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Liposomes of enviroxime and phosphatidylcholine: definition of the drug–phospholipid interactions

Nathalie M. Garcon[1], Howard R. Six[1], James Frazer[3], Carlton Hazlewood[2], Brian E. Gilbert[1] and Vernon Knight[1]

Departments of [1]Microbiology and Immunology and [2]Physiology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030 and [3]Department of Surgery, University of Texas Health Science Center at Houston, M.D. Anderson Hospital, Houston, TX 77030, U.S.A.

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Summary

Interaction of the antiviral compound, enviroxime (E), with natural and synthetic phosphatidylcholines in organic and aqueous media was studied. Although insoluble in chloroform, E dissolved in chloroform solutions containing phosphatidylcholines. Solvation was directly related to the length of the fatty acid chains of the phospholipid. Proton spin resonance studies suggested an interaction of the fatty acid chains with the aromatic rings of E. Suspension of E-phosphatidylcholine mixtures of molar ratios up to 0.7:1.0 in aqueous media resulted in the formation of multilamellar liposomes. Liposomes containing E were more stable permeability barriers than those prepared with phospholipid alone, a property previously observed with cholesterol. Competition experiments suggested that E bound to the same sites in lipid bilayers as does cholesterol. These data indicate that E is incorporated into lipid bilayers of liposomes and that it alters the physical properties of the liposomes in a manner similar to that of cholesterol.

Enviroxime; Liposome; Aerosol; Lecithin

Correspondence to: H.R. Six, Department of Microbiology and Immunology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, U.S.A.

Introduction

Enviroxime, a potent anti-rhinovirus compound in vitro, failed to show efficacy consistently in the treatment of infections in humans in earlier studies (Hayden and Gwaltney, 1982; Levandowski et al., 1982; Miller et al., 1985). These results were due in part to difficulties in delivery of the water-insoluble compound. Our recent studies have shown that this drug is associated with liposomes prepared with egg yolk phosphatidylcholine (EYPC) and that the liposomal form retains its antiviral activity (Wyde et al., 1988). This feature has allowed administration of the drug as a small particle aerosol and preliminary study in humans has shown no side effects (Gilbert et al., 1988). The potential use of enviroxime-liposomes in humans makes it desirable to understand the nature of the interaction of the drug with the phospholipids in the liposomes.

Here we present data on the chemical nature of the binding that exist between enviroxime and phospholipids in liposomes. A relationship between carbon chain length of the fatty acid moieties in the phospholipid and strength of binding enviroxime is described. Data on association and disassociation of drug from the liposomes is also presented.

Materials and Methods

Chemicals

Phosphatidylcholines and cholesterol were obtained from Avanti Polar Lipids (Birmingham, AL). ^{3}H-[1,2-H] cholesterol (specific activity, 71 μCi/μg) was from New England Research Products (Boston, MA). Enviroxime (E) and ^{14}C-E (specific activity 38.6 μCi/μg) were provided by Eli Lilly and Company (Indianapolis, IN). Umbelliferone phosphate, sodium salt, was from Polysciences Inc. (Warrington, PA). Alkaline phosphatase was from Sigma Chemical Company (St. Louis, MO). Organic solvents were of analytical or HPLC grade and were purchased from Matheson Coleman Bell (Norwood, OH).

Solubility of E in chloroform solutions of synthetic phosphatidylcholines

Micronized crystals of enviroxime (20 mg) were added to chloroform solutions (2 ml) containing 10 or 20 mg/ml of phosphatidylcholines with different fatty acid chains. The extent of E solvation was estimated by visual comparison of the residual crystalline volume to E standards under chloroform solvent. Standards containing 10, 7.5, 5, 2.5 and 0 mg/ml of enviroxime were defined as 0, 1+, 2+, 3+ and 4+, respectively.

Nuclear magnetic proton resonance spectra

Proton spectra were recorded on a Varion (EM360-L) instrument operating at 60 MHz and a (XL-200) instrument operating at 200 MHz. Tetramethylsilane was used as an internal reference. Deuterated dimethylsulfoxide and deuterated chloroform were used as solvents.

