ELSEVIER

International Journal of Pharmaceutics 241 (2002) 311–317

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Nebulization of niosomal all-trans-retinoic acid: an inexpensive alternative to conventional liposomes

Tejas R. Desai, Warren H. Finlay *

Department of Mechanical Engineering, Aerosol Research Laboratory of Alberta, University of Alberta, Edmonton, Alta., Canada T6G 2G8

Received 9 October 2001; received in revised form 2 April 2002; accepted 2 May 2002

Abstract

In this study we have demonstrated the potential of encapsulating all-trans-retinoic acid (ATRA) in niosomes and delivering it as an inhaled aerosol. Niosomes may provide a means to reduce the toxicity of ATRA and alter the pharmacokinetics in a manner similar to liposomes. In addition, the low cost of the surfactants used for preparing niosomes and their greater stability compared with liposomes makes them an attractive alternative. Various nonionic surfactants were used to achieve optimum encapsulation and nebulization efficiencies, and the best formulations were obtained with combinations of (Span 20 + Tween 80) and (Span 60 + Tween 80) using an ATRA concentration of 1 mg/ml. The aerosol produced with the selected niosomal formulations upon nebulization in PARI LC STAR nebulizers driven by a Pulmo-Aide compressor was subsequently analyzed for the determination of size distribution and entrapment efficiencies on each stage of an Anderson cascade impactor operated in a manner that avoids spurious sizing due to droplet evaporation. Mass median aerodynamic diameters (MMADs) of 3.7 ± 0.3 and 3.58 ± 0.03 μm, geometric standard deviation (GSD) values of 1.59 ± 0.17 and 1.51 ± 0.01 and entrapment efficiencies well above 50% were obtained for the optimized formulations. The results are very encouraging and offer an alternative approach to the respiratory delivery of ATRA by aerosolization.

Keywords: Niosomes; All-trans-retinoic acid; Aerosols; Nebulization

1. Introduction

The use of aerosols for the treatment of diseases has increased dramatically in the last few years due to several advantages of this application route (Finlay, 2001). For locally active drugs, a significant advantage of the aerosol mode of delivery is its ability to give much higher local levels of the drug than those achieved by systemic administration (Gilbert et al., 1991). Aerosolized liposomes offer the additional advantages of targeted drug delivery and amplified therapeutic effect (Weinstein and Leserman, 1984). Although the application of liposomes for improved aerosol drug delivery is encouraging, the nebulization of liposomes exhibits some difficulties, including the

* Corresponding author. Tel.: +1-780-492-4707; fax: +1-780-492-2200

E-mail address: warren.finlay@ualberta.ca (W.H. Finlay).

0378-5173/02/$ - see front matter
PII: S0378-5173(02)00245-4

instability of aqueous dispersions on storage and the leakage of the encapsulated drug on increase of the air flow pressure (Niven et al., 1992). Moreover, the high cost of synthetic phospholipids and variable purity of natural phospholipids have raised concerns over the adoption of liposomal drug delivery systems.

An alternative approach that overcomes several of these problems associated with liposomes involves the formation of liposome-like vesicles from nonionic surfactants, commonly referred to as niosomes (Baillie et al., 1985; Uchegbu et al., 1995). Preliminary in vivo studies indicate that niosomes behave like liposomes, prolonging the circulation of the entrapped drug and altering its organ distribution and metabolic stability (Azmin et al., 1975; Rogerson et al., 1988). In addition, greater chemical stability, ease of production and extremely low cost make niosomes an attractive colloidal carrier compared with liposomes. Niosomal delivery of drugs has been extensively explored previously for many therapeutic agents (Rogerson et al., 1987, 1988; Jain and Vyas, 1995; Pillai and Salim, 1999). However, the feasibility of aerosolizing niosomes has not been explored to the authors' knowledge.

