3.4-, 5.7-, 7.4-, 9.6- and 9.8-fold higher than that in the absence of PCPE, respectively. The $(\Delta\pi)_{max}$ values in the presence of 5% and 10% PCPE were found to be 8.4 ± 0.2 and 8.5 ± 0.3 mN/m, respectively, and were

Table 1 Effect of four different fluidizing agents on $(\Delta\pi)_{max}$

Fluidizer (0.1%)	$(\Delta\pi)_{max}$ at 20 mN/m (mN/m)		$(\Delta\pi)_{max}$ at 30 mN/m (mN/m)	
	Cancer	Normal	Cancer	Normal
Control	0.89 ± 0.09	1.72 ± 0.10	0.70 ± 0.10	0.87 ± 0.1
PCPG	3.61 ± 0.10*	3.61 ± 0.10*	1.42 ± 0.09*	1.97 ± 0.09*
PCPE	2.95 ± 0.20*	6.47 ± 0.20*	1.17 ± 0.08*	1.74 ± 0.06*
ALEC	1.91 ± 0.10*	2.29 ± 0.20*	0.71 ± 0.10	1.23 ± 0.08*
Exosurf	2.23 ± 0.10*	3.39 ± 0.10*	0.65 ± 0.10	1.40 ± 0.08*

Data expressed as mean ± SD from three trials. *Significantly different compared to the control

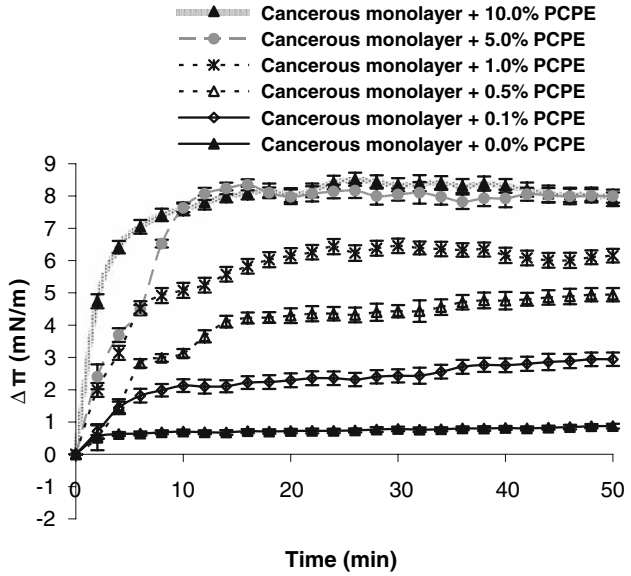

Fig. 5 Effect of amount of fluidizing agent on paclitaxel penetration to cancerous cervical monolayers at 20 mN/m. Data are expressed as mean ± SD. Each curve is a mean of three different trials

not significantly different as evidenced by ANOVA and Newman-Keuls test ($p < 0.05$).

Effect of Liposomal Formulations on Paclitaxel Penetration

In cervical cancerous model membranes at initial surface pressure of 20 mN/m, PCPG produced 1.6, 1.5 and 1.2 times higher drug penetration compared to Exosurf, ALEC and PCPE fluidizing agents, respectively. In normal cervical membranes, PCPE gave the maximum drug penetration in comparison with other fluidizing agents. Thus, PCPG and PCPE were the preferred fluidizers for the cervical model membranes. Hence, the feasibility of using them as liposomal carriers was also evaluated as part of this study. Particle size analysis showed uniform size distribution and the mean particle size of the liposomes to be 120.6 ± 5.4 nm. At 20 mN/m the PCPG liposomal formulation containing 1 mg lipid increased penetration compared to the free drug in cancerous cervical model membranes. The $\Delta\pi$ value was found to increase with time until ~30 min and to stabilize by 50 min. Similar results were obtained at initial surface pressure of 30 mN/m also.

In cancerous model membranes PCPG liposomes (1 mg lipid) gave $(\Delta\pi)_{max}$ values of 1.87 ± 0.2 and 0.94 ± 0.2 mN/m at initial surface pressures of 20 and 30 mN/m, respectively, and were 2.1 and 1.3 times higher compared to the corresponding free drug values. This improvement in penetration was statistically significant by one-way ANOVA followed by Newman-Keuls test. For PCPE liposomes containing 1 mg lipid, 4.1-fold higher penetration was found compared to the free drug in cancerous model membranes at initial surface pressure of 20 mN/m. Thus, PCPG and PCPE liposomes showed a promising and significant increase in cancer model membrane penetration compared to the free drug at physiologically relevant initial surface pressures.

