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Inhibition of Na^+/H^+ Exchanger Activity by an Alkyl-Lysophospholipid Analogue in a Human Breast Cancer Cell Line

Pierre Besson, Jacques Goré, Emmanuelle Vincent, Claude Hoinard and Philippe Bougnoux*

LABORATOIRE DE PHYSIOLOGIE ET BIOPHYSIQUE CELLULAIRES, FACULTÉ DE PHARMACIE AND LABORATOIRE DE BIOLOGIE DES TUMEURS, FACULTÉ DE MÉDECINE, UNIVERSITÉ DE TOURS, FRANCE

ABSTRACT. The mechanisms by which ET-18-OCH3 (1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphocholine) and other analogues of alkyl-lysophospholipids exert their antineoplastic effects are not yet fully elucidated. Possible interference with mechanisms involving intracellular pH (pH_i) regulation was examined by measuring the effect of ET-18-OCH3 on the activity of the Na^+/H^+ exchanger in the breast cancer-derived cell line MCF-7. When ET-18-OCH3 was added to culture medium at 10 μM (determined as a noncytotoxic but cytostatic concentration), it led to an intracellular acidification (0.15 pH unit). It also decreased the rate of pH_i recovery by Na^+/H^+ exchange following artificial acidification. Kinetic parameters of the exchange indicated that this was due to a decrease in the affinity of the exchanger for both transported ions, rather than to a decrease in the number of exchanger proteins in the membrane (same maximal efflux rate for treated and untreated cells). These results suggest that Na^+/H^+ exchanger inhibition and subsequent cytoplasmic acidification participate in the mode of action of ET-18-OCH3, and could be used for modulation of tumor-cell chemosensitivity or their subsequent commitment into programmed cell death. BIOCHEM PHARMACOL 51;9:1153–1158, 1996.

KEY WORDS. edelfosine; sodium-hydrogen antiporter; antineoplastic phospholipid ethers; intracellular hydrogen-ion concentration; membrane lipids

Antineoplastic ether phospholipids are metabolically stable analogues of ether phospholipids naturally present in membranes [1, 2]. The synthetic ether lipid ET-18-OCH3†, (edelfosine) is one of these new antineoplastic agents. Their antiproliferative mode of action is novel because, in contrast to a number of antineoplastic drugs, the target is not DNA. Their site of action is localized at their site of integration—the membranes—and their structure confers fusogenic and cytolytic properties on them, similar to those of natural alkyl-lysophospholipids, which may explain their cytotoxicity at high doses [3]. At lower and nontoxic concentrations, they have cytostatic properties as well as a selective action towards cancer cells, making them a valuable therapeutic tool [4]. Their mechanism of action, however, is not yet fully elucidated. Ether lipids have been reported to affect various aspects of membrane function by disturbing phospholipid metabolism [5] or by inhibiting enzyme activities, as has been shown for protein kinase C, Na/K ATPase [6], or phosphatidylinositol phospholipase C [7]. It has also been reported that ET-18-OCH3 inhibits the transport of a number of nutrients in cell lines, thereby inhibiting their proliferation [8].

There is no known example of an inhibitory effect of alkyl-lysophospholipids on proton transport. The pH_i is involved in the early signals leading to the proliferative response [9] and in the control of a large number of metabolic processes [10, 11]. Thus, the Na^+/H^+ exchanger participates indirectly in the control of cell proliferation by its regulation of pH_i. The sodium-proton exchanger includes approximately 10 predicted transmembrane segments [12, 13]. Its large contact surface with membrane lipids makes this protein a candidate to be functionally affected by the physical state of the membranes in which the lipids are included [14].

These arguments have led us to examine whether or not the ether phospholipid analogue ET-18-OCH3 could alter the functioning of the Na^+/H^+ exchanger. We used ET-18-OCH3 at a concentration that was inhibitory but not cytotoxic. The pH_i of cells grown in the presence of ET-18-OCH3 was lower than that of control cells and the capacity of the Na^+/H^+ exchanger to regulate pH_i was diminished. The study of the exchanger's kinetic properties showed a reduction in its affinity for the two ions transported, rather

* Corresponding author: Pr. J. Goré, J.E. 313 "Lipides et croissance," Faculté de Médecine, 2 bis boulevard Tonnellé, 37032 Tours Cedex, France. FAX (33) 47366226.

