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Effects of antineoplastic ether lipids on model and biological membranes

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Differential scanning calorimetry and electron spin resonance were utilized to measure the effects of di-ether glycerophospholipid analogs (EL) on the physical properties of model membranes and on the membrane fluidity of HL60 leukemic cells. 1-Octadecyl-2-methyl-*rac*-glycero-3-phosphocholine (ET-18-OMe) and 1-thiohexadecyl-2-ethyl-*rac*-glycero-3-phosphocholine (ET-16S-OEt) lower the transition temperature of dimyristoylphosphatidylcholine vesicles in a range of concentrations between 0.5 and 15 mol %. Studies conducted on the interaction of EL with a wide spectrum of different phospholipids, namely dipalmitoylphosphatidylcholine, 1-hexadecyl-2-palmitoylphosphatidylcholine, dipalmitoylphosphatidylethanolamine, and dielaidoylphosphatidylethanolamine confirmed the ability of EL to effect the physical properties of model membranes. Changes in calorimetric enthalpy were observed only with phosphatidylethanolamine-containing phospholipids. ET-18-OMe and ET-16S-OEt increased the membrane fluidity of HL60 leukemic cells labeled with the fatty acid spin label probe 5-nitroxystearate. These data demonstrate the ability of EL to partition into phospholipidic domains and to change their physical properties. Furthermore, they affect the membrane fluidity of whole cells. These effects indicate an interaction between EL and the plasma membrane which may be of importance in determining the cytotoxic activity against tumor cells exerted by EL.

Introduction

Di-ether lipid (EL) analogs of platelet activating factor (1-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF) possess a wide range of biological activities including the inhibition of tumor cell growth in vitro and in vivo [1]. One of the most unusual characteristics of these compounds is that they are believed to act through an interaction with the plasma membrane. 1-Octadecyl-2-methyl-*rac*-glycero-3-phosphocholine, ET-18-OMe, which is the most widely studied analog, accumulates at the plasma membrane level [2,3]. EL also affect membrane-associated metabolic systems, such as phospholipid metabolism [4–7], phosphocholine synthesis [8,9], protein kinase C activity [7,10], and membrane transport [2].

We are particularly interested in the mechanism of action of EL as related to their antitumor properties and we have developed a series of compounds active against various experimental tumors to characterize their pharmacological properties [11–14]. The interaction of EL with the plasma membrane requires further investigation. Scattered evidence is available on the membrane morphologic damage to cells exposed to EL [15–18] and is generally contained in papers on other topics. Membrane fluidity changes have also been suggested [3,19], but the data seem to be controversial [3].

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As part of the comprehensive effort which includes a scanning and transmission electron microscopy study (SEM/TEM) [20] and in expansion of preliminary work [21], this paper presents data obtained using differential scanning calorimetry (DSC) and electron spin resonance (ESR). These are commonly used techniques to investigate the physical properties of model and biological membranes, respectively [22–24]. We combined several analogs with different phospholipid vesicle preparations in order to explore the ability of the EL to penetrate into lipid domains (DSC and model membranes). We also measured the EL-induced changes in membrane rigidity of whole leukemic cells labeled with the fatty acid probe 5-nitroxystearate (ESR and biological membranes).

Materials and Methods

Lipids

Dipalmitoyl-L-α-phosphatidylcholine (DPPC), dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylethanolamine (DPPE), and dielaidoyl-L-α-phosphatidylethanolamine (DEPE) were purchased from Avanti Polar Lipids, Birmingham, AL. 1-Hexadecyl-2-palmitoyl-*rac*-glycero-3-phosphocholine (HPPC) was obtained from Berchtold Biochemisches Labor, Bern, Switzerland. The purity of the lipids was checked by thin layer chromatography on a silica gel plate where they migrated as single spots in a system containing chloroform/ methanol/ acetic acid/water (50 : 25 : 8 : 2, v/v). The narrow phase transition profile was also good evidence for phospholipid purity.