The bar diagram in Figure 6 depicts the effect of increasing amount of liposomal lipid on drug penetration to cancerous cervical model membranes at initial surface pressure of 20 mN/m. The $(\Delta\pi)_{max}$ values due to penetration of liposomal paclitaxel with 0.5, 1, 5 and 10 mg lipid into cancerous cervical model monolayers were 1.5-, 3.6-, 4.9- and 5.9-fold higher than that due to free drug, respectively.

Calcein Release Study

Figure 7 depicts the effect of PCPE on calcein permeability of the encapsulated liposomes. The control cancerous liposomes without fluidizer PCPE had a 12.5% calcein release at 15 min, and in the presence of PCPE the release was found to be increased. Calcein permeability was found to be directly proportional to the amount of the fluidizing agent added. In the presence of 0.1%, 0.5%, 1%, 5% and 10% of PCPE, the percentage calcein release was 1.4-, 1.9-,

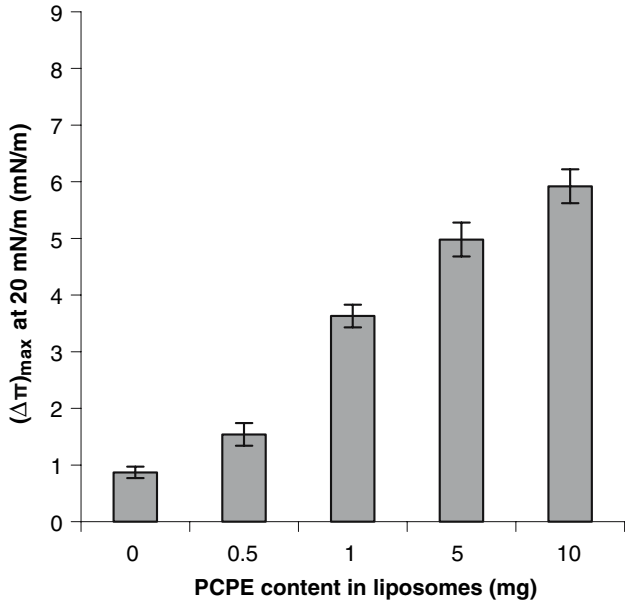

Fig. 6 Effect of fluidizer content in paclitaxel liposomes on penetration to cancerous cervical monolayer. Each bar is a mean of three different trials

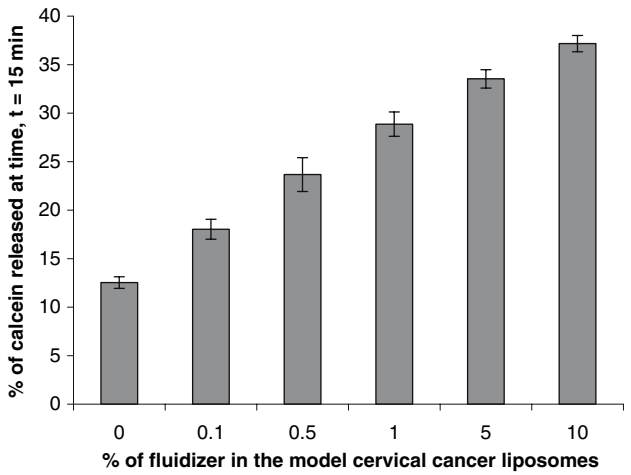

Fig. 7 Effect of fluidizing agent PCPE on calcein release from model cervical cancer liposomes. Data are expressed as mean ± SD from three trials

2.3-, 2.7- and 3.0-fold higher, respectively, compared to the calcein release from control liposomes.

Atomic Force Microscopy

Figure 8 depicts AFM images (2 x 2 μm) of cancerous cervical lipid monolayers with and without 1% PCPE. Cancerous cervical lipid monolayers without PCPE had a root mean square surface roughness of 8.3 nm/μm^2, whereas those with 1% PCPE had a root mean square surface roughness of 10.5 nm/μm^2. Also, in the monolayer with fluidizer more aggregates were observed as opposed to the control monolayer.