† *Abbreviations*: ET-18-OCH3, 1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphocholine; BCECF/AM, acetoxymethyl ester of 2′,7′-bis (carboxyethyl)-5(6)-carboxyfluorescein; MIA, 5-(N-methyl-N-isobutyl)-amiloride; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; pH_i, intracellular pH; β_i, intracellular buffering capacity; J_{H^+}, proton efflux rate; J_{max}, maximal proton efflux rate; K, apparent transport constant.

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than a reduction in the number of exchanger proteins present in the membrane.

MATERIALS AND METHODS

Materials

MIA was purchased from Research Biomedicals Incorporated (Bioblock Scientific, Illkirch, France). BCECF/AM was from Calbiochem (France Biochem, Meudon, France). ET-18-OCH3 was a generous gift of Dr. Paola Principe and Dr. Jean-Michel Mencia-Huerta (Institut Henri Beaufour, Les Ulis, France). Culture medium and reagents were purchased from GIBCO BRL Life Technologies (Cergy Pontoise, France). Hanks' medium, EDTA, and choline chloride solutions were prepared as previously described [15].

Cell Culture

MCF-7 breast cancer-derived cells were routinely cultured as previously described [15]. For experiments, the cells were seeded in routine culture medium in 175-cm^2 flasks and 150-mm diameter Petri dishes, for pH_i experiments and lipid analyses, respectively, at a density of 4×10^6 cells per flask or dish, and allowed to grow for 2 days. At day 2, medium was replaced with culture medium supplemented, or not, with the alkyl-lysophosphatidylcholine analogue. Cells were harvested at day 4. Stock solution of ET-18-OCH3 in ethanol-phosphate buffered saline (60:40, v/v) was stored at −20°C and was added at the desired concentration into culture medium. The final ethanol concentration was less than 0.2% of volume in culture medium.

Lipid Extraction, Purification and Analysis

Total lipid extracts of MCF-7 cells, phospholipid purification, and fatty acid analysis were performed as previously described [15]. Phospholipid samples were divided into two aliquots, one of which was used for fatty acid analysis and the other for phospholipid quantification by phosphorus assay based on the method of Bartlett [16]. Each fatty acid was expressed as nanomoles by comparison of the peak surface to that of tricosanoic acid (23:0) added to the sample as an internal standard before the derivatization stage.

Insertion of ET-18-OCH3 into Membranes

Cells were cultured with 10 μM ET-18-OCH3 for 48 hr. The insertion of the ether lipid into membranes was indirectly assessed by calculation of the ratio of the molar amount of fatty acids in membrane phospholipids to the molar amount of phosphorus in membrane phospholipids. To calculate the molar amount of whole fatty acids, unidentified fatty acids were given a mean molecular weight of 292 g/mol, based on a number of carbon atoms ranging from 16 to 22 (mean = 19) and a number of double bonds ranging from 1 to 5 (mean = 3).

Measurement of the Antiproliferative Effect and Toxicity of ET-18-OCH3

Cell proliferation was determined using the MTT reduction method [17]. Cells were seeded in 96 microwell plates (10^4 cells/well) and allowed to start growth for 48 hr. ET-18-OCH3 was added, and culture continued for 48 hr. On the day of cell quantification, MTT was solubilized in culture medium and added to the wells. After 4-hr incubation, medium was discarded and MTT-formazan crystals were solubilized with DMSO. Absorbance was measured in a multiwell plate spectrophotometer at 570 nm (Molecular Devices model Thermomax microplate reader, B. Braun-Science Tec, Less Ulis, France). To distinguish between a cytostatic effect and a cytotoxic effect, cell viability at 48 hr of treatment was assessed by the Trypan blue exclusion method.

Na^+/H^+ Exchanger Activity

Na^+/H^+ exchanger activity was determined as previously described [15] by measuring pH_i with the fluorescent pH-sensitive probe BCECF.

Statistics

Linear regressions, analysis of variance (ANOVA), Student's *t*-test, and the Mann-Whitney test were performed with an IBM-compatible PC using the P.C.S.M. programs (Deltasoft, Grenoble, France). Unless otherwise stated, values are presented as means ± SE. A P value < 0.05 was considered statistically significant.