Retinoids, the natural and synthetic analogs of Vitamin A, represent a potentially useful class of drugs in chemoprevention and treatment of cancer, due to their ability to regulate cell proliferation and differentiation (Meng-er et al., 1988; Hong and Itri, 1994; Sun and Lotan, 1998; Lokshin et al., 1999). All trans-retinoic acid (ATRA) and its derivatives are potentially useful therapeutic agents and have been extensively studied for lung cancer and malignant disorders (Kalemkerian et al., 1994; Athanasiadis et al., 1995; Ou et al., 1996; Treat et al., 1996; Lokshin et al., 1999). Treat et al. (1996) reported the phase II clinical trial of ATRA in metastatic non-small cell lung cancer (NSCLC) with 28 patients. However, like many other anticancer drugs, chronic administration of retinoids in patients is associated with toxic effects (Lippman et al., 1987). Liposomal incorporation has been proposed as an alternative way of delivering retinoids to reduce these toxic effects (Lopez-Berestein et al. 1994; Wasan et al., 1998; Wallace et al., 2000), and an ATRA-liposomal formulation is in phase II clinical trial for the treatment of lung cancer. Parthasarathy et al. (1999) reported the delivery of liposomal ATRA by aerosolization that offers an effective way to deliver high levels of drug to the lung without apparent toxic effects. In this paper, we explore the possibility of incorporation of ATRA in niosomes and investigate the in vitro delivery of niosomal ATRA by nebulization.

2. Materials and methods

2.1. Materials

Spans 20, 40, 60, ATRA, Tweens 20 and 80 were purchased from Aldrich Chemicals (Milwankee, WI, USA). Cholesterol, sodium chloride and stearylamine were purchased from Sigma Chemicals (St. Louis, MO, USA).

2.2. Methods

2.2.1. Preparation of niosomes

Niosomes were prepared by the lipid hydration method (Pillai and Salim, 1999). The method briefly was as follows: surfactants and ATRA were dissolved in chloroform in a round bottom flask, and the solvent was removed under vacuum in a rotary vacuum evaporator on a water bath at 40 °C to form a thin film on the wall of the flask. Residual chloroform was evaporated in a vacuum oven for 4–8 h at ambient temperature. The film was then hydrated with 0.9% saline, and the dispersion was then sonicated in an ultrasound bath at 55 °C for 30 min. The dispersion was then left to stand at room temperature (23 ± 1 °C) for 1 h. The unentrapped drug was removed by centrifuging (Beckman Coulter, CA, USA) the dispersion at 15 300 rpm and 4 °C for 90 min. The supernatant was then removed and the dispersion was reconstituted at its original concentration to achieve an encapsulation efficiency of at least 95%. The encapsulation efficiency was determined by measuring the amount of ATRA in the supernatant and residue by UV absorption at 350 nm using a UV Spectrophotometer (Hewlett Packard 8452A).

2.2.2. Nebulization of niosomal ATRA

A volume fill of 2.5 ml of niosomal ATRA (1 mg ATRA per ml) was nebulized with Pari LC STAR (Pari, Starnberg, Germany) jet nebulizers, using a DeVilbiss Pulmo Aide Compressor (Model 5650D). Three different nebulizer units were used in this study. The Pari LC STAR nebulizer is reported to have shown superior performance in previous studies (Finlay and Wong, 1998; Finlay et al., 2000). The aerosol was collected on Respigard filters (Marquest Medical Products, Englewood, CO). Usually two to three filters, connected to each other, were required for this purpose, since the surfactants tend to make the filters more hydrophilic. The filter contents were extracted with 10 ml of 0.9% saline to determine the nebulization efficiency. Nebulization efficiency of a niosomal formulation is defined as the total output of drug (ATRA) collected on the filters from the nebulizer calculated as a percentage of the total submitted to nebulization. In other words, after assaying ATRA content extracted from the filters, nebulization efficiency may be determined as:

$$\text{Nebulization efficiency } (\%) = \frac{\text{Aerosolized ATRA (i.e. collected on the filters)}}{\text{Total ATRA nebulized}} \times 100$$

To determine the leakage during nebulization, a portion of the sample was centrifuged at 15 300 rpm and 4 °C for 90 min. The supernatant was assayed for free ATRA, while the pellet was dissolved in methanol and assayed for encapsulated ATRA. The percentage of the drug encapsulated was calculated as the ratio of the drug in the pellet to the sum of the drug in the pellet and drug in the supernatant. Samples of the original unnebulized preparations were submitted to the same procedure.

