[CANCER RESEARCH 52, 6045-6051, November 1, 1992]

Membrane Peroxidative Damage Enhancement by the Ether Lipid Class of Antineoplastic Agents[1]

Brett A. Wagner, Garry R. Buettner, and C. Patrick Burns[2]

Department of Medicine [B. A. W., C. P. B.] and the Electron Spin Resonance Facility [G. R. B.], The University of Iowa College of Medicine, Iowa City, Iowa 52242

ABSTRACT

The ether lipid antineoplastic agents have no known interaction with DNA, but rather they appear to target membranes. The primary mechanism of action is unknown but effects on membrane biology are documented. We have studied the effect of two ether lipids on membrane lipids and examined the hypothesis that membrane peroxidative damage may be involved in their mechanism of action. With the use of cells having membranes enriched in polyunsaturated fatty acids of the ω-3 family of fatty acids, we have demonstrated that the prototypical ether lipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine and a thioether lipid analogue, 1-*O*-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine, increase membrane lipid peroxidation and cytotoxicity in a time- and drug concentration-dependent manner. The oxidative cofactors Fe^{2+} and ascorbic acid were required. The pattern of cell death did not fully correspond to the peroxidation, since cofactors were required for peroxidation but not cytotoxicity. However, the rate of decrease in cell viability after exposure to the drug and cofactors corresponded to the peroxidation rate. In addition, when L1210 cells modified with the monounsaturated fatty acid oleic acid or unmodified cells were used, there was no ether lipid-enhanced peroxidation, and the cells were significantly less sensitive to the drug, with or without cofactors. The lipid-soluble antioxidant vitamin E inhibited 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine peroxidation and cytotoxicity in a concentration-dependent manner in the presence of cofactors but not consistently without them. Depletion of cellular glutathione content of L1210 cells using L-buthionine-(*SR*)-sulfoximine resulted in 40% augmentation of cofactor-facilitated cytotoxicity of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine and a borderline effect on peroxidation. Another ether lipid, the thio compound 1-*O*-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine, enhanced peroxidation in the presence of cofactors with kinetics corresponding to those of cytotoxicity. In the presence of ether lipid and cofactors the intensity of ascorbate free radical increased, consistent with oxidative stress. We conclude that the ether lipids stimulate membrane lipid peroxidation in a time- and drug concentration-dependent manner in the presence of oxidative cofactors. Even though peroxidation may not fully explain the cytotoxic effect of the ether lipid class of anticancer drugs, this observation provides further information on the nature of the membrane damage induced by the drugs. Since the ether lipids generate no known free radical intermediates directly, this suggests that membrane damage indirectly results in a process involving a peroxidative reaction.

INTRODUCTION

The ether lipid class of investigational drugs are alkyl-linked glycerophospholipids structurally similar to the naturally occurring bioactive compound platelet-activating factor. Ether lipids contain ether linkages in positions normally occupied by ester linkages on phospholipids. Ether lipids that contain amido nitrogens, thio linkages, or other substituents and linkages have been synthesized and they vary in biological activity (1-9). They have been investigated *in vitro* and *in vivo* with solid tumors as well as leukemic cells (1, 2, 6, 9-15). Ether lipids are especially interesting because they seem to affect neoplastic cells to a greater extent than normal cells (9, 10, 13). These drugs are currently in clinical testing (16-19).

Ether lipids influence membranes and may exert their cytotoxic effect in that way; they do not directly damage or interact with DNA. However, the mechanism by which they affect membranes is unknown. Ether lipids have been reported to affect various aspects of membrane function by disturbing phospholipid metabolism (1, 9, 20), altering protein kinase C activity (5, 8, 21-25), inhibiting growth factor-dependent inositol phosphate calcium signaling (26), inhibiting phosphatidylinositol-specific phospholipase C (27), inducing cellular differentiation (1, 10, 22, 23), and activating macrophages (9).

Previously we have utilized a lipid-modification model to study membrane function. Biochemical modification of membrane fatty acids results in changes in physical properties such as membrane fluidity (28) and drug transport (29-31). These changes affect membrane dynamics and result in increases in cellular susceptibility to antineoplastic drugs (30), hyperthermia (28, 32, 33), and induction of differentiation (34, 35) and enhanced susceptibility to lipid peroxidation (36). We have now utilized this model to explore the manner by which ether lipids damage cell membranes. Specifically, we examined the possible role of peroxidative damage. Since lipid peroxidation can affect many cellular functions, ether lipids could exert their effects directly or indirectly by predisposing cells to free radical generation and membrane damage. This could explain the diversity of mechanisms proposed for this class of agents. Peroxidative damage could affect many aspects of membrane function.

