Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 254 (2003) 49–53
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Note

In vitro and in vivo aspects of *N*-acyl-phosphatidylethanolamine-containing liposomes

Charlotte Vermehren[a,*], Harald S. Hansen[b], Brian Clausen-Beck[a], Sven Frøkjær[a]

[a] *Department of Pharmaceutics, The Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen, Denmark*
[b] *Department of Pharmacology, The Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen, Denmark*

Received 1 September 2002; accepted 11 November 2002

Abstract

Incorporation of the phospholipid, *N*-acyl-phosphatidylethanolamine (NAPE), has shown to increase the liposomal stability towards plasma components in vitro. Besides increasing the circulation-time, NAPE has been shown to contain fusiogenic properties. Hence, fusion between NAPE-liposomes and target cells may be expected, resulting in a favorable delivery of drug to the target cell. In this study, NAPE has been tested as a potential liposomal component of phosphatidylcholine-liposomes. The liposomes were characterized by size, long-term stability and phase transition temperature (T_m). In vivo behavior of NAPE-liposomes was determined by the blood-circulation half-life in mice.

A characterization of the liposomes revealed that high content of NAPE resulted in liposomes of increased size compared to pure phosphatidylcholine-liposomes. However, the liposomes showed only a slight increase in size during storage for 5 weeks. Determination of T_m for NAPE-liposomes showed increasing values of T_m by increasing percentage of NAPE in the liposomal bilayer, due to the higher T_m of NAPE compared to phosphatidylcholine. Blood-clearance studies showed an initial increase in blood-circulation of liposomes containing high amounts of NAPE.

Thus, these results suggest that liposomes containing high percentage of NAPE may be a promising candidate for long-circulating liposomes, possibly in combination with other stabilizing components, e.g. cholesterol.

Keywords: NAPE; Blood clearance

Using liposomes as drug delivery system, you have the advantages of manipulating the behavior of conventional liposomes by changing the liposomal composition. Hence, liposomes have the potential to overcome many of the requirements of a drug delivery system for the treatment of diseases like cancer and inflammation. Due to the membrane stabilizing effect and the fusiogenicity of the phospholipid *N*-acyl-phosphatidylethanolamine (NAPE), the incorporation of NAPE into liposomal compositions may result in long-circulating liposomes with increased delivery of the drug directly to the target cell compartment.

NAPE is a negatively charged phospholipid that contain a third fatty acid linked to the amine group. NAPE is found in small amounts in normal tissue. However, NAPE is found to accumulate at pathological conditions (Schmid et al., 1990). Thus, it has been suggested that NAPE may act as protective agents (Hansen et al., 1995, 1997).

* Corresponding author. Tel.: +45-3530-6000; fax: +45-3530-6030.
E-mail address: cv@dfh.dk (C. Vermehren).

0378-5173/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0378-5173(02)00681-6

Beside this, NAPE has been suggested as a fusiogen (Shangguan et al., 1998; Mora et al., 2000). *N*-Acylation can convert the H-II phase-generating lipid, phosphatidylethanolamine, into a bilayer-preferring lipid, NAPE (Shangguan et al., 1998). However, the interaction with Ca^{2+} can reverse the lamellar NAPE into a non-lamellar lipid, which have been proposed as intermediate for membrane fusion processes (Cullis and De Kruijff, 1979). Thus, NAPE may have a potential as component of liposomes in order to fuse and deliver drug into cells.

In general, fusion-mediating lipids show membrane destabilizing effects rather than stabilizing the membrane. However, NAPE has been found to stabilize a bilayer conformation in conditions in which fusion may be favored (Morillo et al., 1998). Until now the liposome stabilizing effect of NAPE has only been tested in vitro. In this study, the in vivo behavior, e.g. blood clearance and biodistribution, of liposomes containing different percentage of NAPE has been investigated, and the liposomes were characterized by determination of size and phase transition temperature (T_m).

