Edelfosine Is Incorporated into Rafts and Alters Their Organization

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Received: March 12, 2008; Revised Manuscript Received: June 26, 2008

The effect of edelfosine (1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine or ET-18-OCH3) on model membranes containing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine/sphingomyelin/cholesterol (POPC/SM/cholesterol) was studied by several physical techniques. The sample POPC/SM (1:1 molar ratio) showed a broad phase transition as seen by DSC, X-ray diffraction, and ^{2}H NMR. The addition of edelfosine to this sample produced isotropic structures at temperatures above the phase transition, as seen by ^{2}H NMR and by ^{31}P NMR. When cholesterol was added to give a POPC/SM/cholesterol (at a molar ratio 1:1:1), no transition was observed by DSC nor X-ray diffraction, and ^{2}H NMR indicated the presence of a liquid ordered phase. The addition of 10 mol % edelfosine increased the thickness of the membrane as seen by X-ray diffraction and led to bigger differences in the values of the molecular order of the membrane detected at high and low temperatures, as detected through the M_1 first spectral moment from ^{2}H NMR. These differences were even greater when 20 mol % edelfosine was added, and a transition was now clearly visible by DSC. In addition, a gel phase was clearly indicated by X-ray diffraction at low temperatures. The same technique pointed to greater membrane thickness in this mixture and to the appearance of a second membrane structure, indicating the formation of two separated phases in the presence of edelfosine. All of these data strongly suggest that edelfosine associating with cholesterol alter the phase status present in a POPC/SM/cholesterol (1:1:1 molar ratio) mixture, which is reputed to be a model of a raft structure. However, cell experiments showed that edelfosine colocalizes in vivo with rafts and that it may reach concentrations higher than 20 mol % of total lipid, indicating that the concentrations used in the biophysical experiments were within what can be expected in a cell membrane. The conclusion is that molecular ways of action of edelfosine in cells may involve the modification of the structure of rafts.

Introduction

Synthetic alkyl-lysophospholipids constitute a family of promising anticancer drugs that, unlike most conventional chemotherapeutic drugs, do not target DNA, but act at the cell membrane level, affecting apoptotic signaling.[1−3] The prototype of these synthetic alkyl-lysophospholipids is 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3, edelfosine), which induces selective apoptosis in a range of tumor cells, whereas normal cells are spared.[4] Edelfosine acts against prostate cancer cells,[5] several human and murine leukemia types,[6,7] and human brain and lung tumors,[8,9] among others.[10−13] It has also been reported that these compounds can inhibit the multiplication of the human immunodeficiency virus type I (HIV-I).[14,15] Edelfosine does not interfere directly with DNA and is not mutagenic,[16] unlike most antitumor agents. The mechanism used by such compounds to inhibit cell proliferation is not well known, probably because of the variety of ways in which they act, for example, in the cellular transport system,[17,18] cytokine synthesis,[19,20] intracellular calcium level modulation,[21] or interaction with enzymes, associated to membranes and involved in lipid metabolism,[22−25] and/or in signaling transducing systems, such as protein kinase C (PKC).[26,27]

Edelfosine promotes selective apoptosis in leukemic cells by its preferential incorporation into cancer cells,[4,28,29] leading to its accumulation in lipid rafts[23,28] and to a reorganization of the lipid and protein composition of membrane rafts.[28,30−32] Recent data suggest that edelfosine partitions in lipid rafts lead to a redistribution of sterols from the plasma membrane.[32] This redistribution of a major lipid raft component is likely to alter the biophysical properties of the membrane raft microdomain, with putative important consequences for cell fate. For all these reasons, we thought it interesting to investigate the action of edelfosine on the biophysical features of lipid rafts.

Ternary lipid mixtures containing sphingomyelin, POPC, and cholesterol are known to form liquid ordered phases, which may coexist with other rigid or fluid phases, depending on the temperature.[33−37] These liquid ordered phases have recently attracted much attention because they are thought to mimic domains present in biomembranes, which are often called rafts and which are supposed to be involved in crucial cellular functions such as signal transduction.[38−40]

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10.1021/jp802165n CCC: $40.75
Published on Web 08/20/2008

The liquid ordered phase (L_o) can be characterized by its fast long axis rotation and lateral diffusion rates,[41] similar to those of the L_α phase, with the acyl hydrocarbon chains being predominantly in an all-*trans* conformation.[42,43] In addition to ^{2}H NMR,[42] many other biophysical techniques have been used to characterize L_o phases, such as X-ray diffraction,[44,45] differential scanning microcalorimetry,[46−48] infrared spectroscopy,[46,49] and fluorescence probes.[41,50] Nevertheless, detailed biophysical study of membranes containing cholesterol has been always difficult due to the fluidity and buffering effect of this molecule.[51] The suggestion that edelfosine may alter the properties of a model liquid ordered membrane was made by Heczkova and Slotte.[52]

In this article, we use a variety of nonperturbing biophysical techniques, such as DSC, X-ray diffraction, and ^{2}H NMR, to characterize the phase behavior of a POPC/SM/cholesterol (1:1:1, molar ratio) mixture at different temperatures. Adding edelfosine to this mixture, results in the destabilization of membrane organization at concentrations that are similar to those estimated in cell experiments, where cell *in vivo* localization showed that edelfosine colocalized with rafts.

Materials and Methods

Materials. 1-Palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl(D31)-2-oleyl-*sn*-glycero-3-phosphocholine (POPC-d_{31}), bovine brain sphingomyelin (SM), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1-*O*-Octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (edelfosine or ET-18-OCH_3) was obtained from Avanti Polar Lipids (Alabaster, Alabama, USA) and from INKEYSA (Barcelona, Spain). All other reagents and solvents were commercial samples of the highest purity.

Multilamellar Vesicle Preparation. Different lipid mixtures were prepared and analyzed by several techniques. Samples of POPC/SM/cholesterol (1:1:1 molar ratio) in the absence and presence of 10, 15, and 20 mol % of edelfosine, POPC/SM (1:1 molar ratio) in the absence and presence of 10, 15, and 20 mol % of edelfosine, pure POPC, and pure SM were mixed and studied by differential scanning calorimetry. Moreover, the same samples, except POPC/SM in the presence of 10 and 15 mol % of edelfosine, POPC/SM/cholesterol in the presence of 15 mol % of edelfosine, pure POPC and pure SM, were analyzed by means of ^{2}H NMR spectroscopy, ^{31}P NMR spectroscopy, and X-ray diffraction. The preparation and the buffers used to form the vesicles were the same for all of the samples, the only difference being the amounts of lipids used and the POPC-d_{31} and deuterium-depleted water used for the ^{2}H NMR spectroscopy experiments.

Appropriate amounts of each lipid were dissolved in chloroform and mixed. The organic solvent was evaporated by drying the mixtures under a stream of oxygen-free nitrogen and then storing under high vacuum for a further 2 h to remove the last traces of chloroform. The multilamellar vesicles (MLVs) were prepared by hydrating the lipid mixtures in a buffer containing 20 mM Tris-HCl at pH 7.5 and 100 μM EDTA at a temperature above the transition temperature in the liquid−crystalline phase and vortexing vigorously.

Differential Scanning Calorimetry. A quantity of 2 mg of lipids dissolved in chloroform were mixed and dried with N_2 and high vacuum. The multilamellar vesicles were prepared by hydrating the lipid mixtures with 1 mL of the buffer. Samples were degassed for 10 min before loading into the calorimeter at a final lipid concentration of 2.7 mM. Thermograms were acquired by means of a Microcal VP Scanning Calorimeter (Microcal, Northampton, MA, USA). The same buffer employed to hydrate the lipids was used in the reference cell. The samples were scanned over a temperature range from −6 to 50 °C and with a scan heating rate of 60 °C/h. The thermogram data were recorded and analyzed with the Microcal Origin 5.0 software. Baselines were created and subtracted, and then the traces were normalized according to the same lipid concentration.