MATERIALS AND METHODS

Cell Culture and Fatty Acid Modification. Stock L1210 murine leukemia cells were maintained in RPMI 1640 supplemented with 5% heat-inactivated characterized fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), at 37°C in a humidified 95% air/5% CO_2 atmosphere. Cells to be lipid-modified were grown for 2 days in RPMI 1640-5% fetal bovine serum supplemented with 32 μM 22:6ω3[3] or 18:1ω9 (Nu Chek Prep, Inc., Elysian, MN). Following modification, cells were pelleted, washed, counted, and resuspended at 1×10^7 cells/ml in Hanks' buffered salt solution without calcium and magnesium but supplemented with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Sigma Chemical Co., St. Louis, MO), pH 7.4, as previously described (36). Cell densities were determined with a Coulter model Z_F counter (Coulter Inc., Hialeah, FL) and viability by trypan blue dye exclusion.

For determination of fatty acid composition, cells were washed and extracted with $CHCl_3$-CH_3OH (2:1, v/v). Phospholipids in the lipid extracts were isolated using silicic acid chromatography. After alkaline

Received 5/11/92; accepted 8/18/92.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by Grant CA31526 awarded by the National Cancer Institute, Department of Health and Human Services. Data analysis utilized the Clinfo system, supported by Grant RR59 from the General Clinical Research Centers Program, Division of Research Resources, NIH.

[2] To whom requests for reprints should be addressed, at The University of Iowa Hospitals, Department of Internal Medicine, Iowa City, IA 52242.

[3] The abbreviations used are: 22:6ω3, docosahexaenoic acid; 18:1ω9, oleic acid; BSO, L-buthionine-(*SR*)-sulfoximine; TBARS, thiobarbituric acid-reactive substances; EPR, electron paramagnetic resonance; ET-18-OCH_3, (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; BM 41.440, 1-*O*-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine; 16:0, palmitic acid; 18:0, stearic acid.

hydrolysis, fatty acids in the saponifiable fraction were methylated and the methyl esters were separated by gas-liquid chromatography (29).

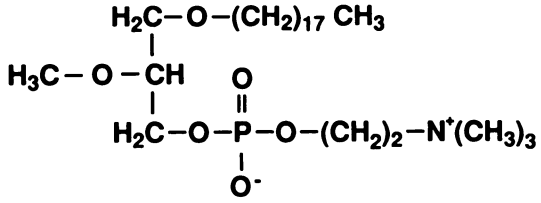
Vitamin E Supplementation. Vitamin E acetate (DL-α-tocopherol acetate) (Sigma) was dissolved in 100% ethanol at stock concentrations needed to supplement media, at 1 μl/ml, to achieve 20–600 μM vitamin E acetate (0.1% ethanol, v/v, in culture medium). Filtered (0.45-μm filters) stock solutions of vitamin E were added to cultures 24 h before cell harvest. Neither vitamin E acetate nor ethanol at the concentrations used affected cell growth rate or viability.

Glutathione Depletion. Cells were incubated for 48 h in fatty acid-supplemented RPMI 1640 with 5% fetal bovine serum. During the last 24 h, BSO (Sigma) at concentrations of 100 and 200 μM was added. We have previously reported that BSO results in glutathione depletion in L1210 cells under similar conditions (37).

Lipid Peroxidation. Two independent methods of measuring lipid peroxidation, ethane and TBARS generation, were used to determine the effect of ET-18-OCH_3 on Fe^{2+}/ascorbic acid-induced lipid peroxidation. Ethane generation, a measure of ω-3 fatty acid peroxidation, was measured as previously described (36). Briefly, 5×10^7 cells suspended in 5 ml Hanks' balanced salt solution were placed into 10-ml disposable syringes (Becton Dickinson and Co., Rutherford, NJ). A 5-ml head space was created by a stream of low-hydrocarbon compressed air (Air Products, Inc., Allentown, PA) into the syringe above the sample. Compressed air was allowed to flush the headspace for 3–5 min, and then the syringe was sealed with a 1-ml syringe plunger septum.