Liposomes composed of dipalmitoylphosphatidylcholine (DPPC) and various amounts (0, 10, 25, 50 mol%) of *N*-palmitoylphosphatidylethanolamine (NPPE) were prepared by dissolving and mixing the phospholipids in chloroform. Dipalmitoylphosphatidyl[*N*-methyl-^{3}H]choline ([^{3}H]DPPC) was used as radioactive marker. Chloroform was removed overnight by rotation evaporation, and the resulting lipid film was first striped with ethanol and then left overnight in the rotation evaporator. The multilamellar liposomes were formed by hydration in buffer for at least 1 h. The hydration temperature was 10 °C above the T_m of the phospholipid. Subsequently, the NAPE-liposomes were sonicated for 10 min in intervals of 30 s using a tip sonicator. One hundred nanometer unilamellar liposomes were made from the multilamellar liposomes by extrusion through 100 nm polycarbonate filters. Size measurements were performed by dynamic light scattering (DLS) using a Zetasizer 4 (Malvern, UK). T_m were determined by Differential Scanning Caleometry (DSC; MicroCal™ Incorporated). NAPE was prepared by *N*-acylation of dipalmitoylphosphatidylethanolamine with palmitic acid anhydride. The reaction was catalyzed by triethylamin, in the molar ratio 1:2:11, and the resulting product was purified by thinlayer chromatography (TLC) (Shangguan et al., 1998).

Blood clearance of the liposomes was performed by i.v. injection of radioactive liposomes of the different compositions into the tail-vein of mice. The injected dose corresponded to 1.5 mg phospholipid and 2.5 μCi [^{3}H]DPPC per 30 g body weight. Blood samples were taken at different time points, and they were bleached with hydrogenperoxide before scintillation counting. At time 360 min the mice were sacrificed and the organs (liver, diaphragm, spleen, lung, heart, kidney, brain, intestine/stomach) were isolated. The organs were solubilized and bleached before radioactivity counting.

The resulting size of DPPC liposomes incl. 0, 10, 25 and 50 mol% NPPE was: 118.5 ± 2.0, 110.0 ± 0.9, 119.1 ± 0.4, 132.1 ± 2.9 nm ($n = 3$). Long-term stability of the liposomes tested at 4 and 25 °C showed for all liposome suspensions only a slight increase in size—between 5 and 12%—during the 5 weeks, and there was found no difference in stability when storing the liposomes at the different temperatures. A characterization of the liposomes by DSC revealed that increasing amounts of NPPE (Fig. 1) in the liposomal bilayer resulted in increasing values of T_m corresponding to 41.8, 55.2 and 60.8 °C for DPPC-liposomes incl. 10, 25 and 50 mol% NAPE.

Blood clearance studies of the ^{3}H-labeled liposomes containing 25 and 50 mol% NAPE showed an initial increase in blood circulation time (Fig. 2) corresponding to $t_{1/2}$ of 40 and 45 min, respectively. However, incorporation of 10 mol% NAPE did not change the clearance profile compared to pure DPPC-liposomes (Fig. 2). The $t_{1/2}$ corresponded to 11 and 13 min, respectively. The tissue distribution of the liposomes was investigated in mice by determination of the content of radioactivity in the isolated organs 6 h after i.v. administration of radioactive labeled liposome suspensions. The liver and stomach/intestine accumulated approximately 40 and 5–7% of the radioactivity, respectively. Only a slight amount of radioactivity was observed in the other organs. The biodistribution data revealed no difference between the different liposome compositions (Fig. 3).

DLS revealed that the sizes of the liposomes containing small amounts of NAPE were almost identical to pure DPPC liposomes. However, incorporating 50 mol% NAPE resulted in liposomes of

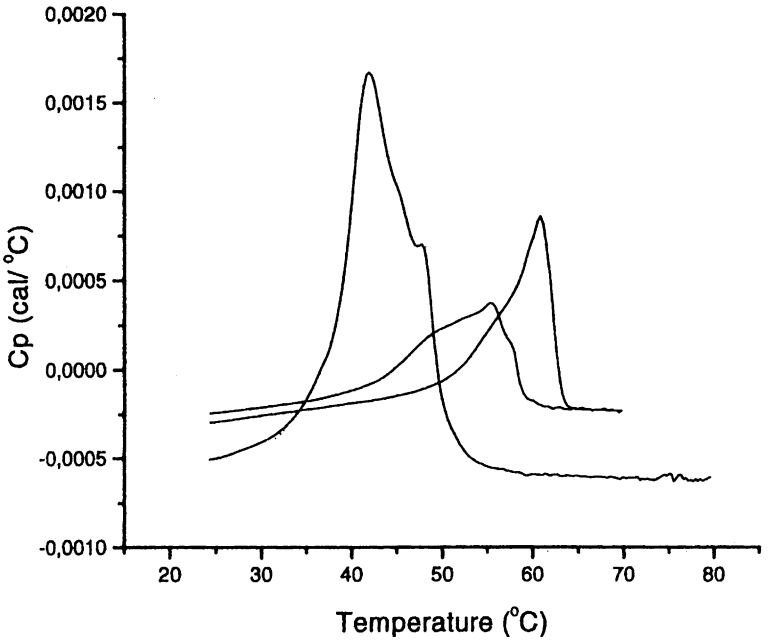

Fig. 1. Phase transition temperatures (T_m) (DSC) of liposomes composed of DPPC and 10, 25 and 50 mol% NAPE corresponding to 41.8, 55.2 and 60.8 °C, respectively.