X-ray Diffraction. X-ray samples were prepared, as described above, using 10 mg of POPC and the appropriate amount of the other lipids. To hydrate the lipid mixture, 30 μL of the buffer was added. Samples were centrifuged at 13,000*g*, and the pellets (lipid concentration, 444 mM) were placed in a steel holder with cellophane windows, which provides a good thermal contact with the Peltier heating unit. X-ray diffraction profiles were acquired at 5, 15, and 45 °C. Every measurement was performed with 10 min of exposure time after 10 min of temperature equilibration. Simultaneous small (SAX) and wide (WAX) angle X-ray diffraction measurements were performed by means of a modified Kratky compact camera (MBraum-Graz-Optical System, Graz, Austria) and using a linear position sensitive detector (PSD; MBraun, Garching, Germany) to monitor the s-range [$s = 2 \sin \theta/\lambda$, 2θ = scattering angle, λ = 1.54 Å] between 0.0075−0.07 and 0.2−0.29 Å^{-1}, respectively. Nickel-filtered Cu Kα X-rays were generated by a Philips (Eindhoven, The Netherlands) PW3830 X-ray generator operating at 50 kV and 30 mA. Calibration of the detectors was carried out by using Ag-stearate (small-angle region, *d*-spacing at 48.8 Å) and lupolen (wide-angle region, *d*-spacing at 4.12 Å) as reference materials. The hydration of the sample was controlled by adding twice as much weight of water and verifying that the *d*-spacings were not modified.

^{2}H NMR Spectroscopy. Typically a quantity of 20 mg of POPC-d_{31} and the appropriate amount of the other lipids dissolved in chloroform were mixed, dried under a stream of nitrogen and then stored under vacuum. Multilamellar vesicles were prepared as previously described, hydrating the lipids with 40 μL of the prepared buffer using deuterium-depleted water, giving a lipid concentration of 666 mM. ^{2}H NMR experiments were carried out on a Bruker Avance 600 instrument (Bruker, Etlingen, Germany) at 92.123 MHz using the standard quadrupole echo sequence.[53] The spectral width was 150 KHz, with a 10 μs 90° pulse, 40 μs pulse spacing, 3.35 μs dwell time, 1 s recycling time, 50 Hz line broadening, and accumulation of 15,000 transients. Spectra were acquired at different temperatures above and below the transition temperature of each pure lipid, raising the temperature in 1 °C steps from 0 to 3 °C and in 2 °C steps from 3 to 49 °C. The first moment, M_1, was calculated for each spectrum of the different mixtures and at each temperature using the follow equation:

$$M_1 = \frac{1}{A} \sum_{\omega=-x}^{x} |\omega| f(\omega)$$

where ω is the frequency shift from the central (Larmor) frequency, $f(\omega)$ is the spectral intensity, and A is defined as

$$A = \sum_{\omega=-x}^{x} f(\omega)$$

^{31}P NMR. Samples for ^{31}P NMR were the same as those for ^{2}H NMR. ^{31}P NMR spectra were obtained in the Fourier Transform mode in a Varian Unity 300 Spectrometer. All chemical shift values are quoted in parts per million (ppm) with reference to pure lysophosphatidylcholine micelles (0 ppm), positive values referring to low-field shifts. All spectra were

obtained in the presence of gated broadband proton decoupling (5W input power during acquisition time), and accumulated free inductive decays were obtained from up to 5.000 scans. A spectral width of 25.000 Hz, a memory of 32.000 data points, a 2 s interpulse time, and an 80° radio frequency pulse (11 μs) were used. Prior to Fourier transformation, an exponential multiplication was applied, resulting in a 60 Hz line broadening.

Edelfosine Uptake. Edelfosine was prepared as a 1 mM stock solution in culture medium. Drug uptake was measured as previously described,[4,28] after incubating 10^6 cells/mL with 0.5 μM edelfosine + 0.05 μCi/mL [^{3}H]Edelfosine for the indicated times, and subsequent exhaustive washing with 2% BSA-PBS. [^{3}H]edelfosine (specific activity, 42 Ci/mmol) was synthesized by tritiation of the 9-octadecenyl derivative (Amersham Buchler, Braunschweig, Germany).

Lipid Raft Isolation. Lipid rafts were isolated from 3−7 × 10^7 cells treated with 10 μM [^{3}H]edelfosine (0.1 μCi) by nonionic detergent lysis and centrifugation on discontinuous sucrose gradients as described.[28,54] Fractions (1 mL) were collected from the top of the gradient, and 20 μL of each fraction were subjected to SDS−PAGE, and 40 μL of each fraction were counted for radioactivity. The location of the ganglioside G_{M1}-containing lipid rafts was determined using the cholera toxin B (CTx B) subunit conjugated to horseradish peroxidase (Sigma).

Fluorescence Microscopy. Cells were treated with 20 μM of the fluorescent analogue all-[*E*]-1-*O*-[15′-phenylpentadeca-8′,10′,12′,14′-tetraenyl]-2-*O*-methyl-*rac*-glycero-3-phosphocholine (PTE-edelfosine)[28] for 7 h and analyzed using a fluorescence microscope (Axioplan 2; Carl Zeiss MicroImaging, Inc.) and a digital camera (ORCA-ER-1394; Hamamatsu). Lipid rafts were visualized using the CTx B subunit labeled with FITC (FITC-CTx B subunit) as described previously.[28,54]

Statistical Analyses. Unless otherwise indicated, the results given are the mean (±S.E.) values obtained from the indicated experiments.

Results

Differential Scanning Calorimetry. It can be observed that pure POPC exhibited a phase transition from L_β to L_α with a T_c at −4 °C and T_m at −3.5 °C (Figure 1). However, pure SM showed an L_β to L_α phase transition with a T_c at 32 °C and a T_m at 38 °C. The mixture POPC/SM (1:1 molar ratio) showed two broad transitions, one with a T_m at −0.2 °C, which must have been produced by a phase rich in POPC and a second very broad one with T_m at 18 °C, where SM must be predominat. However, the addition of cholesterol, producing the mixture POPC/SM/cholesterol (1:1:1 molar ratio), completely abolished the phase transition in the range of temperatures studied (Figure 1). When edelfosine was added to POPC/SM (1:1 molar ratio), it produced a shift in the transition of POPC and SM so that both phospholipids tended to have temperatures closer to those when they are pure. This effect was bigger with 20 mol % than with 10 mol %. In this way, T_m values were −0.2, −1.2, −2.7, and −3.4 °C for the POPC-rich phase in POPC/SM, POPC/SM plus 10 mol % edelfosine, POPC/SM plus 15 mol % edelfosine, and POPC/SM plus 20 mol % edelfosine, respectively. Similarly, the SM rich phase showed T_m of 18 °C, 22.7 °C, 26.6 and 26.7 °C for POPC/SM, POPC/SM plus 10 mol % edelfosine, POPC/SM plus 15 mol % edelfosine, and POPC/SM plus 20 mol % edelfosine, respectively.

This effect of edelfosine may have been due to its interaction with both phospholipids so that the shift could have been the result of the interaction of both SM and POPC with edelfosine and also due to both POPC and SM being free of each other.