Experiments were initiated by injecting through the plunger septum 50 μl of each reactant, 0.9% (w/v) NaCl, or ethanol where appropriate. The samples were mixed and incubated at 37°C prior to analysis of the headspace. ET-18-OCH_3 (edelfosine) was kindly supplied by Dr. Wolfgang Berdel (Freie Universitat, Berlin, Germany), Dr. Edward Modest (Boston University), and Medmark Pharma GmbH (Dr. R. Nordstrom). BM 41.440 (ilmofosine) was a generous gift from Dr. Wolfgang Berdel. Their chemical structures are shown in Fig. 1. Stock solutions were prepared in ethanol or 0.9% NaCl and stored frozen at −20°C for not more than 1 month. $FeSO_4 \cdot 7H_2O$ (Fisher Scientific, Fairlawn, NJ) and L-ascorbic acid (Mallinckrodt, Paris, KY) aqueous solutions were prepared fresh and used within 30 min. At indicated experimental times the 5-ml headspaces were directly injected into a Hewlett-Packard model 5710A gas chromatograph modified with an external heated sample loop for hydrocarbon detection, as described previously (36). Ethane quantitation was accomplished by separation of C1-C4 hydrocarbons on a 5-m x 3.2-mm stainless steel column packed with 80/100 mesh Poracil C (Supelco, Inc., Bellefonte, PA) run isothermally at 50°C with N_2 (20 ml/min) as carrier gas and flame ionization at 150°C to

ET-18-OCH_3



BM 41.440

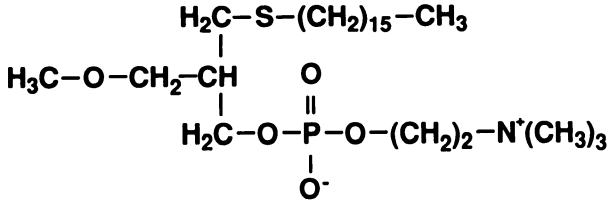


Fig. 1. Chemical formulas of the ether lipids studied.

Table 1 *Fatty acid composition of phospholipids in L1210 cells*

L1210 cells were grown for 48 h in medium supplemented with 18:1ω9 or 22:6ω3, at 32 μM, prior to determination of fatty acid composition.

Fatty acid	Fatty acid composition (%)		
	22:6ω3-modified	18:1ω9-modified	Unmodified
Individual acids[a]			
14:0	1.2 ± 0.7	1.9 ± 0.3	0.8 ± 0.3
16:0	22.7 ± 1.0[b,c]	14.0 ± 0.6	14.3 ± 0.7
16:1	3.5 ± 0.3[c]	4.4 ± 0.4	5.6 ± 0.2
18:0	21.3 ± 0.3[b]	11.0 ± 0.4[c]	19.9 ± 0.3
18:1	20.7 ± 2.0[b,c]	63.5 ± 0.8[c]	52.5 ± 1.8
18:2	1.3 ± 0.4	0 ± 0	0.2 ± 0.2
20:1	0 ± 0[b,c]	1.9 ± 0.3	0.8 ± 0.1
20.4	4.9 ± 1.2	2.7 ± 0.4[c]	5.1 ± 0.2
22:6	23.1 ± 1.7[b,c]	0.3 ± 0.1	0.2 ± 0.2
Others	1.3	0.3	0.6
Polyunsaturated	30.5 ± 1.7[b,c]	3.0 ± 1.0	5.9 ± 0.5
Monounsaturated	24.1 ± 2.3[b,c]	69.9 ± 0.8[c]	58.9 ± 1.6
Saturated	45.1 ± 1.6[b,c]	26.9 ± 0.8[c]	35.0 ± 1.0
Double bonds[d]	1.91 ± 0.08[b,c]	0.83 ± 0.02	0.82 ± 0.01
ω-3	24.2 ± 2.0[b,c]	0.3 ± 0.1	0.2 ± 0.2

[a] Expressed as percentage of total fatty acids. Fatty acids are designated as number of carbon atoms:number of double bonds. Values are the mean ± SE of at least three determinations.
[b] Significantly different from 18:1ω9-modified at $P < 0.01$ level.
[c] Significantly different from unmodified at $P < 0.01$ level.
[d] Mean number of double bonds per fatty acid.

detect and quantify peak areas. C1-C6 *n*-paraffins (10–20 ppm each in N_2; Supelco, Inc.) were used to generate standard curves for quantitation of ethane peak areas. Following injection of the headspace, the remaining aqueous sample was shaken, the cell density and viability were determined, and 100 μl of 20 mM butylated hydroxytoluene were added to the remaining sample. The samples were then frozen at −20°C until the thiobarbituric acid assay.