Discussion

We evaluated the effect of fluidizing agents and liposomal formulation on paclitaxel penetration into cervical model membranes. We used four fluidizing agents: PCPG (9:1 DPPC:PG), PCPE (9:1 DPPC:DOPE), ALEC (7:3 DPPC:PG) and Exosurf (13.5:1.5:1.0 DPPC:hexadecanol:tyloxapol). The selection of DPPC as a primary fluidizing agent in this study was based on a recent report of its excellent emulsifying effect with reference to the controlled release of paclitaxel from biodegradable nanospheres (Feng & Huang, 2001). The presence of DOPE in liposomal formulations is known to enhance the hydrophobicity of the liposomal membrane, thereby facilitating energetically favorable interactions (Simoes et al., 2004). ALEC and Exosurf are commercial lung surfactants. Presently, lung surfactants are being evaluated as drug delivery systems. For example, a recent study by Vermehren et al. (2006) established the promising role of HL10, a commercial lung surfactant, as a drug delivery system for amphipathic drugs. The presence of PG in ALEC and of hexadecanol in Exosurf is known to improve the adsorption of DPPC in exogenous surfactants due to their fluidizing effects (Bangham et al., 1984; Yu et al., 1984). Paclitaxel liposomal formulations containing PC and PG in 7:3 and 9:1 ratios have been reported to have favorable pharmacokinetic and pharmacodynamic properties (Singla, Garg & Aggarwal, 2002). The above-cited literature justifies our selection of materials in specific ratios. Our results reveal that all the agents used increased paclitaxel penetration into cervical model membranes. To enhance the penetration of drugs through the stratum corneum of the skin, usually penetration enhancers are used. For example, phloretin and 6-ketocholestanol are penetration enhancers for percutaneous delivery of certain topically applied drugs, and the penetration-enhancing effect of phloretin and 6-ketocholestanol is believed to be due to their increase of fluidity of the intercellular lipid bilayers of the stratum corneum (Auner et al., 2005). Similarly, permeation of the binary melt system of a drug, lidocaine, into a skin model membrane was reported to be increased by addition of propylene glycol (Kang, Jun & McCall, 2000). However, this concept of improving drug penetration using fluidizing agents has not been explored for other applications. Our study revealed the drug penetration-enhancing effects of the above four materials and suggests that they may be used as fluidizing agents to enhance paclitaxel penetration in cervical membranes.

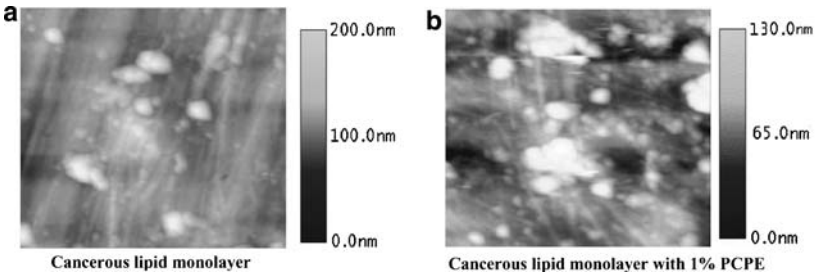

Fig. 8 AFM images of cancerous cervical lipid monolayers with and without fluidizing agent. AFM images are of 2 x 2 μm size

PCPG and PCPE were found to be good paclitaxel penetration enhancers for cervical lipid model membranes with severalfold higher $(\Delta\pi)_{max}$ compared to those in the absence of fluidizing agents. These phospholipid-based fluidizing agents may be coadministered with paclitaxel to improve its penetration in cancerous membranes.

The fluidizing agents used in this study not only showed greater drug penetration compared to free drug but also helped to overcome the adverse reorganization, due to the penetrated drug, in cervical model membranes. This fact is revealed by either constant or continuously increasing $\Delta\pi$ values with respect to time in the presence of fluidizing agents. When present in the cervical model membranes, these fluidizing agents may allow faster penetration of a large number of drug molecules so as to minimize the reorientation effect, thereby giving a penetration equilibrium or increase in penetration. The membrane destabilization effect of the four agents as evidenced by the negative $\Delta\pi$ values in the absence of drug also reveals their fluidizing effect. Further, the drug penetration enhancement was directly proportional to the amount of PCPE fluidizer added until 5%.

Another possible means of using the penetration enhancers is by developing their liposomal formulations. PCPG and PCPE liposomes showed increased penetration compared to free paclitaxel into cancerous cervical model membranes at 20 and 30 mN/m. Similar enhancement of penetration by liposomally entrapped calcein through keratin membrane has been reported (Kamagami et al., 1991). Also, an O/W emulsion formulation of the drug ibuprofen showed penetration enhancement of three- and tenfold compared to an aqueous ibuprofen suspension and a commercial alcohol-based ibuprofen formulation, respectively (Woolfson et al., 2000). Thus, formulations of drugs are known to increase their transport through membranes. Our results suggest that PCPG and PCPE liposomes of paclitaxel are promising formulations for improved drug penetration through cancerous cervical model membranes.