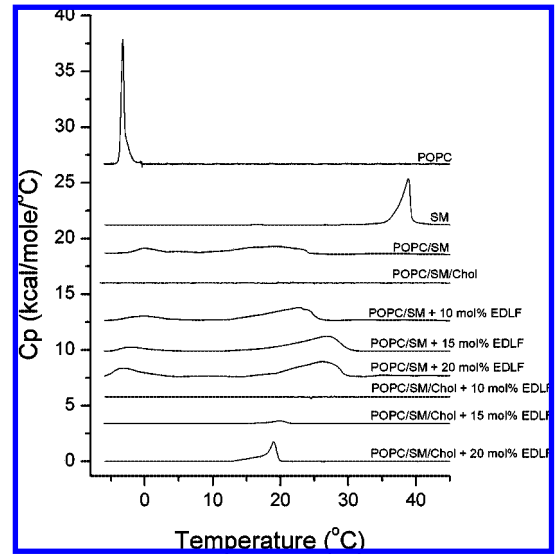

Figure 1. DSC heating thermograms of aqueous dispersions of pure POPC, pure SM, POPC/SM (1:1 molar ratio), POPC/SM/cholesterol (Chol) (1:1:1 molar ratio), POPC/SM (1:1 molar ratio) + 10 mol % edelfosine (EDLF), POPC/SM (1:1 molar ratio) + 15 mol % edelfosine (EDLF), POPC/SM (1:1 molar ratio) + 20 mol % edelfosine (EDLF), POPC/SM/cholesterol (Chol) (1:1:1 molar ratio) + 10 mol % edelfosine (EDLF), POPC/SM/cholesterol (Chol) (1:1:1 molar ratio) + 15 mol % edelfosine (EDLF), and POPC/SM/cholesterol (Chol) (1:1:1 molar ratio) + 20 mol % edelfosine (EDLF). The concentrations of the lipid mixtures were normalized to 9.8 mM. The heating rate was 60 °C/h.

The effect of edelfosine on the phase transition of phospholipids has been described as a small decrease of about 1 °C in the range of concentrations used here.[55] However, the effect of demixing could be to increase the T_c of the SM-rich phase and to decrease the temperature of the POPC-rich phase. Since when POPC and SM are mixed the phase rich in SM has a bigger variation in its T_m with respect to pure SM than that shown by the POPC rich phase with respect to pure POPC, it can be expected that the demixing process will produce a bigger shift in the T_m of the SM-rich phase than in that of the POPC-rich phase, which is exactly what was observed.

Although a certain increase in the size of the two main peaks as edelfosine was increased can be seen, the total ΔH of the broad transitions did not significantly change (results not shown), and therefore this effect is due to the POPC-SM demixing discussed above.

The addition of 10 mol % of edelfosine to the ternary system POPC/SM/cholesterol did not produce any effect inasmuch as just as in its absence, no transition was seen. However, the addition of 20 mol % of edelfosine to this same ternary system produced a striking effect, and a sharp transition appeared, with a T_m at 18.5 °C. This transition peak was localized at a temperature similar to that of the second transition observed for the POPC/SM sample, indicating that edelfosine had, at least partially, abolished the effect of cholesterol. It can be seen that the transition peak corresponding to the POPC-rich phase was not observed, which might be explained by taking into account the different sensitivities of POPC and SM to cholesterol. It has been described that the phase transition of POPC is almost totally abolished by 13 mol % of cholesterol,[56] whereas in the presence of 25 mol % of cholesterol, a clear (although broad) phase transition can still be observed for SM.[57] In our case, we included 20 mol % of edelfosine versus 24 mol % of cholesterol in the final mixture so that there was a certain excess of cholesterol.

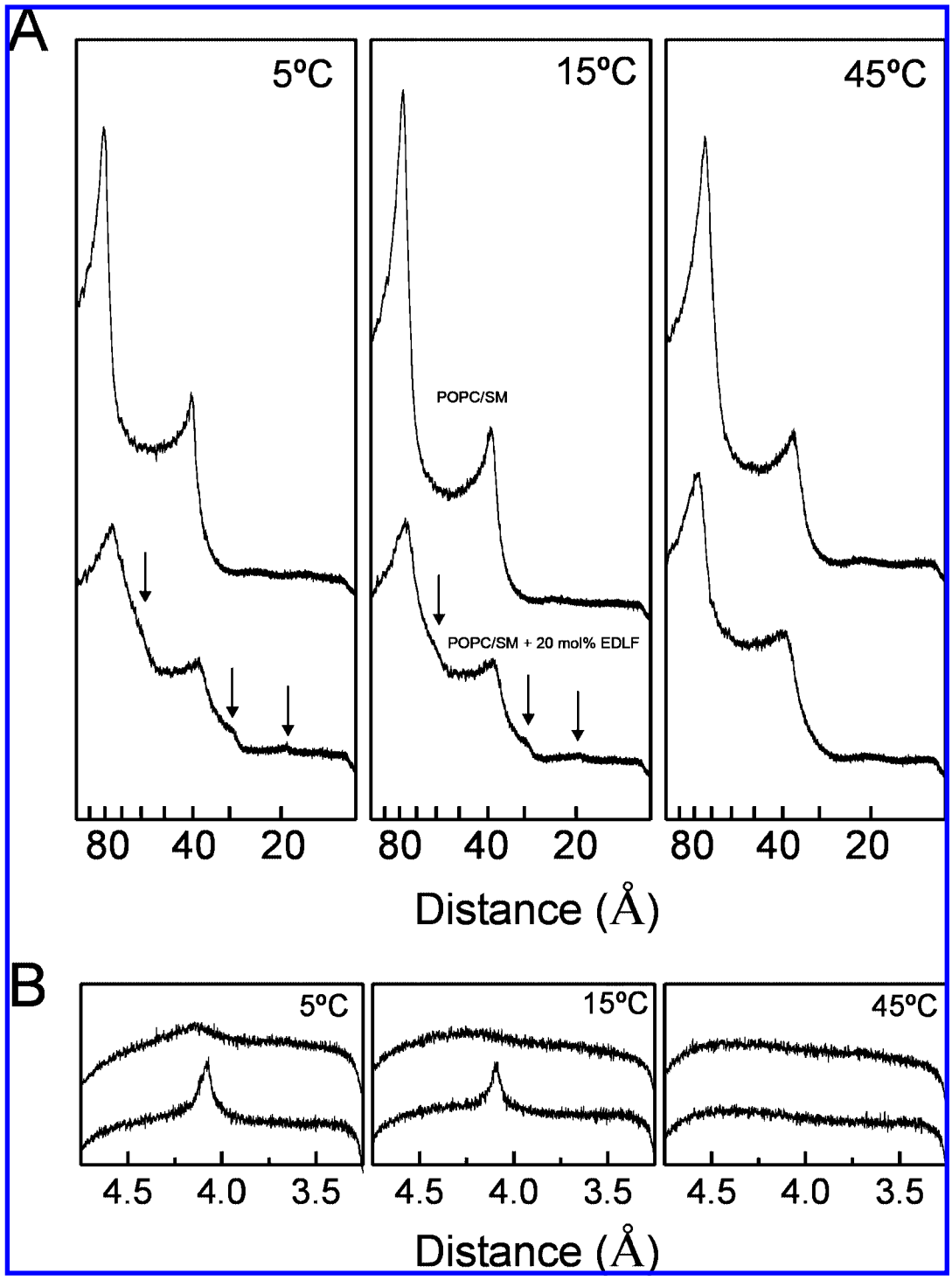

Figure 2. Small-angle (A) and wide-angle (B) X-ray diffraction profiles at the indicated temperatures of the mixtures (top to bottom) POPC/SM (1:1 molar ratio) and POPC/SM (1:1 molar ratio) + 20 mol % of edelfosine (EDLF). Arrows indicate additional *d*-spacings arising after the addition of edelfosine (EDLF).

Small- and Wide-Angle X-ray Scattering. Small-angle X-ray diffraction (SAXD) was used to check the phase behavior of the lipid mixtures. This technique not only defines the macroscopic structure itself, but also provides the interlamellar repeat distance in the lamellar phase. The first order reflection component corresponds to the interlamellar repeat distance, which is composed of the bilayer thickness and the thickness of the water layer between bilayers. In addition, wide-angle X-ray diffraction (WAXD) measurements were also obtained, providing information on the packing characteristics of the acyl chains.