Preparations of liposomes

Multilamellar liposomes were prepared from EYPC as previously described (Kinsky, 1974). When cholesterol was to be added, an amount in chloroform was added to EYPC in the same solvent. When E was to be included, the quantity of drug as a micronized powder was added to EYPC in chloroform solution. Varying molar ratios of lipid to chol or E were used. Exact ratios are given in the appropriate table and figure legends. The solvent was removed on a rotovap (Buchi 421 rotavapor) at 52°C under vacuum. The dried lipid film was dissolved in some instances in T-butanol and again dried under vacuum. Liposomes were then formed by mechanical shaking of the dried residue in sterile water, phosphate buffered saline or 100 mM umbelliferone phosphate (UMP), depending upon the purpose of the experiment.

Electron microscopy

Liposomes prepared from EYPC and E (14 mg and 5 mg/ml, respectively) were examined by electron microscopy. Samples (5.0 mg enviroxime per ml) were mixed with an equal volume of 2% phosphotungstic acid in phosphate buffer, pH 6.3. A drop of the mixture was placed on copper mesh grids coated with Formvar, allowed to air dry, and examined in a Joel 100 electron microscope. Random fields were selected and photographed for assessment of liposome formation.

Competition assay

To determine if chol and E bound to similar sites in the EYPC bilayer, four different groups of liposomes were prepared. These preparations represented various combinations of unlabeled chol or E used to inhibit the association of radiolabeled chol or E with the lipid bilayer. Unlabeled compounds were added at concentrations ranging from 0 to 20 mg/ml. When used, ^{14}C-E was added at 100 000 cpm/ml and ^{3}H-Chol was added at 40 000 cpm/ml. The liposomes were centrifuged at 8000 rpm for 10 min in a Sorval RC2B centrifuge, and the pellets were resuspended in PBS and recentrifuged. Supernatant and pellets were individually mixed with aquasol and counted to establish the amounts of ^{3}H-Chol or ^{14}C-E associated with the bilayer. Micronized E did not form a stable pellet under these conditions.

Entrapped volume

Aqueous volume inside the liposome was assayed using UMP as the trapped marker (Six et al., 1974). Free (untrapped) UMP was determined with 10 µl of liposome suspension diluted in 1.0 ml of 1 M Tris-buffered saline, pH 8.0. After addition of alkaline phosphatase, umbelliferone (UM) was quantitated by comparison with UM standards, 10^{-5} to 10^{-8} M in a fluorometer. Total UMP was determined after destruction of liposomes. Chloroform (2 ml) was added to 10 µl of liposomes, mixed and removed, by evaporation under a nitrogen stream. UMP was then measured as UM. Entrapped UMP was determined by subtraction of the untrapped from the total UM.

TABLE 1

Solubility of enviroxime in chloroform solutions of synthetic and natural phosphatidylcholines[a]

Phosphatidylcholine in chloroform	No. carbon atoms:No. double bonds (position of double bonds)	Extent of enviroxime solubilization at the indicated temperature[b]	
		22°C	37°C
none	–	0	0
dimyristoyl	14:0	0	0
dipalmitoyl	16:0	1	1
distearoyl	18:0	2	2
dioleoyl	18:1(cis,9)	2	3
dilinoleoyl	18:2(cis,9,12)	2	3
diarachidonyl	20:0	4	4
EYPC[c]	Heterogeneous:heterogeneous	1	1

[a]Solutions contained 20 mg/ml of the indicated phosphatidylcholine.
[b]Solubilization was measured after 3 h of incubation, the scale ranged from 0 (completely insoluble) to 4 (completely soluble) as defined in Materials and Methods.
[c]EYPC: egg yolk phosphatidylcholine.