RESULTS

Antiproliferative Effect of ET-18-OCH3 on MCF-7 Cells

ET-18-OCH3 inhibited the multiplication of MCF-7 cells in a dose-dependent manner, with a concentration causing 50% inhibition (IC_{50}) of approximately 6 μM (Fig. 1). In the following experiments, 10 μM was chosen as a working concentration for its efficiency in reducing cell proliferation and its low killing effect. Indeed, the Trypan blue exclusion test showed that after 48-hr treatment, 90 to 80% of cells were viable at concentrations ranging from 10 to 15 μM. For concentrations > 20 μM, cell viability dropped dramatically (below 10%).

Insertion of ET-18-OCH3 in Membranes

When MCF-7 cells were grown for 48 hr with ET-18-OCH3, there was a reduction in the mean total fatty acid: phosphorus ratio from 2.07 ± 0.22 to 1.67 ± 0.21 (control and treated cells respectively, mean ± SD, $n = 3$). The fatty acid composition of total membrane phospholipids (Table 1) shows a reduction in the proportion of all saturated fatty acids and an increase in the quantities of all polyunsaturated fatty acids in the n-6 and n-3 series. It was at a

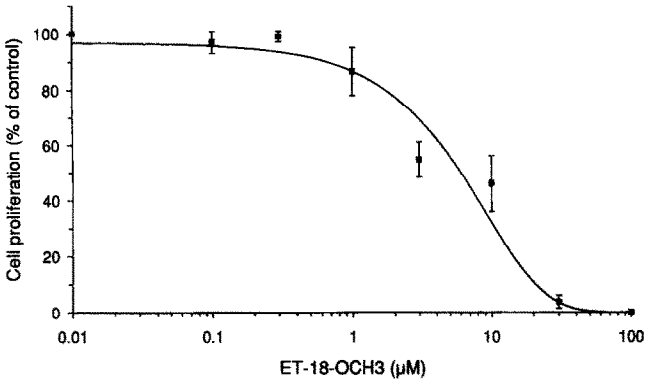

FIG. 1. Dose-dependent inhibition of MCF-7 cell proliferation by ET-18-OCH3. Cells were seeded in 96-well plates at a density of 10,000 cells per well (31,000 cells/cm^2) in culture medium and allowed to grow for 48 hr. ET-18-OCH3 in solution in culture medium was then added at the indicated concentration and the culture was continued for an additional 48 hr. Cell proliferation was assessed by the MTT reduction colorimetric assay. Each data point is the mean ± SE of 3 experiments carried out in 8 replicates for each ether lipid concentration.

quantifiable level for 20:2n-6, 20:5n-3, and 22:5n-3, which were detectable at only trace levels in control cells. The levels of monounsaturated fatty acids were unchanged by treating cells with ET-18-OCH3.

Intracellular pH (pH_i) and Intracellular Buffering Capacity (β_i)

The steady-state pH_i of cells charged with BCECF in HEPES buffer (37°C, pH 7.30, without $HCO3^-$) was significantly lower in cells treated with ET-18-OCH3 than in controls (Table 2). The recovery of pH_i was also determined in cells that had been acidified with 30 mM NH_4Cl for 5 min. The addition of NaCl (140 mM final) to the incubation medium (choline chloride buffer) caused a rapid realkalinization of the cytoplasm of control cells to a steady-state pH_i value of 7.17 ± 0.03 (n = 57) and a slower realkalinization of treated cells to an equilibrium value of 6.97 ± 0.07 (n = 29). The pH_i value in control cells after realkalinization was not significantly different from that in cells that had never been acidified. Inversely, in cells treated with ET-18-OCH3, the pH_i after realkalinization was significantly lower than the equilibrium pH_i of cells that had not been acidified ($P < 0.001$). When MCF-7 cells were treated with ET-18-OCH3, the intracellular buffering capacity was significantly increased (Table 2). Under the two culture conditions, β_i was constant over the pH_i range studied (6.4 to 7.2). Thus, the variations of the rate of proton efflux were directly related to variations of pH_i.

Kinetics of Na^+/H^+ Exchange

The kinetics of exchanger operation as a function of either intracellular protons or of extracellular Na^+ was significantly inhibited by treatment with ET-18-OCH3 (ANOVA, $P < 0.001$). Figure 2 shows how growth with ET-18-OCH3 changes the regulation of pH_i in MCF-7 cells as a function of the extent of cytoplasmic acidification. Kinetics of proton transport were modeled by the Hill equation as previously described [15]. The value of J_{max} (maximal rate of proton efflux) was similar, whether or not the cells were treated with ET-18-OCH3 (Table 2). On the other hand, the value of the apparent transport constant was almost doubled in cells treated with ET-18-OCH3 in comparison to control cells (329 ± 149 nM and 173 ± 14 nM, respectively).