Figure 2 shows the SAXD and WAXD diffraction pattern profiles corresponding to POPC/SM (1:1 molar ratio) and the same mixture with 20 mol % edelfosine added, at several temperatures. At 5 °C, the SAXD of the POPC/SM sample showed diffraction profiles with sharp Bragg reflections and with distances related as 1:1/2, which are characteristic of the multilamellar organization.[58,59] The first *d*-spacing was located at 72.6 Å (Table 1). At 15 °C, the characteristics of the SAXD diffractogram did not change very much, although the first *d*-spacing was now located at 70.6 Å. At 45 °C, changes were more evident inasmuch as the first *d*-spacing now appeared at

TABLE 1: Repeat Spacing *d* (nm), in the Low-Angle and Wide-Angle Regions of Different Lipid Mixtures, Obtained after X-ray Scattering

		low-angle		wide-angle
mixture	temperature (°C)	H,k	d (nm)[a]	d (nm)
POPC/SM	5	1.0	7.262	0.413 (w)
		2.0	3.633	
	15	1.0	7.061	0.423 (w)
		2.0	3.516	
	45	1.0	6.332	
		2.0	3.318	
POPC/SM + 20 mol % EDLF	5	1.0	6.809 - 5.410	0.408 (s)
		2.0	3.406 - 2.614	
		3.0	1.734	
	15	1.0	6.870 - 5.450	0.409 (s)
		2.0	3.437 - 2.624	
		3.0	1.758	
	45	1.0	7.061	
		2.0	3.532	
POPC/SM/cholesterol	5	1.0	6.631	0.435 (w)
		2.0	3.318	
		3.0	2.208	
		4.0	1.659	
	15	1.0	6.574	0.435 (w)
		2.0	3.289	
		3.0	2.185	
		4.0	1.645	
	45	1.0	6.436	
		2.0	3.220	
		3.0	2.246	
		4.0	1.614	
POPC/SM/cholesterol + 10 mol % EDLF	5	1.0	7.000	0.435 (w)
		2.0	3.500	
		3.0	2.321	
		4.0	1.750	
	15	1.0	6.902	
		2.0	3.453	
		3.0	2.300	
		4.0	1.726	
	45	1.0	6.661	
		2.0	3.332	
		3.0	2.214	
POPC/SM/cholesterol + 20 mol % EDLF	5	1.0	6.965 - 5.333	0.409 (s)
		2.0	3.484	
		3.0	2.328	
		4.0	1.754	
	15	1.0	6.965 - 5.333	0.410 (s)
		2.0	3.484	
		3.0	2.328	
		4.0	1.754	
	45	1.0	6.902 - 5.333	
		2.0	3.453	

[a] Doubles entries correspond to two independent patterns.

only 63.3 Å, indicating a substantial decrease in the thickness of the membrane characteristic of a transition to fluid phase. At 5 °C, the WAXD experiment showed a broad peak centered at 4.1 Å, and a similar one at 4.2 Å at 15 °C, indicating that the samples contained gel phases but not only this type of phase; in fact, DSC showed that they were undergoing a transition process at these temperatures. At 45 °C, a broad scattering band characteristic of a fluid phase was observed.

The addition of 20 mol % edelfosine produced substantial changes in the scattering patterns: at 5 °C, the first *d*-spacing was broad and appeared to 68.09 Å, with a second order at 34.1 Å. However, other *d*-spacings apparently not related to the lamellar ones described could also be discerned at 54.1, 26.1 and 17.3 Å (see arrows in Figure 2 and Table 1), which are related between them as 1:1/2:1/3, suggesting the coexistence of another lamellar structure as a result of the addition of edelfosine, which may have given rise to an interdigitated phase, as has been seen to be caused by edelfosine previously.[55] Given the small size of the peaks, this second lamellar phase must be formed by a low percentage of the total lipids. At 15 °C, this sample did not appreciably change so that SAXD showed a first *d*-spacing at 68.7 Å with the second lamellar structure also giving the *d*-spacings at 54.5, 26.3, and 17.6 Å (Table 1). At 45 °C, the SAXD pattern did not change very much with respect to the first *d*-spacing, which appeared at 63.32 Å. The nonappearance of a second lamellar structure was consistent with the

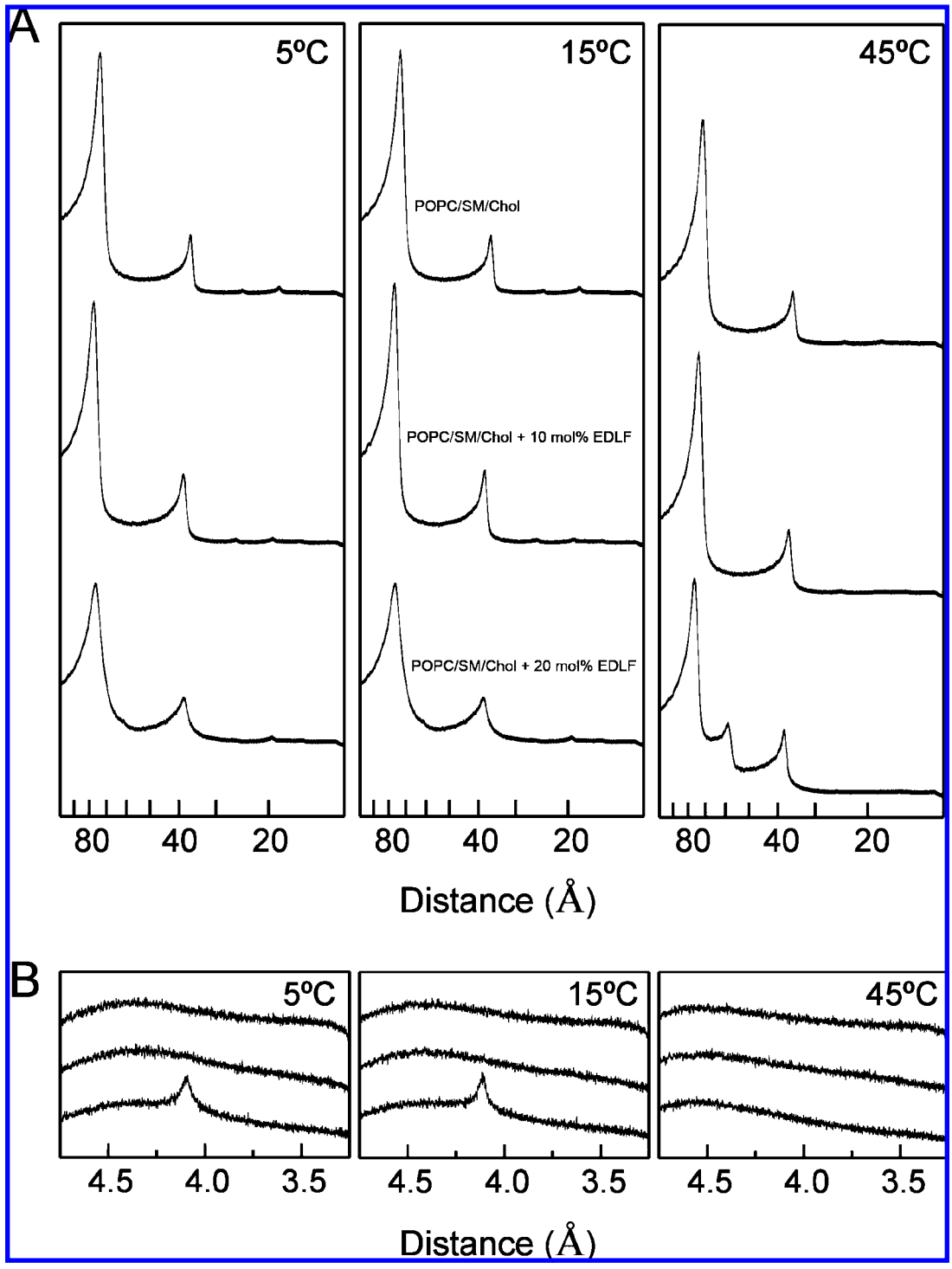

Figure 3. Small-angle (A) and wide-angle (B) X-ray diffraction profiles at the indicated temperatures of the mixtures (top to bottom) POPC/SM/cholesterol (Chol) (1:1:1 molar ratio) and the same lipid mixture + 10 mol % edelfosine (EDLF) or + 20 mol % edelfosine (EDLF).

assignment of this second lamellar structure to an interdigitated phase, which, whereas expected to occur in the gel phase, is not expected when the membrane is fluid. WAXD diffractograms clearly showed a gel phase at 5 and 15 °C, with a peak at a *d*-spacing of 4.1 Å, which is typical of lipid bilayers in the lamellar gel phase with tilted hydrocarbon chains and pseudohexagonal chain packing.[60] At 45 °C, the diffractogram did not show the sharp peak of the lower temperatures but just a broad and diffuse peak indicating a fluid state.