The 2-thiobarbituric acid assay (38, 39) was used to determine TBARS from supernatants of sonicated samples, using 1,1,3,3-tetramethoxypropane hydrolyzed in trichloroacetic acid as standard.

Electron Paramagnetic Resonance. EPR was used to study the rate of free radical oxidations during membrane lipid peroxidation by direct detection of the ascorbyl free radical. The ascorbyl radical EPR signal intensity was used as a general index of the oxidation rate of the experimental system (40). For EPR analysis of ascorbyl radical intensity, unmodified, 18:1ω9-modified, or 22:6ω3-modified cells (5×10^6 cells/ml in 0.9% NaCl) were placed in a glass test tube prior to the addition of ascorbic acid (100 μM, final). Fe^{2+} (20 μM, final) was quickly added, 4 ml of the cell reaction mixture were drawn (by vacuum) into an EPR flat cell, and consecutive 20-s EPR scans were taken. EPR scans were started 30 s after the addition of Fe^{2+}. At the end of the ninth scan (≃190 s), ET-18-OCH_3 (40 μM, final) was added to the remaining cells in the glass test tube, mixed, and introduced into the EPR flat cell, replacing the previous sample. The introduction of the ET-18-OCH_3-treated cells to the flat cell occurred after the ninth scan and before the tenth scan. EPR spectra were obtained on a Bruker ESP-300 spectrometer operating at 9.79 GHz and 100-kHz modulation frequency. Sequential EPR spectra were obtained at 25°C in a quartz flat cell centered in a TM_{110} cavity. EPR settings were as follows: 40-mW nominal microwave power, 0.631-G modulation amplitude, and 328-ms time constant, with scanning at 6 G/21 s and a receiver gain of 6.3×10^5.

RESULTS

Lipid Modification. The fatty acid composition of the cellular phospholipids is shown in Table 1. The cells grown in medium containing 22:6ω3 were enriched in 22:6ω3, compared to unmodified cells or those grown in 18:1ω9. They also contained a higher percentage of polyunsaturated fatty acids and greater

mean number of double bonds per fatty acid. There were significant differences in other fatty acids, particularly higher percentages of 16:0 and lower 18:1ω9. Cells grown in 18:1ω9 contained higher percentages of 18:1ω9 and total monounsaturates, compared to cells grown in 22:6ω3 and the unmodified cells. The 18:1ω9 cells were otherwise similar to the unmodified cells but contained lower percentages of 18:0 and total saturated fatty acids. These changes are similar to those reported previously in this cell line (28, 30, 36).

Effect of Fatty Acid Modification on Peroxidation and Survival. The effect of type of fatty acid alteration on ET-18-OCH_3-enhanced peroxidation is shown in Fig. 2. ET-18-OCH_3 in the presence of cofactors resulted in the generation of appreciable levels of both peroxidative products, ethane (Fig. 2, *upper*) and TBARS (Fig. 2, *inset*), from 22:6ω3-enriched L1210 cells; there was little generated from the 18:1ω9-enriched or unmodified cells regardless of the presence of cofactors. Only trace amounts of ethane and TBARS were generated by ET-18-OCH_3 from 22:6ω3-modified cells in the absence of cofactors and only small amounts by cofactors alone (Fig. 2, *upper* and *inset*).

The effect of type of fatty acid modification on ether lipid-enhanced cytotoxicity was studied utilizing L1210 cells enriched with various fatty acids (Fig. 2, *lower*). The 22:6ω3-enriched cells with or without cofactors were more sensitive to ET-18-OCH_3 than cells enriched with the monounsaturated fatty acid or unmodified cells. This differential cytotoxicity could be explained by the greater number of double bonds in the 22:6ω3-enriched cells.