The EL analogs employed in this study are listed in Table I, where chemical structures and antitumor activity against the HL60 human leukemic cell line are also reported. Except for ET-18-OMe, which was a kind gift of Dr. W.E. Berdel, Technical University, Munich, F.R.G., and lyso-PAF which was provided by Sigma Chemical Company, St. Louis, MO, all the EL were synthesized in a collaborative program by Dr. C. Piantadosi and his colleagues at the University of North Carolina at Chapel Hill [11–14]. All the EL were racemic. The EL were stored in the dark at −20 °C in a desiccator. Compounds 1–8 have ID_{50} values against HL60 between 1.6 and 3.7 μM, while lyso-PAF, a 2-hydroxy ether lipid with one log less inhibitory activity (ID_{50} = 43 μM), served as a 'negative' control.

TABLE I

CHEMICAL STRUCTURES AND ANTINEOPLASTIC ACTIVITY OF THE EL EMPLOYED IN THIS STUDY

This was determined using $0.5 \cdot 10^6$ cells/ml, and a trypan blue dye exclusion cytotoxicity test. See Refs. 13 and 14 for details.

Code	Chemical structure	Antineoplastic activity (ID_{50} against HL60 leukemia; μM)
1. ET-18-OMe	$CH_2O(CH_2)_{17}CH_3$ | $CHOCH_3$ | CH_2PC	2.5
2. ET-18-H	$CH_2O(CH_2)_{17}CH_3$ | CH_2 | CH_2PC	3.7
3. ET-18-OEt	$CH_2O(CH_2)_{17}CH_3$ | $CHOCH_2CH_3$ | CH_2PC	2.0
4. ET-16S-OMe	$CH_2S(CH_2)_{15}CH_3$ | $CHOCH_3$ | CH_2PC	2.2
5. ET-16S-OEt	$CH_2S(CH_2)_{15}CH_3$ | $CHOCH_2CH_3$ | CH_2PC	1.7
6. ET-18S-OMe	$CH_2S(CH_2)_{17}CH_3$ | $CHOCH_3$ | CH_2PC	2.0
7. ET-18S-OEt	$CH_2S(CH_2)_{17}CH_3$ | $CHOCH_2CH_3$ | CH_2PC	1.3
8. Amido-18-OEt	$CH_2NHCO(CH_2)_{16}CH_3$ | $CHOCH_2CH_3$ | CH_2PC	1.6
9. Lyso-PAF	$CH_2O(CH_2)_{15}CH_3$ | $CHOH$ | CH_2PC	>10

The fatty acid spin label probe 5-nitroxystearate, [2(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl], was purchased from Syva Associates, Palo Alto, CA. A 10 mM stock solution in ethanol was prepared and kept in the dark at −20°C.

Cells

HL60 human leukemic cells were obtained from the American Type Culture Collection, Bethesda, MD. They were propagated in suspension culture in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM L-glutamine supplement (all from GIBCO) at a density ranging between 0.5 and $1 \cdot 10^6$ cells/ml.

DSC experimental

DSC samples were prepared according to the method of Wright and White [25] with minor modifications. Briefly, appropriate amounts of drugs in ethanol were combined with 7 mg of phospholipid in chloroform (for the PCs), or in methanol at 50°C (for DEPE and DPPE). Samples were then evaporated to dryness under a gentle stream of nitrogen. To remove the excess of organic solvent overnight vacuum desiccation was performed at room temperature. Dry lipids were then rehydrated with 100 μl of 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes, pH 7.4), and kept in a water bath at 10°C above the transition temperature of the phospholipid for about an hour, during which time the samples were vortexed every 15–20 min in order to obtain a homogeneous suspension of multilamellar vesicles. For DSC measurements, a 75 μl aliquot of the suspension, containing 5 mg of phospholipid, was pipetted into stainless steel pans (Large Volume Caps, Perkin-Elmer, Norwalk, CT) which were then hermetically sealed. A DuPont 1090 thermal analyzer equipped with a DuPont 910 differential scanning calorimeter and a DuPont 1091 disk memory were used (DuPont Instruments Company, Wilmington, DE). Indium and gallium standards were used for the instrument calibration. Temperature scans were programmed as follows: DMPC, from 5°C to 35°C at 1 C°/min; DPPC and HPPC, from 20°C to 60°C at 1 C°/min; DEPE, from 10°C to 70°C at 1 C°/min; DPPE, from 25°C to 75°C at 1 C°/min.