Figure 3 shows the result of adding cholesterol to these mixtures. SAXD showed that the POPC/SM/cholesterol (1:1:1) sample at 5 °C has a first order *d*-spacing at 66.3 Å, which is lower than that in the POPC/SM (1:1 molar ratio) where it was 72.6 Å (Figure 2). The *d*-spacings at 33.2, 22.1, and 16.6 Å correlated with the first on at 1:1/2:1/3:1/4 indicating a lamellar structure. At 15 °C, the pattern was very similar with a first order scattering peak at 65.7 Å. At 45 °C, there were no significant changes, with the first order *d*-spacing of SAXD appearing at 64.4 Å. Note that the pronounced decrease in the repetitive distance upon heating from 15 to 45 °C observed for the POPC/SM sample (Figure 2) was totally absent here, as a consequence of the presence of cholesterol. For this sample, WAXD showed diffractograms with a broad and diffuse scattering with a maximum at 4.4 Å at all temperatures, which

is similar to that found for other systems containing cholesterol, such as DPPC with 40 mol % cholesterol, indicating the formation of liquid-ordered phases.[61]

When 10 mol % of edelfosine was added to the POPC/SM/cholesterol (1:1:1) sample, SAXD showed an increase in the distance at which the scattering peaks appeared so that at 5 °C, the diffraction first order was observed at 70.0 Å, i.e., an increase of 4 Å (Table 1), which means an increase in the thickness of the membrane (Figure 3). Up to three additional *d*-spacings were correlated with sequence 1: 1/2:1/3:1/4 corresponding to a lamellar phase. Note that the effect of edelfosine is to reverse the effect of cholesterol so that the thickness is similar to that observed for POPC/SM at low temperature.

At 15 °C, the pattern was almost the same, with a first *d*-spacing at 69.0 Å. At 45 °C, a small decrease in thickness was observed so that the first order was appearing now at 66.6 Å. WAXD again showed broad scatterings at all temperatures and was centered at 4.4 Å.

More drastic changes were observed when the proportion of edelfosine was raised to 20 mol % (Figure 3). At 5 °C, the first *d*-spacing appeared at 69.7 Å, again indicating an increase in the thickness of the membrane with respect to the ternary system POPC/SM/cholesterol in the absence of edelfosine (66.3 Å). In addition to the second, third, and fourth orders of the lamellar system, a smaller *d*-spacing peak was also detected at 53.3 Å. Exactly the same pattern was found at 15 °C. At 45 °C, the pattern differed because a clear and distinct peak could now be observed at 53.3 Å. WAXS showed a sharp peak at 4.1 Å at 5 °C and at 15 °C, indicating the existence of a gel−crystalline phase. This peak was not seen at 45 °C, at which the broad scattering observed for other samples was again detected. It seems that the effect of edelfosine was to give rise to a gel phase at low temperature as if it had neutralized the cholesterol. The *d*-spacing at 53.3 Å indicated that phase separation occurred even at high temperatures in the fluid phase (indicated by DSC and also by WAXD) and that this *d*-spacing may have been caused by edelfosine associated with cholesterol.

^{2}H NMR Studies. This technique was used to characterize the coexistence of different phases, such as liquid ordered, disordered, and gel-crystalline phases. The information is provided by the palmitoyl chain of POPC. Figure 4 shows that the POPC/SM (1:1 molar ratio) sample at 0 °C possesses a gel-crystalline pattern in which deuterons do not undergo axially symmetric motion on the NMR time scale because the acyl chains are closely packed. The system undergoes evolution to reach a fluid state (L_α) at 23 °C, at which there is a superposition of Pake doublets, indicating that the acyl chains undergo rapid, axially symmetric reorientation about the bilayer normal. Between 0 and 23 °C, there is a progressive fluidification, which agrees with the DSC results shown above, reflecting that the first transition detected is due to a POPC-rich phase, whereas the second one is probably due to a phase rich in SM.

The POPC/SM (1:1 molar ratio) sample (Figure 4) with 20 mol % of edelfosine added also showed a gel−crystalline pattern at low temperatures, but when the temperature was raised to above 33 °C, the intensity of the spectra decreased, and an isotropic component arose superimposed on a fluid pattern, indicating that POPC is now incorporated into small vesicles or micelles in the presence of edelfosine.

The POPC/SM/cholesterol (1:1:1) sample (Figure 5) differed from the above samples because the presence of cholesterol produced the wider spectrum characteristic of a liquid-ordered (L_o) phase. Nevertheless, this phase was mixed with others. It has been described[36] that this ternary lipid system contains a

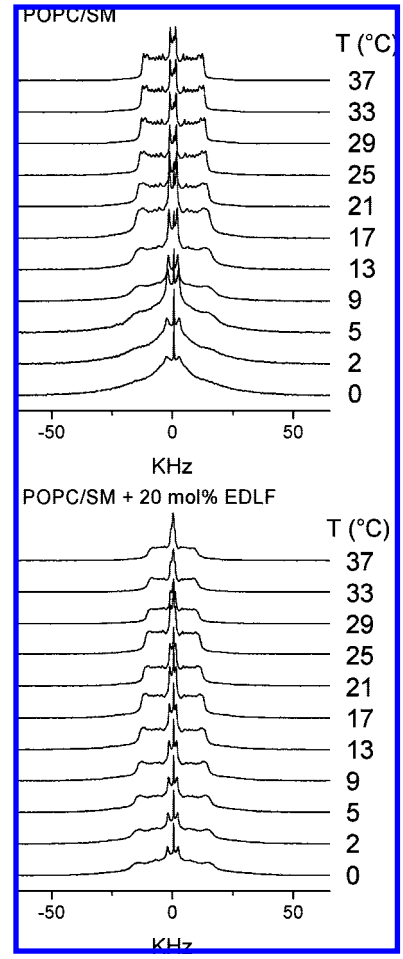

Figure 4. ^{2}H NMR spectra of POPC-d_{31}/SM (1:1 molar ratio) and POPC-d_{31}/SM (1:1 molar ratio) + 20 mol % edelfosine (EDLF) vesicles as a function of temperature. The temperatures are shown on the right-hand side of each spectrum.

mixture of gel−crystalline one (L_β) and L_o and that this would explain the low resolution of the Pake doublets and the width of the spectra. The higher resolution reached at about 9 °C probably indicated the presence of L_β, L_o, and L_α. Another change occurred at 27 °C, with what can be interpreted as the presence of an L_o/L_α mixture, and remained up to the maximum temperature studied (49 °C).

The addition of 10 mol % of edelfosine (Figure 5) to the former sample meant that at temperatures as low as 3 °C $L_\beta/L_o/L_\alpha$ mixtures existed but that at 11 °C the L_β one disappeared and L_o/L_α phases were the only ones to remain. At temperatures above 31 °C, L_α was clearly predominant, but the spectra were still wider than those for a pure L_α phase (see, e.g., POPC/SM at 49 °C).

The POPC/SM/cholesterol (1:1:1 molar ratio) sample containing 20 mol % of edelfosine (Figure 5) showed a clear phase transition from an L_β/L_o phase to an L_o/L_α mixture at 13 °C. This transition seemed to involve both SM and POPC because no phase separation was observed in DSC. A new transition occurred at 31 °C, although the spectra at higher temperatures continued to be too wide for a pure L_α phase. It is interesting that the isotropic phase detected when 20 mol % of edelfosine was added to POPC/SM (1:1) was absent here, indicating that the effect of edelfosine was neutralized by cholesterol.