Results

Interaction of enviroxime with phospholipids in organic medium

Although completely insoluble in chloroform alone or in solutions of dimyristoylphosphatidylcholine, E was soluble in solutions of PC that contained fatty acids with chain lengths of 16 carbons or greater (Table 1). A direct relationship between increasing carbon chain length and the ability to solubilize E was noted with PC containing fatty acids of 16 to 20 carbons. The presence of one or two double bonds in the fatty acid moiety did not markedly affect the solubility of E and el-

TABLE 2

Liposome formation with enviroxime or cholesterol and egg yolk phosphatidylcholine (EYPC)

Amount of E or chol added (mg)[a]	Percentage umbelliferone phosphate entrapped in EYPC vesicles containing	
	Enviroxime	Cholesterol
0	3.1	–
1	2.4	2.4
2	3.2	3.3
5	2.8	3.3
10	1.1	1.8
15	0	0.4
20	0	0

[a]E or chol in the indicated amount was added to 15 mg EYPC in chloroform and the solvent was removed under vacuum.
[b]The lipid film was suspended in 1 ml of 0.1 M umbelliferone phosphate and untrapped marker was removed by centrifugation.

evated temperature (37°C) had little influence. PC purified from egg yolk had a limited capacity to solubilize E. Other experiments established that the extent of E solubility was related to PC concentration (data not shown).

A proton spin resonance spectrum for enviroxime is shown in Fig. 1A. The molecular formula for E (shown in the insert) predicts a spectrum for 18 protons and integration of the peaks was consistent with this number. The terminal methyl