The main phase transition temperature was defined as the peak of the gel to liquid-crystalline endotherm, and was calculated as T_c, which is the point where the slope of the downward deflection of the thermographic peak intersects the base-line. This point was then projected on the centigrade temperature axis. Calorimetric enthalpies (ΔH, in kcal/mol) were evaluated by planimetry (Neumonics model 1250, Landsdale, PA) using the internal power signal as a reference. The width at half-height ($\Delta T_{1/2}$) of the phase transition profile was also calculated and expressed in °C. Variations in these parameters are indications of the interaction occurring between the drug and the phospholipid. When the calorimetric enthalpy is not changed in the presence of the drug, a buffer/phospholipid partition coefficient can be calculated on the basis of the calorimetric data according to Kamaya et al. [26]. Higher values of this coefficient can be considered a good indication of the increased ability of the drug to partition into phospholipid model membranes. DSC calculations were made according to Mabrey and Sturtevant [23]. Lipid phosphorus assays were performed on randomly selected samples according to the method of Chalvardjian and Rudnicki [27], to assess the quantitative delivery of lipid to the sample pans.

ESR experimental

HL60 leukemic cells at a concentration of $2 \cdot 10^6$/ml were incubated for 2 h at 37°C in a total volume of 10–40 ml of medium without (control) and in the presence of 8 μM ET-18-OMe or 1-thiohexadecyl-2-ethyl-*rac*-glycero-3-phosphocholine (ET-16S-OEt), which corresponds to 2 $\mu M/0.5 \cdot 10^6$ cells. The ID_{50} values listed in Table I were obtained incubating $0.5 \cdot 10^6$ cells/ml for 48 h [14]. These two analogs are not toxic after 2 h incubation at the concentrations used in this study. The samples were centrifuged for 10 min at 2500 rpm, the supernatant discarded and the cells resuspended in 0.5 ml of phosphate-buffered saline (PBS, pH 7.4). The cells were labeled with 2.5 μl of the 10 mM stock solution of probe, gently shaken, and analyzed immediately.

The ESR measurements were carried out with a Bruker ER 200 D spectrometer equipped with a variable temperature-controlled unit, Bruker ER 4111 VT. Between 10 and 20 spectra were collected for each sample using an IBM Aspect 2000 computer. The instrument parameters were set as follows: field modulation, 100 kHz; microwave power, 6.3 mW; scanning time, 20 s; sweep width; 200 G; time constant, 200 ms; modulation amplitude, 1 G. Spectra were collected at 25 °C.

The order parameter is a measure of relative membrane rigidity and is calculated from the outer and inner hyperfine splitting of the spectra (measured using the Aspect 2000 computer) according to Gaffney [28]. Completely free probe signals were obtained when ESR spectra were collected using the probe and the drug at the concentrations employed in the experiments.

Results

Fig. 1 shows typical thermograms obtained with the phospholipid vesicle preparations with and without ET-18-OMe.

Fig. 2 illustrates the ability of two EL active against HL60 to decrease the phase transition temperature of DMPC liposomes. This decrease is concentration-dependent and linear between 0 and 5 mol % for ET-18-OMe (correlation coefficient $R = -0.980$) and between 0 and 3 mol % for ET-16S-OEt ($R = -0.992$). The EL also broaden the thermographic peak (increased $\Delta T_{1/2}$), but do not affect the calorimetric enthalpy, with the exception of both EL at 10 mol % (data not shown). These experiments using a wide range of concentrations indicated that 3 mol % is the only point at which the two compounds having different inhibitory activities (Table I) show a significant difference and where the thio analog has a stronger effect on the transition temperature (Fig. 2 and Table II). Therefore we decided to run the DSC analyses with the complete pattern of phospholipids and with the entire spectrum of analogs at 3 mol %.

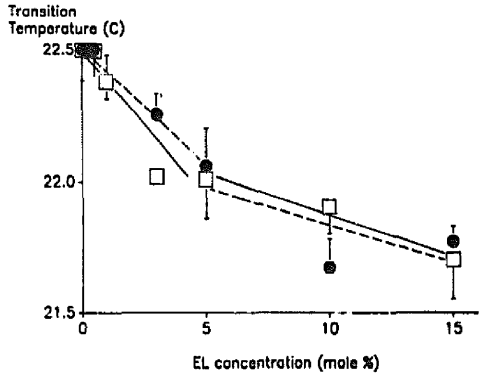

Fig. 2. Concentration-dependent decrease of the transition temperature of DMPC vesicles in the presence of two EL. Mean ± S.D., $n = 3$. ●— - - —●, ET-18-OMe; □———□, ET-16S-OEt.