***M$_1$* of the ^{2}H NMR Spectra.** The first spectral moment M_1 measures the average spectral width, and since each phase has a distinct spectral width, it will also have a characteristic M_1,

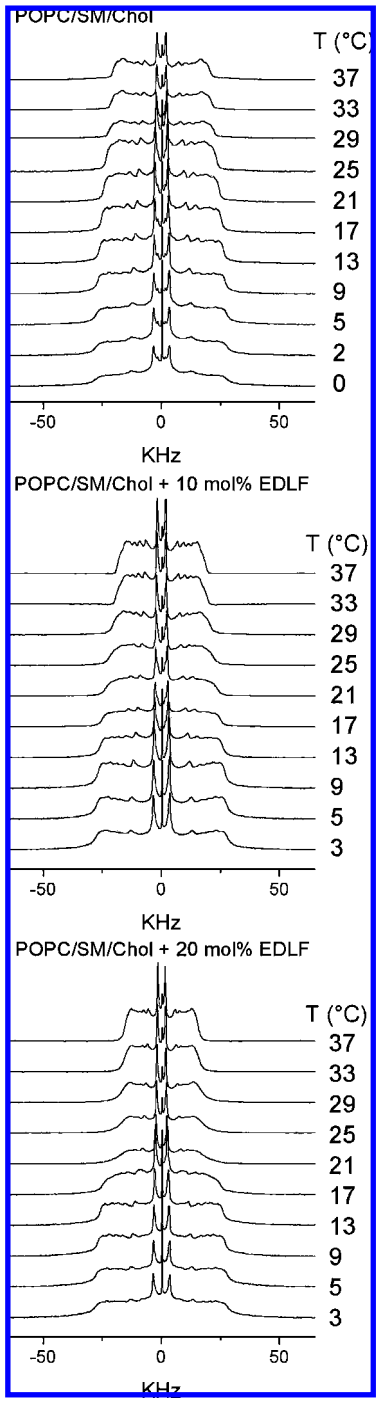

Figure 5. ^{2}H NMR spectra of POPC-d_{31}/SM/cholesterol (Chol) (1:1:1 molar ratio), POPC-d_{31}/SM/cholesterol (Chol) (1:1:1 molar ratio) + 10 mol % edelfosine (EDLF), and POPC-d_{31}/SM/cholesterol (Chol) (1:1:1 molar ratio) + 20 mol % edelfosine (EDLF) vesicles as a function of temperature. The temperatures are shown on the right-hand side of each spectrum.

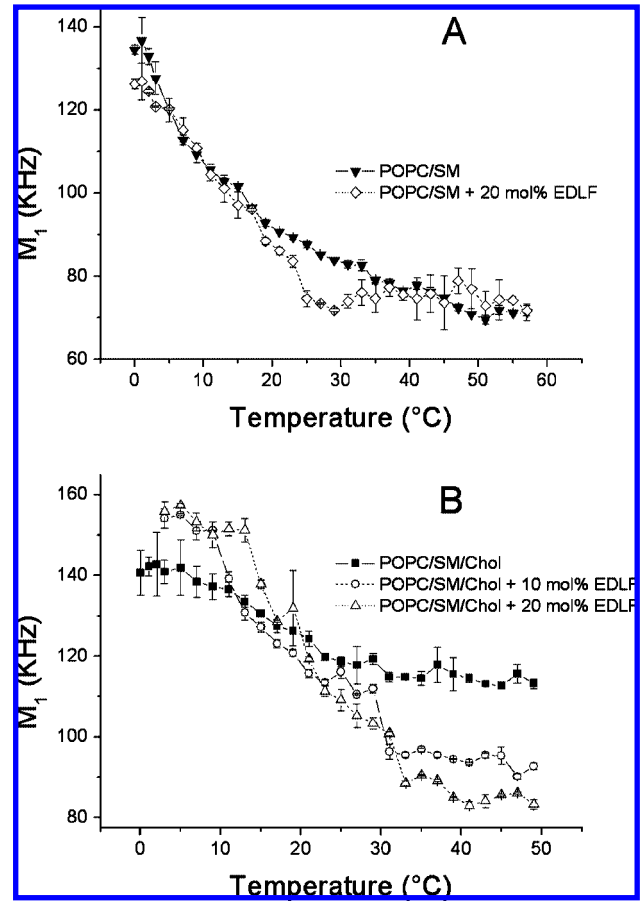

Figure 6. (Panel A) Temperature dependence of M_1 for POPC-d_{31}/SM (1:1 molar ratio) and POPC-d_{31}/SM (1:1 molar ratio) + 20 mol % edelfosine (EDLF). (Panel B) Temperature dependence of M_1 for POPC-d_{31}/SM/cholesterol (Chol) (1:1:1 molar ratio), POPC-d_{31}/SM/cholesterol (Chol) (1:1:1 molar ratio) + 10 mol % edelfosine (EDLF), and POPC-d_{31}/SM/cholesterol (Chol) (1:1:1 molar ratio) + 20 mol % edelfosine (EDLF).

which is proportional to the average order parameter. The variations in M_1 with temperature can be used to characterize membrane phase transitions and the molecular order of a membrane. Figure 6A shows that the POPC/SM (1:1 molar ratio) sample has an M_1 of 134 kHz at 0 °C, which is characteristic of a gel phase membrane. Upon increasing the temperature, there is a decrease in M_1, which finishes at about 13 °C, indicating a transition, and a second decrease starting at 15 °C corresponding to a second transition. These transitions must correspond to those previously observed by DSC and ^{2}H NMR, i.e., a phase rich in POPC and the second a phase rich in SM, although there are small differences in the temperatures observed with the different techniques. At high temperatures, values of approximately 70 kHz were observed for M_1, which is characteristic of a fluid phase. When 20 mol % edelfosine was added, there were again two transitions, the first ending at 13 °C and the second starting at about 16 °C and ending at 29 °C. These two transitions were therefore similar to those detected in the absence of edelfosine and probably correspond to the same two phases.

Figure 6B shows the variations in M_1 when cholesterol was present. In the ternary system, POPC/SM/cholesterol (1.1:1 molar ratio) M_1 values were 145 kHz at 0 °C, i.e., values indicative of a gel phase and similar to those seen for POPC/SM at this temperature, although these values were lower than those in the presence of added edelfosine (see below). A change started at about 10 °C, with a decrease in M_1 that ended at about 29 °C with values of about 115 kHz, a value which is indicative of intermediate fluidity.

The addition of 10 mol % edelfosine induced an increase in M_1 values at low temperatures, reaching 153 kHz at 3 °C and indicating a rigidification of the membrane. A transition giving rise to a decrease in M_1 was detected at 9 °C and ending at about 30 °C, with values of about 95 kHz at temperatures higher than 30 °C, which is lower than the M_1 value seen for the ternary system POPC/SM/cholesterol in the absence of edelfosine, indicating a decrease in molecular order at high temperatures.