Figure 3 shows how growth with ET-18-OCH3 modified the regulation of pH_i in MCF-7 cells as a function of extracellular Na^+. Experimental data fitted to the Michaelis-Menten model showed that the apparent transport constant (K) of the exchanger for Na^+ increased when cells were grown with ET-18-OCH3 in comparison to control cells (72 ± 57 mM and 22 ± 6 mM, respectively). The maximal rate of proton efflux, on the other hand, was unchanged (Table 2).

DISCUSSION

The Na^+/H^+ exchanger plays an essential role in the regulation of pH_i and, thus, participates in the control of most

TABLE 1. Membrane fatty acid composition of MCF-7 cells treated, or not, with 10 μM ET-18-OCH3*

Fatty acid	Control	ET-18-OCH3
14:0	2.50 ± 0.95	1.85 ± 0.41
16:0	19.81 ± 1.08	13.38 ± 0.56†
18:0	6.44 ± 1.14	5.40 ± 0.59
Total saturates‡	30.62 ± 1.81	22.20 ± 1.20†
14:1	0.55 ± 0.03	0.49 ± 0.13
16:1	17.44 ± 3.19	17.39 ± 0.95
18:1	34.61 ± 0.19	33.63 ± 1.44
24:1	1.54 ± 0.25	2.05 ± 0.06†
Total monounsaturates	54.85 ± 2.83	54.96 ± 1.77
18:2n-6	2.29 ± 0.55	5.29 ± 1.01†
20:2n-6	trace	0.27 ± 0.08†
20:3n-6	0.50 ± 0.09	0.81 ± 0.18†
20:4n-6	2.27 ± 0.07	4.89 ± 0.36†
Total polyunsaturates n-6	5.09 ± 0.72	11.25 ± 1.55†
18:3n-3	0.22 ± 0.03	0.34 ± 0.03†
20:5n-3	trace	0.26 ± 0.17†
22:5n-3	trace	0.20 ± 0.12†
22:6n-3	1.52 ± 0.16	2.08 ± 0.40
Total polyunsaturates n-3	1.73 ± 0.14	2.88 ± 0.14†
20:3n-9	0.24 ± 0.05	0.19 ± 0.04
Others	9.45 ± 0.49	8.53 ± 0.92

* Results are mean ± SD of 3 determinations of fatty acid levels in unfractionated phospholipids purified by thin layer chromatography. Values expressed as a percent of total peak area. Trace indicates level below 0.1%. †$P < 0.05$ compared to the control value (Mann-Whitney test). ‡Total, in each fatty acid class, comprises: Saturates, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0, and 24:0; Monounsaturates, 14:1, 16:1n-7, 18:1 (n-9 and n-7), 22:1 and 24:1; Polyunsaturates, n-6: 18:2n-6, 20:2n-6, 20:3n-6, 20:4n-6; Polyunsaturates, n-3: 18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3; Others, unidentified fatty acids and 20:1 poorly resolved from an unidentified peak.

TABLE 2. pH_i and characteristics of Na^+/H^+ exchanger in MCF-7 cells treated, or not, with ET-18-OCH3*

	Control	ET-18-OCH3 (10 μM)
Steady-state pH_i†	7.19 ± 0.07 (n = 54)	7.04 ± 0.05 (n = 32)
Cytoplasmic buffering capacity†		
β_i (mM H^+/pH unit)	42 ± 2 (n = 68)	51 ± 2 (n = 42)
Sensitivity to intracellular H^+‡	(n = 53)	(n = 33)
J_{max} (μM H^+/s)	576 ± 59	580 ± 262
K (nM)	173 ± 14	329 ± 149
h	2.75 ± 0.78	2.10 ± 0.71
Sensitivity to extracellular Na^+‡	(n = 19)	(n = 35)
J_{max} (μM H^+/s)	387 ± 29	382 ± 130
K (mM)	22 ± 6	72 ± 57

* pH_i, intracellular pH; β_i, intracellular buffering capacity; J_{max}, maximal efflux rate; K, apparent transport constant; h, Hill coefficient. Results are mean ± SE of n experiments. †$P < 0.001$, ET-18-OCH3 compared to control (Student's t-test); ‡$P < 0.001$, ET-18-OCH3 compared to control (ANOVA).

cell functions, including proliferation. In this study, we have shown that at a concentration that is noncytotoxic but inhibitory for the proliferation of MCF-7 cells, the analogue of alkyl-lysophosphatidylcholine, ET-18-OCH3, leads to a reduced functionality of the sodium-proton exchanger and to the acidification of cell cytoplasm.