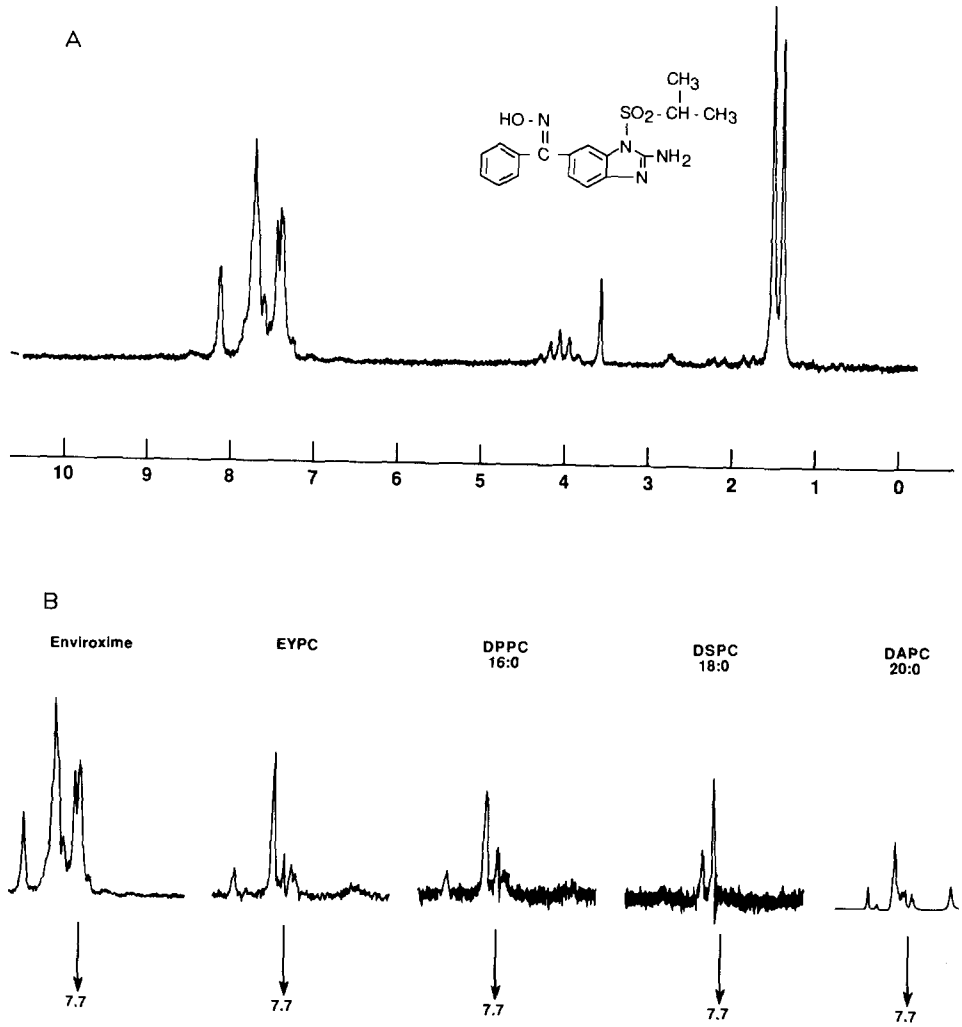

Fig. 1. (A) NMR proton spectra (60 MHz) of enviroxime 100 mg/ml in deuterated DMSO. The chemical structure of enviroxime (syn form) is shown in the insert. (B) NMR proton spectra (60 MHz) of enviroxime in deuterated chloroform containing (left to right) egg yolk phosphatidylcholine (EYPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and diarachidonylphosphatidylcholine (DAPC), respectively. Numbers on the abscissa are in parts per million.

protons (e) show a doublet at 1.3 ppm. The -CH proton (c) appears as quintuplet at 3.8 ppm. The amine group (d) shows a single peak at 3.2 ppm, whereas the two double rings (b) show two broad peaks at 7.2 ppm. The hydroxide group shows a peak at 8.2 ppm. As shown in Fig. 1B, addition of phosphatidylcholines containing fatty acids of 16 carbons or longer both dampened and shifted the peaks resulting from resonance of the protons on the aromatic rings. These data suggest a direct interaction of E with fatty acids of the PC molecule and they further indicate that carbon chain length of the fatty acids was important in this interaction.

Interactions of enviroxime with phospholipid in aqueous media

To determine whether liposomes were formed in the presence of E, dried lipid films composed of EYPC (15 mg/ml) and varying concentrations of drug were dispersed in aqueous solutions. Construction of a functional permeability barrier was used to assess this property. UMP, a molecule known to be entrapped in the aqueous compartments of liposomes, was used as a marker (Six et al., 1974). As shown in Table 2, liposome formation was confirmed at drug concentrations as high as 5 mg/ml. Low levels of entrapment were observed at 10 mg/ml of drug. Inconsistent results were obtained at a concentration of 15 mg/ml and entrapment

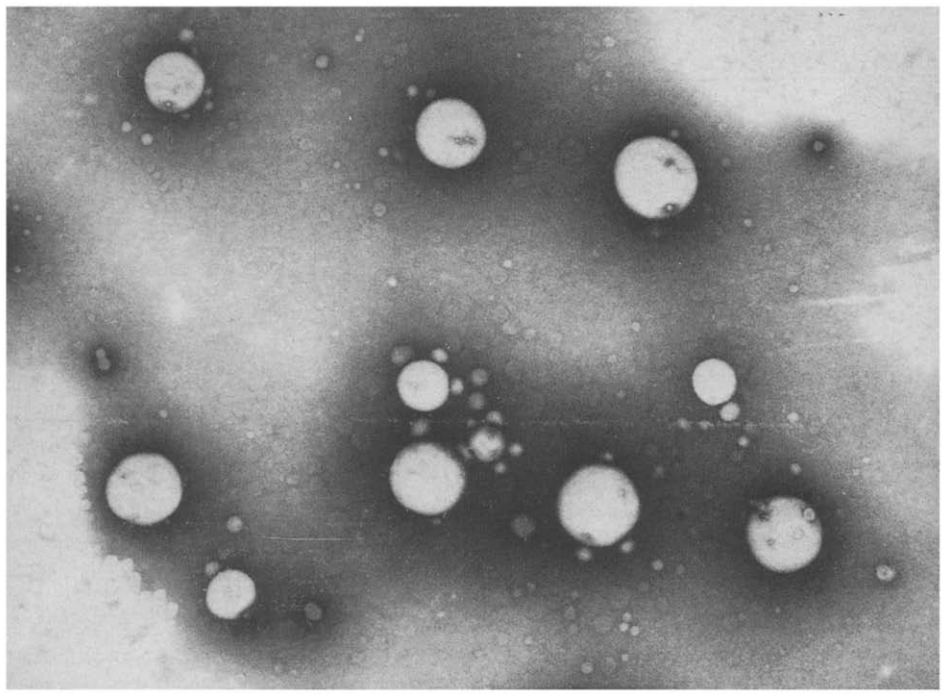

Fig. 2. Liposomes prepared with 14.2 mg of EYPC and 5 mg E were diluted in water and mixed with an equal volume of 2% phosphotungstic acid in phosphate buffer, pH 6.3. After drying on Formvar coated copper grids, observation was performed in a Joel 100 C electron microscope.