2.2.3. Particle size measurement of the nebulized niosomes

To determine the particle size distribution, the nebulizers were connected directly to an Andersen Cascade Impactor (Andersen Mark II, Graseby Andersen, Smyrna, GA), running the nebulizers intermittently to avoid droplet shrinkage due to cooling in the impactor (Finlay and Stapleton, 1999). The impactor flow rate was calibrated to 28.3 l/min using a dry gas meter (DTM-115, Singer, American Motor Division). Each LC Star nebulizer was filled with 2.5 ml of niosomal ATRA preparation and connected to the Pulmo-Aide compressor. Each sample was nebulized for 30 s and then the sample was allowed to equilibrate to ambient temperature for the following 9 min 30 s, before the next cycle. Five such cycles were performed. As mentioned above, this procedure eliminated the cooling effect that otherwise compromises particle size measurements of nebulizers with the Anderson cascade impactor (Finlay and Stapleton, 1999). The mass median aerodynamic diameters (MMAD) of aerosol droplets were determined by extracting the content of each plate and assaying for ATRA by UV Spectrophotometry. To determine the entrapment efficiency of ATRA on the various stages of the impactor, the fractions collected on each plate were extracted with 0.9% saline. These extracts were then centrifuged at 15 300 rpm and 4 °C for 90 min, and the resulting supernatant and pellet were assayed separately for ATRA using UV Spectrophotometry ($\lambda_{max} = 350$ nm). Statistical tests were performed using single factor analysis of variance (ANOVA) and Tukey HSD means comparisons. Results are presented in Table 2 as mean $\pm$ S.D.

2.2.4. Transmission electron microscopy (TEM)

The morphology of the hydrated niosome dispersions was examined using TEM. A drop of niosome dispersion was applied to a carbon-coated copper grid and left for 1 min to allow some of the particles to adhere to the carbon substrate. The excess of dispersion was removed by absorbing the drop with a piece of filter paper. A drop of 2% phosphotungstic acid solution was applied and again excess solution was removed by absorbing the liquid with the tip of a filter paper and the sample was air-dried. The sample was then observed under a Hitachi H-7000 electron microscope at 75 kV.

3. Results and discussions

Various niosomal formulations were prepared and compared in terms of encapsulation efficiency, leakage upon nebulization and nebulization efficiency. Niosomal preparation containing Span 20 and cholesterol at a concentration of 30 mM, and molar ratio of 1:1, formed large agglomerates during nebulization, thereby showing extremely low nebulization efficiency. Previously, similar problems associated with nebulization of liposomal formulations containing cholesterol were observed by Lange et al. (2001). Due to the anionic nature of ATRA, it may be assumed that incorporation of positive charge to the niosomes will increase its uptake, since it is known that the inclusion of a charged lipid into the lipid bilayers causes the electrostatic separation of bilayers, and is a method by which uptake of drugs may be improved (Johnson, 1973; Alpar et al., 1981). To verify this assumption, various formulations with Spans (20,40 and 60) with stearylamine in a molar ratio of 9:1 were prepared. For all the formulations, the concentration of ATRA was kept constant at 1 mg/ml. These formulations indeed showed high encapsulation efficiency, but showed poor nebulization efficiencies.

In an attempt to achieve a nebulizable formulation, the water soluble nonionic surfactant Tween 80 was incorporated in Spans (20,40 and 60). After optimization of the various parameters, two formulations, shown in Table 1, were obtained that showed excellent nebulization efficiencies. These two formulations were evaluated in terms of nebulization efficiency, encapsulation efficiency of nebulized niosomes collected on the filter and encapsulation efficiency of the unnebulized preparation left in the reservoir after nebulization. The results are shown in Table 2.

Table 1
Formulations used for nebulization of niosomal ATRA

Component	Formulation 1 concentration (mg/ml)*	Formulation 2 concentration (mg/ml)*
Span 20	13.24	0.00
Span 60	0.00	7.24
Tween 80	8.12	8.12
ATRA	1.00	1.00

*, Values give concentration of surfactants before centrifugation.

Table 2
Comparison of various parameters following nebulization of niosomal Formulations 1 and 2 (Mean ± S.D., $n = 3$)

Parameter	Formulation 1	Formulation 2
Nebulization efficiency (%)	36.9 ± 2.6	41.5 ± 5.4
Entrapment of ATRA in nebulized niosomes (%)	73.4 ± 1.9	58.2 ± 1.9
Entrapment of ATRA in unnebulized dispersion (%)	89.0 ± 0.9	91.8 ± 2.6
MMAD (μm)	3.7 ± 0.3	3.58 ± 0.03
Geometric standard deviation (GSD)	1.59 ± 0.17	1.51 ± 0.01