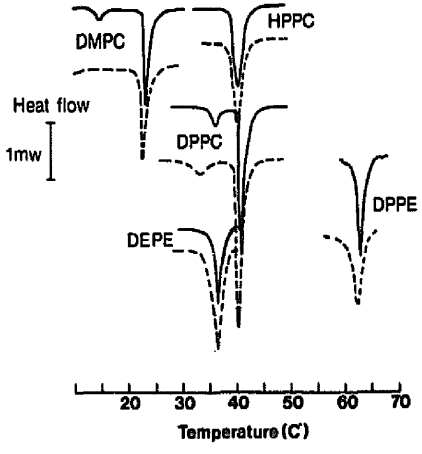

Fig. 1. DSC thermograms of pure phospholipid preparations (control; ———) and in the presence of 3 mol %. ET-18-OMe (— — —).

When DPPC and ET-18-OMe concentrations varying from 0.5 to 10 mol % were used, the same type of phenomenon was found, and the resulting decrease in transition temperature was as follows: 0.5 mol % = no decrease; 1% = −0.1 C°; 5% = −0.4 C°; 10% = −0.5 C°.

When pure DMPC and DPPC were used, a pretransition peak was clearly evident in both cases at 13 °C and 34.5 °C, respectively (Fig. 1). Addition of EL resulted in a significant downward shift of the pretransition. For example, with DMPC and ET-18-OMe (0.5 mol%), the T_c of the pretransition was lowered by 1.5 C°. The pretransition peak disappeared when 3 mol % EL was added to DMPC (Fig. 1) and when 5 mol % of the

TABLE II

ALTERATION OF THE CALORIMETRIC PARAMETERS OF DMPC VESICLES IN THE PRESENCE OF 3 mol % OF EL

Values are given as the mean ± S.D.; $n = 3$.

Code	T_c (°C)	$T_{1/2}$ (°C)	ΔH (kcal/mol)
Control	22.50 ± 0.12	0.80 ± 0.06	5.81 ± 0.47
1. ET-18-OMe	22.25 ± 0.08	1.10 ± 0.17	5.47 ± 0.27
2. ET-18-OEt	22.00 ± 0.17	1.10 ± 0.10	6.37 ± 0.64
3. ET-18-H	22.33 ± 0.15	1.10 ± 0.15	5.86 ± 0.99
4. ET-16S-OMe	22.20 ± 0.06	1.03 ± 0.06	5.92 ± 0.20
5. ET-16S-OEt	22.02 ± 0.17	1.20 ± 0.10	6.31 ± 0.33
6. ET-18S-OMe	22.10 ± 0.10	1.07 ± 0.06	6.18 ± 0.08
7. ET-18S-OEt	22.00 ± 0.10	1.20 ± 0.10	5.80 ± 0.39
8. AM-18-OEt	22.10 ± 0.14	1.08 ± 0.04	5.48 ± 0.59
9. Lyso-PAF	22.05 ± 0.05	1.10 ± 0.06	6.00 ± 0.32

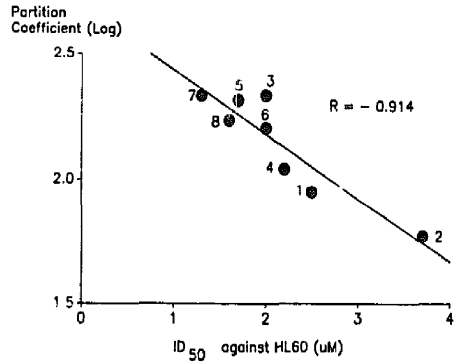

Fig. 3. Relationship between the buffer/phospholipid partition coefficient of DMPC and activity against HL60 cells expressed as ID_{50} of the eight analogs with ID_{50} values lower than 10 μM. The numbering of the analogs is given in Table I.

analog was added to DPPC liposomes (data not shown).