TABLE 3

Competition between enviroxime and cholesterol for binding sites in phosphatidylcholine bilayers

Amount of unlabeled E or Chol added (mg)	Radiolabeled Chol		Radiolabeled E	
	Chol	Env.	Chol	Env.
1	85.5[a]	83.8	88.1	91.2
2	91.4	90.2	84.3	96.0
5	94.8	87.4	76.1	95.2
10	94.7	88.8	68.3	94.6
15	92.8	91.1	58.6	90.1
20	11.7	89.6	51.0	16.6

[a]Percentage radioactivity associated with the liposome.

did not occur at a concentration of 20 mg/ml. Similar results were obtained when cholesterol was added to the EYPC.

Examination of EYPC and E (15 and 5 mg, respectively) preparations in an electron microscope showed the presence of vesicles that were highly heterogeneous in size (Fig. 2). These vesicles exhibited the multilamellar appearance characteristic of liposomes at higher magnification (data not shown). The ability to entrap aqueous markers and the morphology of these preparations indicated that EYPC and E mixtures were forming liposomes.

Several lines of evidence suggested that E was incorporated into EYPC bilayers in a manner similar to cholesterol. To determine whether these molecules were binding to the same sites, competition assays were performed. Table 3 shows a comparison of four different preparations of liposomes. Liposomes composed of 15 mg of EYPC and radiolabeled chol were able to accommodate an additional 15

TABLE 4

Incorporation of enviroxime or cholesterol into egg yolk phosphatidylcholine liposomes[a]

Amount of E or chol added (mg)	Percent of enviroxime or cholesterol recovered in liposomes at the indicated hours after formation[b]					
	Enviroxime			Cholesterol		
	0	24	72	0	24	72
1	91	89	87	86	78	72
2	96	95	94	91	85	78
5	95	94	93	95	87	80
10	95	93	93	95	91	89
15	90	87	86	93	85	81
20	17	16	16	12	10	9

[a]The indicated amount of E or Chol was added to 15 mg of EYPC in chloroform and the solvent was removed under vacuum.

[b]Lipid film was suspended in sterile saline. Aliquots were removed at 0,24, and 72 h and unassociated E or Chol removed by centrifugation. ^{3}H-Chol or ^{14}C-E added prior to liposome formation facilitated quantitation.

mg of unlabeled chol but at a concentration of 20 mg much of the binding was inhibited. However, addition of 1 to 20 mg of E failed to inhibit incorporation of chol into liposomes. Both chol and E inhibited the binding of ^{14}C-E to liposomes, but unlabeled E did so more efficiently at 20 mg/ml. Once incorporated into the lipid bilayers both chol and E remained in the liposome for more than 72 h (Table 4).

Enviroxime did not passively adsorb to liposomes when a reaction vessel was coated with ^{14}C-E and liposomes (EYPC in PBS) were added (data not shown). Samples of the liposomes were removed at intervals up to 72 h and the amount of E associated with the liposomes never exceeded 6%.