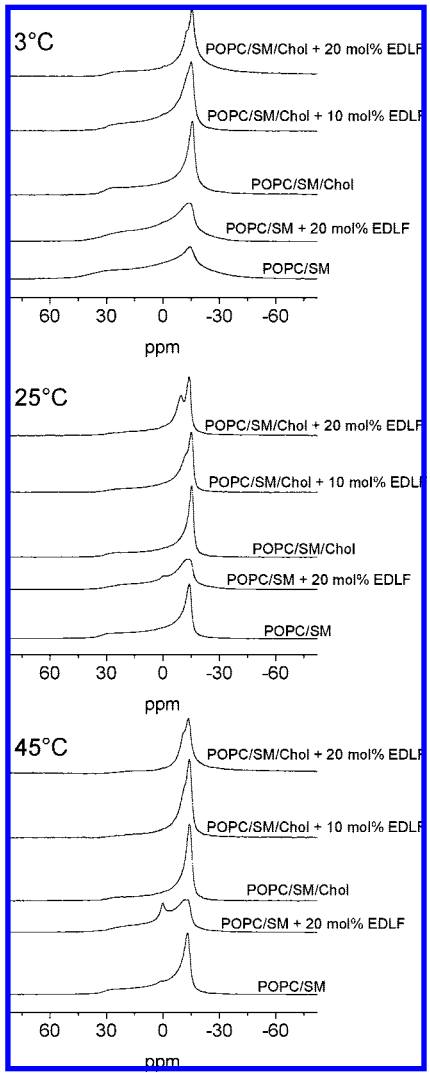

Figure 7. ^{31}P NMR spectra at 3°, 25°, and 45 °C of the samples POPC/SM (1:1 molar ratio), POPC/SM (1.1 molar ratio) plus 20 mol % of edelfosine (EDLF), POPC/SM/cholesterol (chol) (1:1:1 molar ratio), POPC/SM/cholesterol (1:1:1 molar ratio) plus 10 mol % edelfosine (EDLF), and POPC/SM/cholesterol (1:1:1 molar ratio) plus 20 mol % edelfosine (EDLF).

When 20 mol % of edelfosine was added, the M_1 values at low temperatures were slightly higher than those in samples with 10 mol % edelfosine or without edelfosine (with 155 kHz at 3 °C indicating a higher order), and a transition started at 12 °C, ending at 31 °C. M_1 values at high temperatures were about 84 kHz, which are lower than those for 10 mol % edelfosine, indicating lower order, although these values were not as low as those found for POPC/SM (1:1 molar ratio), which were about 72 kHz at 55 °C, indicating that the presence of cholesterol was partially but not totally compensated by edelfosine.

^{31}P NMR. The ^{31}P NMR spectra of the different samples were obtained as previously described[62] and are depicted in Figure 7. It can be seen that at 3 °C the spectrum of POPC/SM (1:1 molar ratio) is asymmetric with a high-field peak and a low-field shoulder, characteristic of an axially symmetrical shift tensor and consistent with the arrangement of the phospholipids in a bilayer configuration. The lineshapes were broad with a chemical shift anisotropy ($\Delta\sigma$) of 59 ppm, indicating the predominance of the gel phase. The addition of 20 mol % of edelfosine produced a significant change since $\Delta\sigma$ was now 50 ppm indicating that the membrane was now more fluid. It is interesting that a very small isotropic peak is visible in this spectrum. The addition of cholesterol to give POPC/SM/cholesterol (1:1:1 molar ratio), produced a membrane with an intermediate fluidity judging from $\Delta\sigma$, which was 49 ppm. When 10 mol % was added to the last sample, the spectrum was not very modified showing a $\Delta\sigma$ of 48 ppm, although a shoulder could already been detected on the main peak toward the low field. This peak was clearly visible when 20 mol % edelfosine was added, and it is probably due to edelfosine. This last sample had a $\Delta\sigma$ of 47 ppm.

At 25 °C (Figure 7) POPC/SM (1:1 molar ratio) showed a spectrum with a narrower line shape ($\Delta\sigma$ of 48 ppm) indicating a fluidification of the membrane. The addition of 20 mol % of edelfosine induced clear changes since an isotropic component was now visible, which can be attributed to small vesicles or a micelle and small vesicle mixture. In addition, the main peak now appeared split due to the resolution of the different phosphorus nuclei. $\Delta\sigma$ of this mixture was 50 ppm, indicating that this membrane was in an intermediate fluidity state, and this is in agreement with the DSC results shown above, which showed a shift toward higher temperatures of the second transition peak when 20 mol % of edelfosine was added. The POPC/SM/cholesterol (1:1:1 molar ratio) had a $\Delta\sigma$ of 48 ppm again indicating an intermediate degree of fluidity. The addition to this sample of 10 mol % of edelfosine reduced this value to 47 ppm, but as in the 3 °C spectrum, it was interesting that a shoulder appeared in the main peak. This shoulder was converted to almost a second peak when 20 mol % of edelfosine was added, with a $\Delta\sigma$ of 43 ppm, indicating a clear fluidification of the sample, although no isotropic peak was detected so that the possible complexes formed by edelfosine with the membrane lipids remained in the membrane and were not solubilized. It is then concluded that cholesterol is capable of protecting from solubilization when up to 20 mol % of edelfosine was added.

Finally, at 45 °C, $\Delta\sigma$ values were 45, 43, 48, 43, and 42 ppm for POPC/SM (1:1 molar ratio), POPC/SM (1:1 molar ratio) plus 20 mol % edelfosine, POPC/SM/cholesterol (1:1:1 molar ratio), POPC/SM/cholesterol (1:1:1 molar ratio) plus 10 mol % of edelfosine, and POPC/SM/cholesterol (1:1:1 molar ratio) plus 20 mol % of edelfosine, respectively. These values indicate that membranes were fluid at this temperature, except POPC/SM/cholesterol (1:1:1 molar ratio), which conserved an intermediate degree of fluidity. It should be remarked that a very clearly visible isotropic peak was now present in the POPC/SM plus 20 mol % of edelfosine sample evidencing a considerable solubilization and/or formation of small vesicles. Therefore in the absence of cholesterol, 20 mol % of edelfosine has a certain solubilization capacity.

Location of Edelfosine in Cells. Human T lymphoid leukemic Jurkat cells take up a significant amount of edelfosine (31 ± 4.2 pmol/10^6 cells after 7 h of incubation with 500 pmol [^{3}H]edelfosine; $n = 4$). After lipid raft isolation in sucrose gradients, we found that about $42.6 \pm 5.2\%$ ($n = 4$) of the edelfosine taken up by the cells accumulated in lipid rafts (Figure 8A and B). This accumulation of edelfosine in lipid rafts was also observed by using the fluorescent analogue PTE-edelfosine (all-[*E*]-1-*O*-[15′-phenylpentadeca-8′,10′,12′,14′-tetraenyl]-2-*O*-methyl-*rac*-glycero-3-phosphocholine), which behaves in a manner similar to that of the parental edelfosine.[28,63] PTE-edelfosine colocalized with lipid rafts, forming large edelfosine-rich lipid raft clusters in cancer cells (Figure 8C). The area comprised by the aggregated lipid rafts is about $25.6 \pm 6.3 \times 10^8$ Å^2/cell ($n = 5$), as assessed by fluorescence microscopy. Taking into account that a lipid molecule averages