The results of the experiments conducted with nine EL and DMPC liposomes are listed in Table II. All the analogs tested induced a significant decrease in the transition temperature and a significant broadening of the thermographic profile in comparison to the values of the control sample (DMPC without the drug). No significant change in the calorimetric enthalpy was observed. These data suggest that all the EL tested are capable of partitioning into lipid domains and can alter the physical properties of these domains. If we consider only the analogs which have good growth-inhibitory activity against HL60 leukemic cells (below 4 μM) (analogs 1–8) and plot partition coefficients, calculated as described in the Methods section, together with antitumor activity expressed as ID_{50} values, we can derive the trend illustrated in Fig. 3. The most active analogs are also those most capable of partitioning into DMPC model membranes.

The experiments with the entire pattern of phospholipids were performed using three of the EL: ET-18-OMe, ET-16S-OEt, and amido (AM)-18-OEt. The T_c and ΔH control values of DMPC, DPPC, DPPE, and DEPE correspond to those reported in the literature [29]; this is, to our knowledge, the first report on the calorimetric parameters of HPPC which are: $T_c = 39.11 \pm 0.15$ °C; $\Delta H = 8.22 \pm 0.88$ kcal/mol. This is consistent with previous observations [30] reporting that ether-ester glycerophospholipids have slightly lower transition temperatures than their di-ester analogs.

All three EL were able to alter T_c as shown in Table III. Differences in chain length (C_{16}-PC vs. C_{14}-PC), in chain composition (di-ester PC vs. ether-ester PC) or in the presence of double bonds (saturated PE vs. monounsaturated PE) of the phospholipids which served as substrates did not substantially affect the behavior of the three EL.

However, differences in the head group seem to result in a stronger interaction. In fact, PE-containing phospholipids were more affected by EL partitioning than PC-containing preparations. As shown in Table III, the transition temperatures of DPPE and DEPE were decreased by more than 1

TABLE III

TRANSITION TEMPERATURE DECREASE (C°) OF THE FIVE PHOSPHOLIPID PREPARATIONS IN THE PRESENCE OF 3 mol % OF EL

	DMPC	DPPC	HPPC	DPPE	DEPE
ET-18-OMe	0.25	0.30	0.22	1.33	1.10
ET-16S-OEt	0.48	0.43	0.32	1.32	1.23
AM-18-OEt	0.40	0.35	0.35	1.60	1.34
Pure phospholipid transition temp. (°C)	22.5	41.1	39.1	62.0	36.1

C°, while the T_c decrease of PCs varied from 0.25 C° to 0.5 C°. In addition, when PC phospholipids were used we did not observe changes in the calorimetric enthalpy, whereas in the case of DPPE and DEPE vesicles, we did observe changes in enthalpy. For example, pure DEPE liposomes have a ΔH corresponding to 5.1 ± 1.69 kcal/mol; when 3 mol % ET-16S-OEt is present this value is increased to 9.08 ± 0.91. Pure DPPE ΔH averages a value of 8.9 kcal/mol, and in the presence of 3 mol % of ET-18-OMe this value is shifted to 6.9 ± 1.17 kcal/mol. These values were carefully checked calculating the exact delivery of phospholipid in the pan by assaying the phosphorus content of these samples. This was a necessary procedure since PE-containing vesicles in methanol are much more heterogeneous than DPPC and DMPC vesicles in $CHCl_3$, and pipetting variability is higher during sample preparation. When PC phospholipids were used and phosphorus assays were performed, we found excellent correspondence between the calorimetric values calculated assuming a 5 mg content of phospholipid and those calculated on the basis of the actual phosporus content. Since the drugs were used at 3 mol %, the contribution of the phosphorus contained in the EL can be considered negligible.

We investigated also the effect of EL on the lamellar to hexagonal phase transition of DEPE. Pure DEPE showed a value for this transition corresponding to 61.8°C which was increased by 3 mol % EL by several degrees. This increase ranges from 1.4 C° to 4 C°. The low calorimetric enthalpy of this phenomenon does not allow very accurate measurements of this parameter.