The penetration of drug depends on the type of monolayer. Zhao & Feng (2004, 2005) reported significant differences in paclitaxel penetration into model membranes of DPPC, DSPC and DMPC, indicating that even the difference in lipid chain length of monolayer material could significantly alter paclitaxel penetration. Thus, different lipid profiles could well give different paclitaxel penetration. We established different lipid profiles for cancerous and normal cervical lipid extracts through our previous studies (Preetha et al., 2006). The cholesterol, PC, phosphatidylethanolamine, phosphatidylinositol, PG, SM and phosphatidylserine levels in cancerous cervical tissues were 1.5, 3.6, 2.0, 2.3, 4.7, 1.7 and 2.2 times higher than those of normal cervical tissues, respectively (Preetha et al., 2005a, 2005b). The difference in paclitaxel penetration into cancerous and normal cervical model membranes could be due to their different lipid profiles. The lowering of paclitaxel penetration in DPPC model membranes due to the presence of cholesterol, which rigidifies the DPPC model membrane, has already been established by Zhao & Feng (2006). Furthermore, our earlier study also revealed the monolayer-rigidifying effect of SM, leading to lower paclitaxel penetration in two-component model membranes of DPPC and SM (Preetha et al., 2006). However, apart from cholesterol and SM, higher levels of other lipids in cancerous membranes may also affect paclitaxel penetration.

The calcein release experiments performed as a confirmation test for our Langmuir monolayer penetration results also revealed the effect of fluidizing agents on drug release. Calcein release from cervical cancerous model liposomes was found to increase with increase in amount of PCPE until 10%, similar to the Langmuir monolayer study where the drug penetration was found to increase until 5% of the fluidizer. In order to confirm the fluidizing effect of the agents on the cancerous cervical lipid extract, AFM imaging was performed. The cancerous cervical lipid extract monolayers with and without 1% PCPE were deposited onto mica sheets, and AFM images were taken. Surface roughness analysis showed higher roughness for the monolayer in the presence of PCPE compared to the control monolayer. A rough surface indicates more disorder in the monolayer packing compared to a smoother surface (Birdi, 2003). A higher number of aggregates was found in the presence of PCPE compared to the control monolayer. Such disorder and aggregation are suggestive of a fluidizing effect, which may account for the improved penetration of drugs through the monolayer.

Conclusion

In conclusion, our study revealed the poor drug penetration of free paclitaxel and enhanced penetration in the presence of fluidizing agents and liposomal formulations. PCPG and PCPE were found to be promising paclitaxel penetration enhancers for cervical lipid model membranes with severalfold higher $(\Delta\pi)_{max}$ values compared to those in the absence of fluidizing agents. PCPG and PCPE liposomal formulations also increased the paclitaxel penetration into cervical model membranes. The fluidizing effect revealed by Langmuir monolayer study was further confirmed by the calcein release from liposomes and surface roughness analysis by AFM. These results may have implications in using the above biocompatible lipids and surfactants as penetration enhancers along with anticancer drugs or as carriers for liposomal formulations of anticancer drugs for improved membrane penetration.

Acknowledgements This work was partly funded by the All India Council for Technical Education in the form of a Career Award for Young Teachers to R. B.

References

Auner BG, O'Neill MAA, Valenta C, Hadgraft J (2005) Interaction of phloretin and 6-ketocholestanol with DPPC-liposomes as phospholipid model membranes. Int J Pharm 294:149–155

Banerjee R, Bellare JR (2001) Comparison of in vitro surface properties of clove oil–phospholipid suspensions with those of ALEC, Exosurf and Survanta. Pulm Pharmacol Ther 14:85–91

Bangham AD, Miller NGA, Davies RJ, Greendig LA, Morley CJ (1984) Introductory remarks about artificial lung expanding compounds (ALEC). Colloids Surf 10:337–341

Birdi KS (2003) Scanning Probe Microscopes: Applications in Science and Technology. Boca Raton, FL: CRC Press, pp 51–93

Brannon-Peppas L, Blanchette JO (2004) Nanoparticle and targeted systems for cancer therapy. Adv Drug Deliv Rev 56:1649–1659

Crosasso P, Ceruti M, Brusa P, Arpicco S, Dosio F, Cattel L (2000) Preparation, characterization and properties of sterically stabilized paclitaxel-containing liposomes. J Control Release 63:19–30

Feng S, Huang G (2001) Effects of emulsifiers on the controlled release of paclitaxel (Taxol®) from nanospheres of biodegradable polymers. J Control Release 71:53–69

Fidelio GD, Maggio B, Cumar FA (1986) Molecular parameters and physical state of neutral glycosphingolipids and gangliosides in monolayers at different temperatures. Biochim Biophys Acta 854:231–239