As can be seen, both Formulations 1 and 2 exhibit excellent nebulization efficiencies of 36.9 ± 2.6 and $41.5 \pm 5.4\%$ respectively, and are comparable to the values reported for other nebulizable liposomal formulations (Lange et al., 2001; Finlay and Wong, 1998). It is also seen from Table 2 that both the formulations show a considerable decrease in entrapment efficiency for the nebulized niosomes extracted from the filter. Figs. 1 and 2 show the distribution of total and encapsulated drug on each stage of Anderson impactor for Formulations 1 and 2, respectively. It has been previously reported that nebulization of liposomal dispersions can cause leakage of the entrapped drug due to vesicle fragmentation, which may occur due to shock waves and kinematic discontinuities associated with droplet impaction on nebulizer baffles (Finlay, 2001, p-209) or as liquid is drawn up the nebulizer liquid inlet tube and mixed with the high speed air-jet (Taylor et al., 1990). Similar fragmentation of niosomal vesicles can be assumed to have occurred. Another cause of leakage could be due to the dilution effect (Taylor et al., 1990), since during extraction of the material deposited on the filter or Anderson impactor, the niosomes were in dilute conditions. To

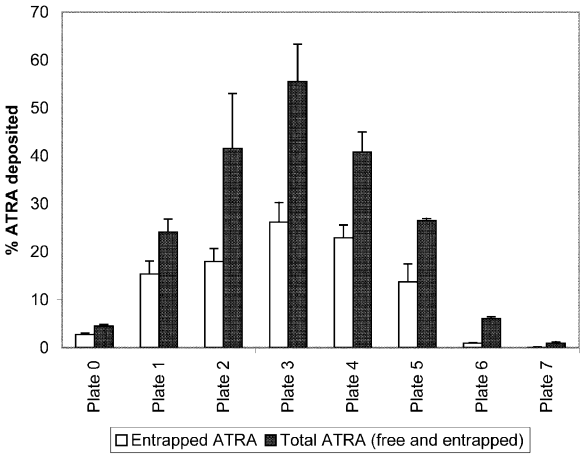

Fig. 1. Deposition of niosome entrapped (□) and total (■) ATRA in the Anderson cascade impactor following nebulization of Formulation 1. Particle size cut-offs are 9.0, 5.8, 4.7, 3.3, 2.1, 1.1, 0.7, 0.4 μm for plates 0–7 respectively.

verify this latter effect, niosomal preparations were subjected to 10 fold dilutions. It was observed that both the preparations showed approximately 10% leakage upon 10 fold dilutions. This observation indicates that dilution is one of the causes of leakage.

Table 2 also shows the MMADs and GSDs of both the formulations. Independent assessment of the size distribution of the aqueous phase and the niosomes in aerosols showed no statistically significant difference ($P = 0.87$) in their MMADs, indicating that the drug is homogeneously distributed in the aerosol droplets.

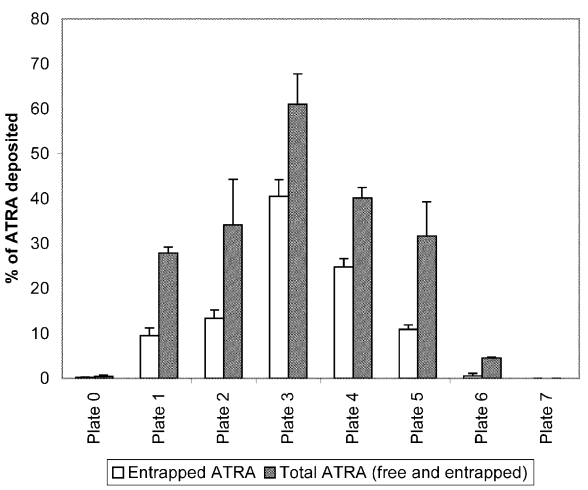

Fig. 2. Deposition of noisome entrapped (□) and total (■) ATRA in the Anderson cascade impactor following nebulization of Formulation 2. Particle size cut-offs of the plates are given in the caption of Fig. 1.