The regulation of pH_i depends on several anion or cation transport systems (Cl^-/OH^-, $Na^+/HCO3^-$). The reduced rate of proton efflux in MCF-7 cells is most probably related to the functioning of the Na^+/H^+ exchanger and is not dependent on the altered activity of other transporters. The latter are, in fact, involved to only a slight extent in the regulation of the pH_i of MCF-7 cells, as shown in a prior study [15]. In addition, measurements of pH_i were carried out in an incubation medium lacking bicarbonates to circumvent the regulatory components involving these ions.

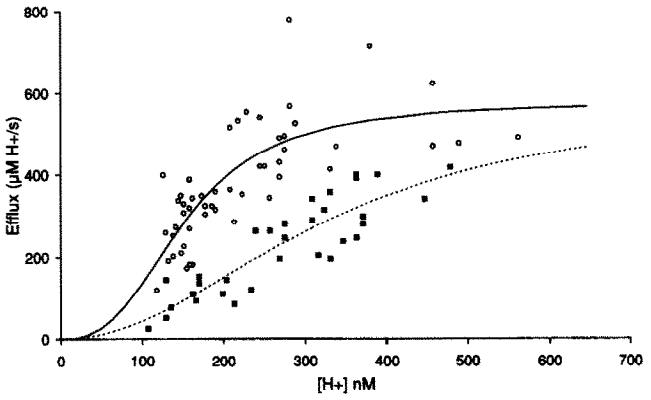

FIG. 2. Kinetics of sodium-proton exchange as a function of cytoplasmic acidity. Proton efflux rate was measured in MCF-7 cells treated for 48 hr with 10 μM ET-18-OCH3 (black squares) or untreated MCF-7 cells (white circles). BCECF-loaded cells were acidified to different levels with the ammonium chloride method and resuspended in sodium-free medium [15]. Proton efflux was initiated by addition of Na^+ and variations of BCECF fluorescence were calibrated to pH values. The initial rate of proton efflux was plotted against intracellular H^+ concentration. Each data point represents one single experiment.

Finally, the type of proton efflux kinetics observed can be fitted to a Michaelis-Menten model when the extracellular concentration of sodium ions is varied, and to an allosteric Hill model as a function of cytoplasmic acidity. This is in agreement with the known mode of functioning of the sodium-proton exchanger. The inhibition of pH_i regulation by ET-18-OCH3 is, thus, probably due to the inhibition of operation of the sodium-proton exchanger.

The inhibitory effect of ET-18-OCH3 is apparently due to the reduced affinity of the exchanger for the two ions transported, rather than to a reduced number of exchanger molecules in the membranes. The apparent transport constant (K) for sodium ions and protons increases, while the maximal theoretical rate of proton efflux (J_{max}), proportional to the number of exchangers, is unchanged by treatment with ET-18-OCH3. The way in which ET-18-OCH3 inhibits the Na^+/H^+ exchanger remains to be elucidated. Inhibition appears to differ from that observed with the amiloride derivative MIA, because the extemporaneous ad-

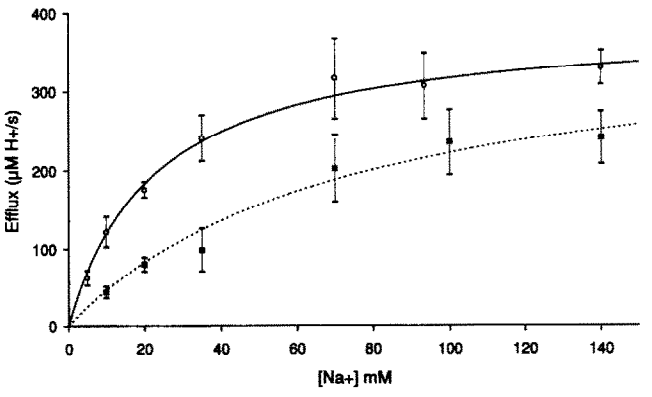

FIG. 3. Kinetics of sodium-proton exchange as a function of extracellular sodium ion concentration. Proton efflux rate was measured in MCF-7 cells treated for 48 hr with 10 μM ET-18-OCH3 (black squares) or untreated MCF-7 cells (white circles). BCECF-loaded cells were acidified to approximately pH_i 6.5. The initial rate of proton efflux was plotted against the concentration of added NaCl. Each data point is the mean ± SE of 3 to 6 separate experiments.