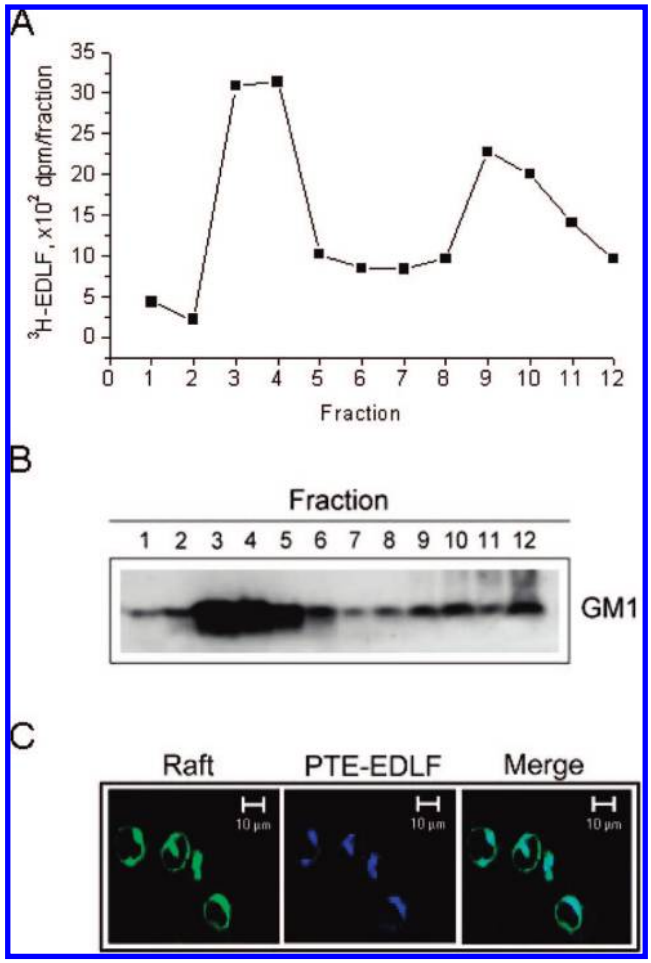

Figure 8. Accumulation of edelfosine (EDLF) in lipid rafts. Jurkat cells treated with 10 μM [^{3}H]edelfosine (EDLF) (0.1 μCi) for 7 h were lysed in 1% Triton and subjected to discontinuous sucrose density gradient centrifugation. The distribution of [^{3}H]edelfosine (EDLF) is expressed in dpm/1-mL fraction (A). Location of the ganglioside G_{M1}-containing lipid rafts was determined using CTx B subunit conjugated to horseradish peroxidase (B). (C) Jurkat cells were incubated with 20 μM PTE-edelfosine (EDLF) (blue fluorescence) for 7 h, and its colocalization with membrane rafts was then examined using the FITC-CTx B subunit (green fluorescence for rafts). Areas of colocalization between membrane rafts and PTE-edelfosine (EDLF) in the merge panel are cyan. Data are representative of four independent experiments. Bar, 10 μm.

70 Å^2, we found that edelfosine accounts for 21.7 $\pm$ 4.3% (n = 5) of the lipid molecules present in membrane rafts.

Discussion

The present findings indicate that edelfosine accumulates in lipid rafts in leukemic cells at a high concentration: 21.7 $\pm$ 4.3% of total lipid content in cell rafts. At such a concentration, edelfosine is able to affect a number of biophysical features of lipid membranes, as has been shown above using different biophysical techniques for model membranes. The POPC/SM/cholesterol system is often taken as a raft model, although it is necessary to be cautious since it has been suggested that cholesterol freely diffuses in the membrane, and it does not form POPC/SM/cholesterol domains.[64] Even more complex systems have been also used as raft models.[65]

The DSC experiments showed that the addition of 20 mol % edelfosine to a POPC/SM/cholesterol mixture, which does not show any phase transition in the range −5 to 45 °C, produced a change in membrane organization, with the appearance of a sharp phase transition. This effect is probably produced by the association of edelfosine with cholesterol. Similarly, the X-ray diffraction studies showed that in the absence of cholesterol the addition of edelfosine widened the peaks observed in the diffractograms, probably indicating a decrease in the membrane order.

It is interesting that, whereas a certain reduction in the d-spacings was observed at low temperatures, the transition to fluid phase involved a reduction in d-spacing in the gel phase but not in the fluid phase so that edelfosine seems to stabilize the repetition distance.

When cholesterol was present, only small changes in d-spacings were observed upon increasing the temperature. A new lamellar phase with reduced d-spacing was now detected but only in the presence of 20 mol % edelfosine, probably due to the stable association between cholesterol and edelfosine. It is also of note that the addition of edelfosine in the presence of cholesterol did not modify the scattering peaks, although it increased their width and reduced their intensities, as occurred in the absence of cholesterol. This may indicate the (at least) partial neutralization of the effect of edelfosine by cholesterol.

^{2}H NMR clearly illustrated the phase heterogeneity present in a POPC/SM/cholesterol mixture, and the addition of edelfosine increases the gel phase so that the fluidity buffering effect of cholesterol is abolished. ^{2}H NMR showed that the addition of edelfosine to POPC/SM induced some disordering, although an isotropic phase appeared at high temperature. However, in the presence of cholesterol, the addition of edelfosine induced an increase in membrane order at low temperatures, whereas at high temperatures, the order decreased, indicating that edelfosine forms a complex with cholesterol. It has to be taken into account that the molar percentages of edelfosine present are always lower than those of cholesterol and that if it is assumed that the interaction is 1:1, not all molecules of cholesterol may be neutralized. Using higher concentrations of edelfosine would produce solubilization/micellization, and furthermore, cell studies have shown that about 20 mol % of total lipids is a concentration likely to occur *in vivo*. Nevertheless, it should be noted that 10 mol % one also altered the membrane organization as shown by ^{2}H NMR. The ^{31}P NMR studies confirmed these conclusions showing that cholesterol protected from the solubilization effect of edelfosine, suggesting the formation of a complex between edelfosine and cholesterol, and these complexes remained in the membrane. The cell studies also showed that up to 20 mol % edelfosine of total lipids in the rafts did not dissolve them inasmuch as they were still clearly separated from other zones of the membranes.

Edelfosine exerts its proapoptotic action in tumor cells in the micromolar range,[4] and the drug is taken up by the cancer cell and accumulated in lipid rafts.[23,28,30] At this micromolar range, our present studies suggest that a high percentage (about 21.7%) of edelfosine accounts for the lipid molecules present in membrane rafts in the tumor cell, thus leading to alteration of the biophysical raft properties. Nevertheless, normal cells do not take up significant amounts of edelfosine.[4,29] A putative protein-dependent process has been proposed as the underlying basis for this differential uptake between malignant and normal cells.[4] Pharmacokinetic analyses in rats show that *in vivo* edelfosine treatment renders micromolar concentrations both in blood and tumors.[66,67] On these grounds, the effects described here of edelfosine on the biophysical traits of rafts are expected to take place under *in vitro* and *in vivo* conditions showing the antitumor activity of the drug.

The data reported here are in agreement with recent evidence showing that edelfosine partitions into lipid rafts and leads to a redistribution of sterols from the plasma membrane into the yeast cells.[32] The results herein reported show that this redistribution of a major lipid raft component alters the biophysical properties of the raft microdomain.

The high affinity of edelfosine for cholesterol demonstrated in this study is in agreement with previous findings that showed a redistribution of cholesterol from the plasma membrane to the cells after edelfosine treatment.[32] Because cholesterol is a major and essential molecule in lipid rafts, the effect of edelfosine on cholesterol will lead to an alteration of the lipid raft composition both in lipids and proteins. In this regard, edelfosine has been shown to recruit a number of proapoptotic molecules, in particular Fas/CD95 death receptor and downstream signaling molecules, into clusters of lipid rafts, leading to the triggering of apoptosis in leukemic cells.[28,30,54] Likewise, edelfosine also alters the lipid raft protein composition in yeasts, leading to the displacement of the essential plasma membrane protein Pma1p out of lipid rafts and subsequently to cell death.[32]

The results herein reported support the idea that the antitumor drug edelfosine acts through reorganizing and modifying lipid rafts and also support the current view of considering lipid rafts as a novel and promising anticancer target.[68]

Acknowledgment. This work was supported by a grant from the Fundación Séneca, Región de Murcia (00591/P1/04) to J.C.G.-F. and grants from Fondo de Investigación Sanitaria and European Comission (FIS-FEDER 06/0813 to C.G. and 04/0843 to F.M.), and Ministerio de Educación y Ciencia (SAF2005-04293) to F.M.

Abbreviations

Chol = cholesterol
DSC = differential scanning calorimetry
EDTA = ethylenediaminetetraacetic acid
ET-18-OCH$_3$ = 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine
^{2}H NMR = ^{2}H-nuclear magnetic resonance
M_1 = first spectral moment
MLVs = multilamellar vesicles
^{31}P NMR = ^{31}P-nuclear magnetic resonance
SM = sphyngomyelin
T_c = transition temperature
Tris = 2-amino-2-(hydroxymethyl)propane-1,3-diol
POPC = 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

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JP802165N