slightly increased size. This is in agreement with Domingo et al. (1993) who have shown that phosphatidylcholine vesicles grow with increasing content of NAPE. The negatively charged NAPE may induce an electrostatic repulsion at the bilayer which may favor a reduced bilayer curvature resulting in a larger cross-sectional area compared to pure phosphatidylcholine-liposomes.

The T_m of NAPE-liposomes increased with increasing amounts of NAPE in the bilayer. The T_m

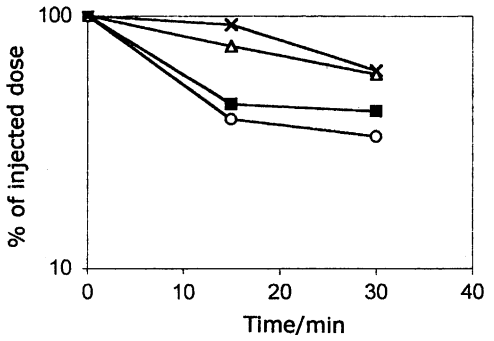

Fig. 2. Comparison of the initial blood clearance of liposomes composed of ^{3}H-DPPC and 0, 10, 25 and 50 mol% NAPE ($n = 6$). Data points give the relative amount of the i.v. administered lipid-associated radioactivity in the blood as a function of the time. (■) 0 mol% NAPE (~100 mol% DPPC), (○) 10 mol% NAPE, (△) 25 mol% NAPE, (×) 50 mol% NAPE.

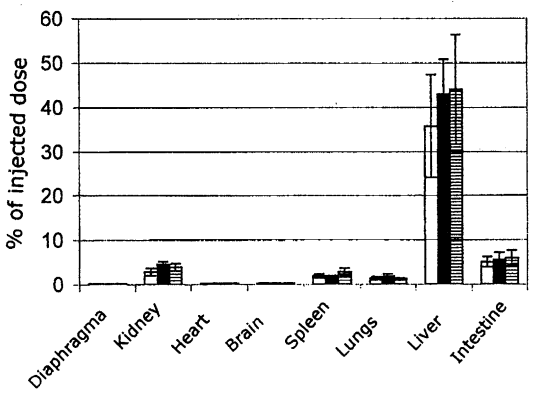

Fig. 3. Biodistribution of liposomes composed of ^{3}H-DPPC and 10, 25 and 50 mol%, respectively, in mice 6 h after i.v. injection ($n = 6$).

increased from 41.8 °C (~10 mol% NAPE) to 60.8 °C (~50 mol% NAPE) (Fig. 1). However, the increase in T_m was expected as the T_m of pure DPPC-liposomes has been determined to 41.2 °C (Vermehren et al., 1998) and the T_m of pure NPPE-liposomes corresponds to 64.0 °C (Lafrance et al., 1990). The increase in T_m may result in a more ordered/rigid system which may be less interactive with degrading components. Thus, NAPE has been found to stabilize a bilayer conformation. The membrane stabilizing effect of NAPE has been suggested to be related to the bilayer penetration of *N*-acyl chains containing more than 10 carbon atoms (Schmid et al., 1990). In this way, the headgroup motion may be reduced, and *N*-acylation of PE was found to reduce the hydrogen bonding between the amino and phosphate groups, resulting in increased hydration of the phosphate group. Hence, the increased hydration level as well as the restriction of the motion of the lipid molecules have been proposed to contribute in the membrane stabilizing effect. Like this, NAPE-liposomes showed increased stability toward serum components in vitro and there was found a synergistic stabilizing effect of NAPE and cholesterol when the liposomes contained between 10 and 22 mol% NAPE (Mercadal et al., 1995). Kára et al. (1994) found a $t_{1/2}$ of 2.5 h within 24 h of i.v. administered plasmanyl-(*N*-acyl)ethanolamine preparation in mice, however, no in vivo data of NAPE-liposomes are available. In the present study, an initial increased circulation time in mice of liposomes containing 25 and 50 mol% NAPE was observed (Fig. 2). However, including cholesterol in NAPE-liposomes is expected to increase the circulation time further. In agreement with Mercadal et al. (1995), the incorporation of 10 mol% NAPE did not influence the circulation time compared to conventional DPPC-liposomes (Fig. 2).