Discussion

The present studies have demonstrated a strong interaction between enviroxime (E) and phosphatidylcholine (PC) in both polar and nonpolar solvents. A direct association between the fatty acids of PC and the two unsaturated carbon rings of E was demonstrated by proton resonance in organic solvents. Length of the carbon chain was an important factor in this binding and fatty acids of 16 carbons or greater were required. A direct relationship between E binding and carbon chain length was noted. Other studies have suggested an interaction between the phosphate group of EYPC and the amine group of E. In organic solvents E bound to PC probably existed as aggregates or micelles, but nonetheless the liquid could be handled as a true solution. In aqueous solutions E and PC mixtures formed multilamellar vesicles with characteristics of liposomes. Moreover, incorporation of E into PC bilayers made them more stable permeability barriers than bilayers made with PC alone. In addition, mixtures of E and dipalmitoyl-PC formed liposomes at ambient temperatures suggesting that E could also reduce the transition temperature of some phospholipids. The effects of E upon liposomal bilayers have been previously observed with cholesterol (Kinsky, 1976 and references cited therein) and our binding studies confirmed that the two compounds competed for similar sites in the PC membrane. Liposomes were consistently formed with mixtures of E and EYPC at ratios up to 5:14 on a mg per mg basis. The molar ratio for these values was 0.74:1.0 which agrees very well with data obtained with cholesterol and EYPC.

We attempted to increase the quantity of E carried by liposomes using synthetic PC with defined fatty acid chains. While liposomes were formed with dipalmitoyl- and distearoyl-PC, the quantities of E incorporated on a molar basis were not increased. Thus, the increased solubility of E in organic solutions of synthetic PC of longer carbon chain fatty acids may reflect the strength (energy) of binding rather than an increase in the number of binding sites. We were able to increase the quantity of drug incorporated into EYPC liposomes by approximately 35% through synthesis of a palmitoyl-E derivative (N.M. Garcon, unpublished data). However, as expected this derivative was completely devoid of antiviral activity in tissue culture systems (Wilkel et al., 1982). Whether the palmitic acid linked to E by a pep-

tide bond will be hydrolyzed in vivo has not been determined, but regeneration of antiviral activity by cleavage of a similar bond has been reported (Cheetham and Epand, 1987).

The quantity of E that was passively adsorbed to preformed liposomes was very small even after 72 h of incubation. We had anticipated that adsorption and possibly intercalation would occur despite the low solubility of E in water (<2 μg/ml); the rationale being that E in solution would intercalate into the liposomal membrane, allowing additional E to be solubilized and the process would continue with time. The reasons for this apparently limited extent of transfer are not presently known but the observation raises the question of how E carried by liposomes enters cells. Since KB cells in tissue culture rapidly attain an antiviral state after addition of liposomes containing E (<1 hour) and molar ratio of E to EYPC (1:3000 to 1:1.3) did not influence the antiviral activity (Wyde et al., 1988; Six et al., 1989) transfer of the drug through the aqueous medium is unlikely. This suggests that the liposomes interact directly with the cells and that drug is released during or after lose of liposome structure. Moreover, the size of the liposomes carrying the drug did not change the antiviral activity as measured in tissue culture (N.M. Garcon and P.R. Wyde, unpublished observations). The absence of a size dependency suggests that fusion may be the dominant mechanism of entry into the cell (Schroit and Pagano, 1978). When liposomes containing both E and a lipophilic derivative of fluorescein were given to mice by small particle aerosol, prompt staining of the tall columnar epithelial cells of the bronchi was observed (Wyde et al., 1988). Fluorescence was strongest on the periphery of the cells suggesting that the liposomes may have fused with the limiting cellular membrane. Such a mechanism would deliver antiviral drug both to the cell membrane and to the cytoplasm. Although not examined in the mouse studies these interactions should also occur with epithelial cells of the upper respiratory tract.

Transport proteins capable of binding a variety of lipids and facilitating their movement between cells through aqueous solutions are found in blood (Tall, 1986). Proteins with specificity for cholesterol have been well described and these proteins can utilize cholesterol from liposomal membranes (Damen et al., 1981). Whether these lipoproteins can bind E and facilitate its movement through aqueous solutions to cellular membranes is unknown but a potential role in facilitating E entry into cells is possible.

We believe that liposomes offer a method for delivery of water insoluble compounds to the respiratory tract for treatment of disease. Suspension of liposomes containing E can be manipulated as though they are true solutions. These liposomes can be used in small particle aerosol generators and the characteristics of the aerosol are similar to that reported for the water soluble antiviral ribavirin (Six et al., 1989; Knight et al., 1986, 1988). Moreover, liposomes may increase the intracellular concentration of such drugs.

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