Fonseca C, Simoes S, Graspar R (2002) Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity. J Control Release 83:273–286

Gambinossi F, Mecheri B, Nocentini M, Puggelli M, Caminati G (2004) Effect of the phospholipid head group in antibiotic-phospholipid association at water–air interface. Biophys Chem 110:101–117

Kamagami S, Okabe H, Kainose H, Kikkawa M, Ishigami Y (1991) Penetration enhancement across a model membrane by liposomally entrapped drugs using N,N′-diacylcystine as a bilayer lipid. FEBS Lett 281:133–136

Kang L, Jun HW, McCall JW (2000) Physicochemical studies of lidocaine–menthol binary systems for enhanced membrane transport. Int J Pharm 206:35–42

Koziara JM, Lockman PR, Allen DD, Mumper RJ (2004) Paclitaxel nanoparticles for the potential treatment of brain tumors. J Control Release 99:259–269

Lopez-Castellano A, Cortell-Ivars C, Lopez-Carballo G, Herraez-Dominguez M (2000) The influence of Span® 20 on stratum corneum lipids in Langmiur monolayers: comparison with Azone®. Int J Pharm 203:245–253

Park DC, Kim JH, Lew YO, Kim DH, Namkoong SE (2004) Phase II trial of neoadjuvant paclitaxel and cisplatin in uterine cervical cancer. Gynecol Oncol 92:59–63

Preetha A, Huilgol N, Banerjee R (2005a) Interfacial properties as biophysical markers of cervical cancer. Biomed Pharmacother 59:491–497

Preetha A, Banerjee R, Huilgol N (2005b) Surface activity, lipid profiles and their implications in cervical cancer. J Cancer Res Ther 1:180–186

Preetha A, Huilgol N, Banerjee R (2006) Comparison of paclitaxel penetration in normal and cancerous cervical model monolayer membranes. Colloids Surf B Biointerfaces 53:179–186

Ruan G, Feng S (2003) Preparation and characterization of poly (lactic acid)-poly (ethylene glycol)-poly(lactic acid) (PLA-PEG-PLA) microspheres for controlled release of paclitaxel. Biomaterials 24:5037–5044

Ruel-Gariepy E, Shive M, Bichara A, Berrada M, Garrec DL, Chenite A, Leroux J (2004) A thermosensitive chitosan-based hydrogel for the local delivery of paclitaxel. Eur J Pharm Biopharm 57:53–63

Savarrese A, Cognetti F (2003) New drugs in the treatment of recurrent or metastatic cervical cancer. Oncol Hematol 48:323–327

Simoes S, Moreira JN, Fonseca C, Duzgunes N, Pedroso-de-Lima MC (2004) On the formulation of pH-sensitive liposomes with long circulation times. Adv Drug Deliv Rev 56:947–965

Singla AK, Garg A, Aggarwal D (2002) Paclitaxel and its formulations. Int J Pharm 235:179–192

Takahashi A, Nemoto T, Fujiwara T (1994) Biophysical properties of protein-free, totally synthetic pulmonary surfactants, ALEC and Exosurf, in comparison with surfactant TA. Acta Paediatr Jpn 36:613–618

Vermehren C, Frokjaer S, Aurstad T, Hansen J (2006) Lung surfactant as a drug delivery system. Int J Pharm 307:89–92

Woolfson AD, Malcolm RK, Campbell K, Jones DS, Russell JA (2000) Rheological, mechanical and membrane penetration properties of novel dual drug systems for percutaneous delivery. J Control Release 67:395–408

Yu S, Harding PGR, Possmayer F (1984) Artificial pulmonary surfactant: potential role for hexagonal H_{II} phase in the formation of a surface-active monolayer. Biochim Biophys Acta 776:37–47

Zhang JA, Anyarambhatla G, Ma L, Ugwu S, Xuan T, Sardone T, Ahmad I (2004) Development and characterization of a novel Cremphor EL free liposome–based paclitaxel (LEP-ETU) formulation. Eur J Pharm Biopharm 10:1–11

Zhao L, Feng S (2004) Effects of lipid chain length on molecular interactions between paclitaxel and phospholipid within model Biomembranes. J Colloid Interface Sci 274:55–68

Zhao L, Feng S (2005) Effects of lipid chain unsaturation and headgroup type on molecular interactions between paclitaxel and phospholipid within model biomembrane. J Colloid Interface Sci 285:326–335

Zhao L, Feng S (2006) Effects of cholesterol component on molecular interactions between paclitaxel and phospholipid within the lipid monolayer at the air–water interface. J Colloid Interface Sci 300:314–326