For all NAPE-liposome compositions the biodistribution study revealed an accumulation of liposome-associated radioactivity in the liver corresponding to approximately 40% of doses, indicating that uptake by the reticuloendothelial system of the different liposome compositions may be independent of the NAPE content (Fig. 3). However, it was expected that the liver uptake may increase with increasing mol% of anionic lipids in the liposomes (Senior, 1987). The relatively high amount of radioactivity in the stomach/intestine may be a result of the metabolism of the liposomes taken up in the liver followed by drain from the bile into the intestine.

Thus, these results suggest that it may be possible to prepare stable NAPE-liposomes of appropriate size. The observed initial increase in circulation-time may be further prolonged by incorporation of other stabilizing agents, hereby optimizing a selective passive retention of NAPE-liposomes in leaky pathologic tissue, e.g. cancer and inflammation tissue.

Acknowledgements

The authors would like to thank “Apotekerfonden af 1991” for funding the Malvern Zetasizer.

References

Cullis, P.R., De Kruijff, B., 1979. Lipid polymorphism and the functional roles of lipids in biological membranes. Biochim. Biophys. Acta 559, 399–420.

Domingo, J.C., Mora, M., Africa de Madariaga, M., 1993. Incorporation of *N*-acylethanolamine phospholipids into eg phosphatidylcholine vesicles: characterization and permeability properties of the binary systems. Biochim. Biophys. Acta 1148, 308–316.

Hansen, H.S., Lauritzen, L., Strand, A.M., Moesgaard, B., Frandsen, A., 1995. Glutamate stimulates the formation of *N*-acylphosphatidylethanolamine and *N*-acylethanolamine in cortical neurons in culture. Biochim. Biophys. Acta 1258, 303–308.

Hansen, H.S., Lauritzen, L., Strand, A.M., Vinggaard, A.M., Frandsen, A., Schousboe, A., 1997. Characterization of glutamate-induced formation of *N*-acylphosphatidylethanolamine and *N*-acylethanolamine in cultured neocortical neurons. J. Neurochem. 69, 753–761.

Kára, J., Zimakova, N.I., Serebryakova, E.A., Dedková, V., Zolotaryov, A.E., 1994. Pharmacokinetics and metabolism of a new antitumor semisynthetic ether phospholipid, ^{14}C-labeled plasmanyl-(*N*-acyl)ethanolamine, in mice bearing sarcoma Mc11. J. Cancer Res. Clin. Oncol. 120, 662–667.

Lafrance, D., Marion, D., Pézolet, M., 1990. Study of *N*-acyldipalmitoylphosphatidylethanolamines in aqueous dispersion by infrared and raman spectroscopies. Biochemistry 29, 4592–4599.

Mercadal, M., Domingo, J.C., Bermudez, M., Mora, M., Madariaga, M.A.D., 1995. *N*-palmitoylphosphatidylethanolamine stabilizes liposomes in the presence of human serum: effect of lipidic composition and system characterization. Biochim. Biophys. Acta 1235, 281–288.

Mora, M., Mir, F., De Madariaga, M.A., Sagristá, M.L., 2000. Aggregation and fusion of vesicles composed of *N*-palmitoyl derivatives of membrane phospholipids. Lipids 35, 513–524.

Morillo, M., Sagristá, M.L., De Madariaga, M.A., 1998. *N*-stearoyl-phosphatidylserine: synthesis and role in divalent-cation-induced aggregation and fusion. Lipids 33, 607–616.

Schmid, H.H.O., Schmid, P.C., Natarajan, V., 1990. *N*-acylated glycerophospholipids and their derivatives. Prog. Lipid Res. 29, 1–43.

Senior, J.H., 1987. Fate and behavior of liposomes in vivo: a rewiew of controlling factors. CRC Crit. Rev. Ther. Drug Carrier Syst. 3, 123–193.

Shangguan, T., Pak, C.C., Ali, S., Janoff, A.S., Meers, P., 1998. Cation-dependent fusiogenicity of an *N*-acyl phosphatidylethanolamine. Biochim. Biophys. Acta 1368, 171–183.

Vermehren, C., Kiebler, T., Hylander, I., Callisen, T.H., Jørgensen, K., 1998. Increase in phospholipase A_2 activity towards lipopolymer-containing liposomes. Biochim. Biophys. Acta 1373, 27–36.