In addition to extensive DSC work, our investigation included whole cell membrane fluidity experiments using ESR techniques on HL60 cells. Table IV describes the order parameter variation of the HL60 plasma membrane, after ESR analysis of 5-nitroxystearate-labeled cells. ET-18-OMe and ET-16S-OEt significantly decrease membrane rigidity at 25°C at a drug/cell ratio of 2 μM/0.5 $\cdot 10^6$ cells. Cell viability assayed by trypan blue dye exclusion after the 2 h incubation was 100%. The spin label dose employed was not toxic to the cells, the cell counts after the ESR analysis being equal to those before the addition of the label.

TABLE IV

ORDER PARAMETER OF 5-NITROXYSTEARATE-LABELED HL60 CELLS AFTER EXPOSURE TO ET-18-OMe AND ET-16S-OEt

Controls received vehicle (PBS). Values are expressed as the mean ± S.D. See text for details.

	Order parameter	n
Control	0.6245 ± 0.0125	10
ET-18-OMe	0.5895 ± 0.0094	11
	$P < 0.005$	
Control	0.6128 ± 0.0069	6
ET-16S-OEt	0.5833 ± 0.0057	6
	$P < 0.005$	

Discussion

The biophysics of naturally occurring ether lipids and their relationship with biological and model membranes has been extensively reviewed by Paltauf [31]. Our work dealt with synthetic di-ether phospholipids and the characterization of their membrane interactions in order to better define their pharmacological properties.

We initiated the first in-depth study with ether lipid analogs involving three approaches to membrane analysis: SEM/TEM [20], DSC and model membranes, and ESR and biological membranes. The high degree of heterogeneity of the membrane environment convinced us of the necessity of a multiple approach to obtain more accurate and complete information. Therefore, we integrated the DSC study with other techniques, such as SEM/TEM and ESR, which allowed the use of viable cells. Thus, the type of information obtained achieves greater biological significance.

DSC

The DSC study clearly indicates that EL in general partition into lipid bilayers and change the physical properties of model membrane preparations, as evidenced by (i) the decrease in the transition temperature values; (ii) the broadening of the thermographic peaks; (iii) the disappearance of the pretransition in DMPC and DPPC models; and (iv) the change in the calorimetric enthalpy values in PE-containing models.

These observations are consistent with previous reports with DPPC and ET-18-OMe, PAF and other related compounds at 10 mol % [21,32], and with DPPC or distearyl-PC (DSPC) and the ether-desoxylysolecithin, 1-hexadecyl-2-propanediol-3-phosphorylcholine, at 5 mol % [33].

Extensive work has been done at 3 mol % because at this concentration differences in the ability of the compounds to induce a transition temperature decrease were detectable as indicated by our preliminary experiments. Furthermore, higher concentrations (10 or 15 mol %), at which a high degree of membrane perturbation occurs (Fig. 2), are outside the range of concentrations which can be reached by the accumulation of EL in the membrane at nontoxic levels and therefore these high EL concentrations cannot, in our opinion, represent a pharmacologically meaningful model for early stage membrane changes.

At 3 mol %, EL induce a significant and reproducible decrease of the main transition temperature of PC-containing liposomes and a broadening of the thermographic signal. This phenomenon is moderate (between 0.3 and 0.5 C°), and the calorimetric enthalpy is not changed. The pretransition phase of DMPC and DPPC was, however, strongly affected.

When PE-vesicles are used, a stronger interaction occurs with 3 mol % EL. The transition temperatures of DPPE and DEPE are lowered by 1 C° or more, and significant changes in calorimetric enthalpy occur. Blume et al. [33] measured significant changes in the calorimetric enthalpy of DPPE and dimyristoyl-PE (DMPE) in the presence of lysophosphatidylcholine. These data confirm that a stronger perturbation of bilayer packing occurs when different head groups interact.

An advantage of including DEPE in this study was to investigate EL effects on the lamellar to hexagonal transition. In fact this phospholipid adopts this configuration at temperatures above 60°C [34] as many lipids do under physiological conditions [35]. DSC is not the best technique to monitor this phase transition due to the low energy involved. However, the control value for the lamellar to hexagonal T_c corresponds to published data [34] and the increase due to the presence of the EL was clearly evident. Epand [34] described a similar increase using lysophosphatidylcholine. The increase of this parameter by EL suggests that the analogs stabilize the bilayer conformation and is another indication of their ability to interfere with the physical properties of PE-containing lipid systems.