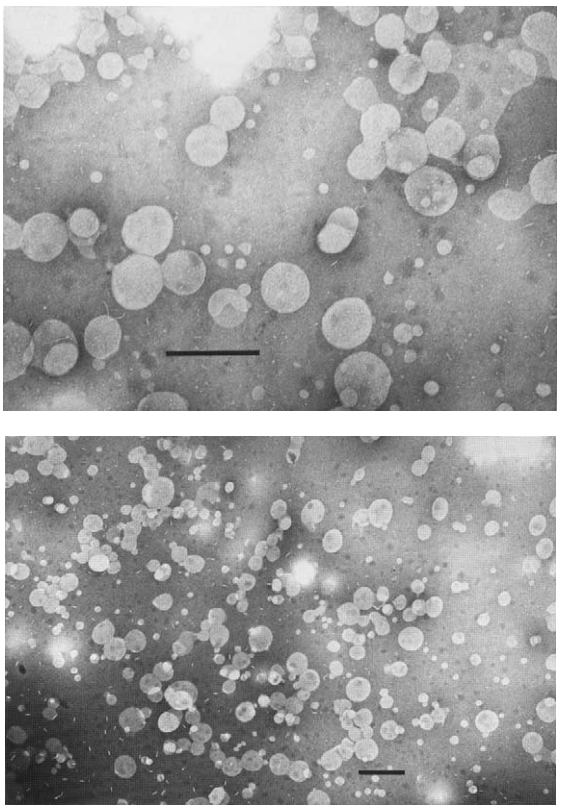

Fig. 3. (a) and (b): Transmission electron micrographs of niosomes obtained from (a) Formulation 1 and (b) Formulation 2. Scale bar indicates 0.5 μm.

Transmission electron micrographs (Fig. 3(a) and (b)) reveal that the niosomes prepared by the hydration method are spherical in shape. The average vesicle sizes, as obtained from the magnification data of electron microscopy, were in the range of 210–270 and 170–250 nm for Formulations 1 and 2 respectively.

4. Conclusions

In this paper, we have shown that ATRA can be entrapped in niosomes in high amounts when particular surfactant combinations (Span 20/60 +

Tween 80) are used. The results also demonstrate that the optimized niosomal formulations can be nebulized efficiently with good entrapment of ATRA (> 50%) in aerosol droplets of appropriate size for inhalation therapy. This opens the door for future in vivo testing of the respiratory delivery of niosomal-ATRA formulations for lung cancer treatment.

Acknowledgements

The authors wish to thank Helena Orszanska for her excellent laboratory help. The authors gratefully acknowledge the financial support by the Natural Science and Engineering Research Council of Canada.

References

Alpar, O.H., Bamford, J.B., Walters, V., 1981. In vitro incorporation of release of hydroxycobalamin by liposomes. Int. J. Pharm. 7, 349–351.

Athanasiadis, I., Kies, M.S., Miller, M., Ganzenko, N., Joob, A., Marymont, M.A., Rademaker, A., Gradishar, W.J., 1995. Phase II study of all-trans-retinoic acid and α-interferon in patients with advanced non-small cell lung cancer. Clin. Cancer Res. 1, 973–979.

Azmin, M., Florence, A., Handjani, R., Vila, R.M., Stuart, J., Vanlerberghe, G., Whittaker, J., 1975. The effect of non-ionic surfactant vesicle (niosomes) entrapment on the absorption and distribution of methotrexate in mice. J. Pharm. Pharmacol. 37, 237–242.

Baillie, A., Florence, A., Hume, L., Muirhead, G., Rogerson, A., 1985. Preparation and properties of niosomes—non-ionic vesicle. J. Pharm. Pharmacol. 37, 863–868.

Finlay, W.H., 2001. The Mechanics of Inhaled Pharmaceutical Aerosols: an Introduction. Academic Press, UK.

Finlay, W.H., Wong, J.P., 1998. Regional lung deposition of nebulized liposome encapsulated ciprofloxacin. Int. J. Pharm. 167, 121–127.

Finlay, W.H., Stapleton, K.W., 1999. Undersizing of droplets from a vented nebulizer caused by aerosol heating during transit through an Anderson impactor. J. Aerosol. Sci. 30, 105–109.

Finlay, W.H., Lange, C.F., King, M., Speert, D.P., 2000. Lung delivery of aerosolized dextran. Am. J. Respir. Crit. Care Med. 161, 91–97.

Gilbert, B.E., Wyde, P.R., Wilson, S.Z., Robins, R.K., 1991. Aerosol and intraperitoneal administration of ribavarin and ribavarin triacetate: pharamcokinetics and protection of mice against intracerebral infection with influenza A/WSN virus. Antimicrob. Agents Chemother. 35, 1448–1453.

Hong, W.K., Itri, L.M., 1994. Retinoids and human cancer. In: Sporn, M.B., Roberts, A.B., Goodman, D.S. (Eds.), The Retinoids: Biology, Chemistry and Medicine, second ed. Raven Press, New York, pp. 597–624.

Jain, C.P., Vyas, S.P., 1995. Preparation and characterization of niosomes containing rifampicin for lung targeting. J. Microencap. 12, 401–407.

Johnson, S.M., 1973. The effect of charge and cholesterol on the size and thickness of phospholipid vesicles. Biochim. Biophys. Acta 307, 27–41.