dition of 10 μM ET-18-OCH3 (slightly above IC_{50}) does not inhibit the exchanger (data not shown) under conditions where 0.33 μM MIA (equal to IC_{50}) instantaneously inhibits its operation [15]. ET-18-OCH3, thus, apparently does not act through the intermediary of the extracellular Na^+ site of the exchanger. The observed inhibition was associated with a reduction in the total fatty acid:phosphorus ratio in phospholipids extracted from the membranes of cells grown with ET-18-OCH3, consistent with the replacement of diacyl-containing phospholipids in membranes by the ether lipid. This makes probable the existence of disturbances in the physical state of membranes that could adversely affect the operation of the Na^+/H^+ exchanger and, perhaps, other membrane proteins as well. It is likely that these disturbances are specifically due to the ether lipid because incubating cells with supplemental fatty acids dramatically changed the fatty acid composition of membrane phospholipids and inhibited cell proliferation, but altered neither the operation of the Na^+/H^+ exchanger nor steady-state pH_i [15].

Our data do not enable us to determine whether or not ET-18-OCH3 acts upon the exchanger directly or indirectly. It has been reported that ET-18-OCH3 inhibits other transport systems, such as the transport of choline and of variety of nutrients [8], as well as the transport of ions in the case of Na/K ATPase [6]. It also alters the internalization of surface molecules, such as the EGF receptor [18], or the activity of enzymes translocated to the membrane, such as PKC [6]. Other membrane proteins and protein-protein interactions could also be affected by changes in the physical properties of the membrane. It has been shown that the correct operation of the sodium-proton exchanger [19–21], of the EGF receptor [22], or the receptor of PKC [23] is related to their interaction with proteins of the cytoskeleton. In the case of adherent cells, the adhesion of cells to each other or to a support is a signal that participates in the control of their proliferation. This signal is transmitted to the cytoskeleton by transmembrane proteins and integrin-type and cadherin-type receptors [24, 25]. By changing the physical properties of membranes, ET-18-OCH3 might disturb interactions among integrins, the cytoskeleton, and the sodium-proton exchanger. As a result, it could affect cell proliferation and reduce the functioning of the sodium-proton exchanger. In addition, it is known that ATP depletion leads to a decrease in the affinity of the exchanger for protons [26], although it does not necessarily lead to a decrease in the phosphorylation state of the protein. In addition to protein-protein physical interaction in the membranes as a mechanism for the inhibition of the exchanger, a stimulatory effect of ET-18-OCH3 on the activity of phosphatases could lower the phosphorylation state of the exchanger and, thereby, its functioning. The understanding of the mechanisms of action of ET-18-OCH3 requires that its effect on each of these potential targets be thoroughly investigated.

Another result of treating MCF-7 cells with ET-18-OCH3 is the increase in cytoplasmic buffering capacity (β_i). This property may reflect a physiological compensation for the inhibition of the sodium-proton exchanger, enabling the increase in β_i to partially oppose the effects of intracellular acidification. There are, in fact, a large number of consequences of cytoplasmic acidification [11] that could result in a reduction of cell proliferation or survival. A more acidic intracellular pH decreases the activity of various enzymes, such as those participating in glycolysis and in protein or nucleic acid synthesis [11]. It could also increase the activity of DNase II, an endonuclease involved in the triggering of apoptosis induced by chemotherapeutic agents [27]. The observation that ET-18-OCH3 induces apoptosis in leukaemic cells [28, 29] provides some grounds for this hypothesis.

pH is also a determining factor in the action of antineoplastic drugs on tumor cells. It has been shown that some antineoplastic substances accumulate in the cell preferentially when the pH is acid [30–32], thereby compensating for the phenomenon of multiple resistance to cytotoxic drugs [33]. It has been reported that analogues of ether lipids act synergistically with other antineoplastic drugs, such as mitotic spindle poisons and drugs interacting with DNA [34]. The acidifying effect of ET-18-OCH3 and the consequences on the regulation of pH_i could, thus, be used to modulate the chemosensitivity of tumors and could play a role in cancer treatment.

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