The EL ability to penetrate a phospholipid domain and affect its physical properties seems to be a property common to both active and less active compounds (e.g., lyso-PAF) and cannot be strictly correlated with cytotoxicity. However, if we consider only the analogs with good inhibitory activity on cancer cells (compounds 1–8), we can speculate that lipophilicity is a requirement for EL designed to be active against cancer cells. All the active inhibitory compounds in fact showed the ability of partitioning into membranes, and the trend depicted in Fig. 3, where only active EL ($ID_{50} < 4\ \mu M$) are included, shows that more lipophilic compounds are better inhibitors of malignant cells.

ESR

In order to investigate EL effects on the physical properties of biological membranes, we performed preliminary ESR experiments measuring the order parameter value of HL60 leukemic cells labeled with the fatty acid probe 5-nitroxystearate. These experiments showed that this value can be lowered by ET-18-OMe and ET-16S-OEt, thus indicating that the membrane fluidity is increased. The magnitude of the variation, although small (5%), is significant from the point of view of membrane fluidity, in which small changes can imply severe alterations in the normal metabolism or homeostasis of the cell [36]. Furthermore, our observations are supported by high statistical significance with very large sample populations.

Our experimental design allowed us to relate the increase of membrane fluidity specifically to the presence of the drug. We used a dose (2 μM) which is cytotoxic when the incubation is carried out for 24–48 h [14], but we incubated for only 2 h, at which time no cytotoxicity has yet occurred. In addition, the uptake of 2 μM ET-18-OMe after 2 h of incubation is 10% of the administered dose (Daniel, L.W., unpublished data), and based on the total phospholipid content of HL60 (Daniel, L.W., unpublished data) we obtain a drug/phos-

pholipid ratio close to 3 mol %, which makes this study consistent with our DSC work.

We think that we can ascribe the decrease in membrane ordering to the plasma membrane. In fact, in order to have some evidence that the ESR measurements were primarily due to the probing of the plasma membrane, we regenerated the spontaneously decayed signal with $K_3Fe(CN)_6$ [36,37], achieving a complete restoration of the spectrum (data not shown). This is a good indication that the spin label probe must be on the cell surface.

This first set of ESR experiments warrants further investigations with various analogs at different cell lines and at different temperatures (25–37°C).

The membrane fluidity changes induced by the antineoplastic EL on tumor cells represent additional evidence of EL–membrane interaction and indirectly give biological significance to the phenomena described in model membranes. Changes in membrane fluidity induced by ET-18-OMe on MO4 mouse fibrosarcoma, on LLC-H61 mouse lung carcinoma, on HL60 human leukemia, and on R1C tumorigenic baby rat kidney cells, as measured by fluorescence polarization with the diphenylhexatriene (DPH) probe, have been reported by Van Blitterswijk et al. [3,19]. These investigators found that isolated plasma membranes may show a fluidity increase or decrease and that the nuclear membrane was fluidized in HL60 and LLC-H61 cells. These inconsistencies may reflect the cell type, culture conditions differences, the relative composition of the isolated preparations, or the small number of experiments done. Under these experimental conditions, cells showed moderate cytotoxicity (25%) when exposed to ET-18-OMe for 48 h. In our experience, treatment of HL60 cells under quite mild conditions (8 h at 2.5 μM ET-18-OMe) equivalent to 25% cytotoxicity resulted in measurable membrane changes (blebs, ruffles and holes) [20]. Thus, the membrane biophysical and drug uptake measurements reported [3] may have been influenced by cell membrane damage.

Conclusions

We conclude that EL showed affinity for membrane phospholipids, and this may be an important requirement for EL activity against tumor cells. Their ability to change model membrane physical properties and biological membrane fluidity at an early stage might be important for their mechanism of action. These data, together with our SEM and TEM results showing a dramatic plasma membrane damage in leukemic cells exposed to EL in vitro and in vivo, indicate that the plasma membrane can be considered a major target of antitumor EL. Membrane composition might then play a role in determining the susceptibility of a certain cell type to EL. We concur with other investigators [3] that EL-induced changes in membrane physical properties do not alone explain or strictly correlate with EL biological effects. Nevertheless, the effects of EL on membrane biophysics may be related to membrane biochemistry alterations in determining the pharmacological action of these compounds.

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