Kalemkerian, G.P., Jasti, R.K., Celano, P., Nelkin, B.D., Marby, M., 1994. All-trans-retinoic acid alters myc gene expression and inhibits in vitro progression in small cell lung cancer. Cell Growth Differ. 5, 55–60.

Lange, C.F., Hancock, R.E.W., Samuel, J., Finlay, W.H., 2001. In vitro delivery and regional airway surface liquid concentration of a liposomal cationic peptide. J. Pharm. Sci. 90, 1647–1657.

Lippman, S.M., Kessler, J.F., Meyskens, F.L., 1987. Retinoids as preventive and therapeutic anticancer agents. Caner Treat. Reports 71, 493–515.

Lokshin, A., Zhang, H., Mayotte, J., Lokshin, M., Levitt, M.L., 1999. Early effects of retinoic acid on proliferation, differentiation and apoptosis in non-small cell lung cancer cell lines. Anticancer Res. 19, 5251–5254.

Lopez-Berestein, G., Rosenblum, M., Sadeghi, T., Mehta, K., 1994. Pharmacokinetics, tissue distribution and toxicology of tretinoin incorporated in liposomes. J. Liposome Res. 4, 689–700.

Meng-er, H., Ye, Y., Shu-rong, C., Jin-ren, C., Jia-Xiang, L., Lin, Z., Long-jun, G., Zheng-yi, W., 1988. Use of all-trans-retinoic acid in treatment of acute promyelocytic leukemia. Blood 72, 567–572.

Niven, R.W., Carvajal, M.A., Schreier, H., 1992. Nebulization of liposomes. III. The effects of operating conditions and local environment. Pharm. Res. 9, 515–520.

Ou, X., Campau, S., Slusher, R., Jasti, R.K., Marby, M., Kalemkerian, G.P., 1996. Mechanism of all-trans-retinoic acid-mediated L-myc gene regulation in small lung cancer. Oncology 13, 1893–1899.

Parthasarathy, R., Gilbert, B., Mehta, K., 1999. Aerosol delivery of liposomal all-trans-retinoic acid to the lungs. Cancer Chemother. Pharmacol. 43, 277–283.

Pillai, G.P., Salim, M.L.D., 1999. Enhanced inhibition of platelet aggregation in-vitro by niosome encapsulated indomethacin. Int. J. Pharm. 193, 123–127.

Rogerson, A., Cummings, J., Florence, A., 1987. Adriamycin loaded niosomes: drug entrapment, stability and release. J. Microencap. 4, 321–328.

Rogerson, A., Cummings, J., Willmott, N., Florence, A., 1988. The distribution of doxorubicin in mice following administration in niosomes. J. Pharm. Pharmacol. 40, 337–342.

Sun, S.Y., Lotan, R., 1998. Retinoids as chemopreventive and therapeutic agents. Drugs Future 23, 621–634.

Taylor, K.M.G., Taylor, G., Kellaway, I.W., Stevens, J., 1990. The stability of liposomes to nebulization. Int. J. Pharm. 58, 57–61.

Treat, J., Friedland, D., Luginbuhl, W., Meehan, L., Gorman, G., Miller, W., Bavaria, J., Kaiser, L., 1996. Phase II trial of all-trans-retinoic acid in metastatic non-small cell lung cancer. Cancer Invest. 14, 415–420.

Uchegbu, I., Jeoma, F., Florence, A., 1995. Nonionic surfactant vesicles (niosomes): physical and pharmaceutical chemistry. Adv. Coll. Int. Sci. 58, 1–55.

Wallace, T.L., Larson, J.L., Bazemore, S.A., Wilson, C.W., Cossum, P.A., 2000. The nonclinical safety evaluation of the anticancer drug ATRAGEN™ (liposomal all-trans-retinoic acid). Int. J. Toxicol. 19, 33–42.

Wasan, K.M., Ramaswamy, M., Ng, S.P., Wong, W., Parrott, S.C., Ojwang, J.O., Wallace, T., Cossum, P.A., 1998. Differences in the lipoprotein distribution of free and liposome-associated aa-trans-retinoic acid in human, dog and rat plasma are due to variations in lipoprotein lipid and protein content. Anitmicrob. Agents Chemother. 42, 1646–1653.

Weinstein, J.N., Leserman, L.D., 1984. Liposomes as drug carriers in cancer chemotherapy. Pharmacol. Ther. 24, 207–233.