Effects of ET-18-OCH_3 on Time Dependence of Lipid Peroxidation and Cytotoxicity. Since maximal oxidative conditions for the generation of hydrocarbon from 22:6ω3-enriched cells include Fe^{2+} and ascorbic acid, we began by examining

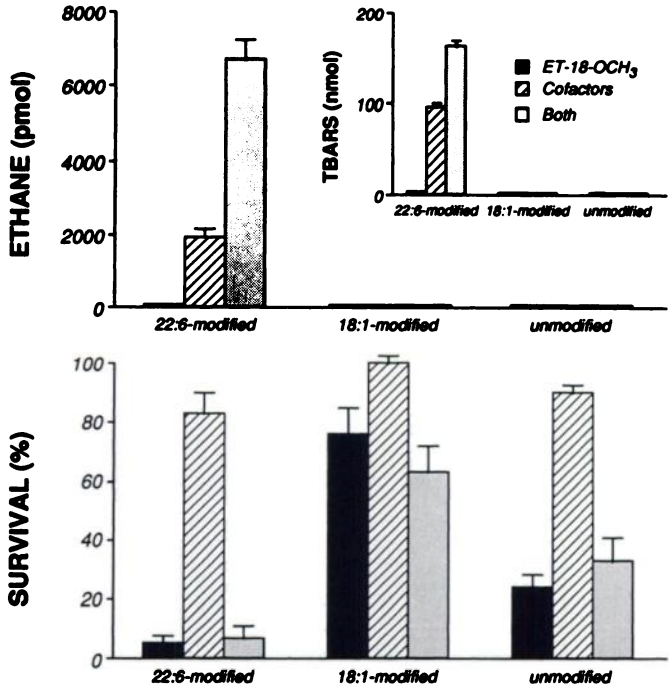


Fig. 2. Effect of fatty acid modification on ether lipid-induced peroxidation and cytotoxicity. L1210 cells were enriched with 22:6ω3 or 18:1ω9 by growing for 2 days in growth medium containing 32 μM fatty acid prior to a 2-h incubation with ET-18-OCH_3 (40 μM), oxidative cofactors ascorbic acid (100 μM) and Fe^{2+} (20 μM), or both ether lipid and cofactors. Peroxidation, as estimated by the generation of ethane (*upper*) and TBARS (*inset*), and immediate cytotoxicity (viable cells by dye exclusion) (*lower*) were determined. Ethane and TBARS are expressed as amounts generated by 5×10^7 cells in 2 h. All values are the mean ± SE of 4–9 determinations.

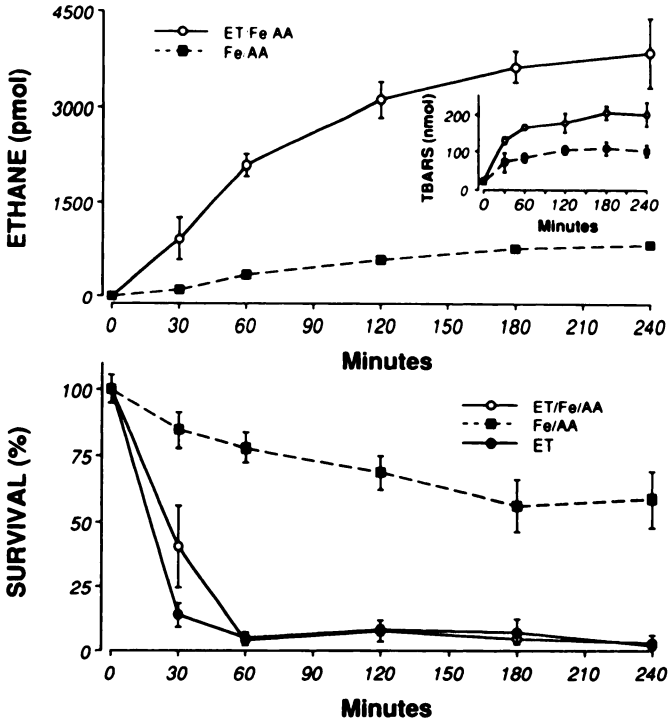


Fig. 3. Time course of peroxidation and cytotoxicity induced by ET-18-OCH_3. *Upper*, ethane generation from 22:6ω3-enriched L1210 cells treated for various times, as shown, with 20 μM ET-18-OCH_3 (*ET*) in the presence of the cofactors Fe^{2+} (*Fe*) (20 μM) and ascorbic acid (*AA*) (100 μM). Also shown is the ethane generation resulting from cofactors alone. Ethane values, in pmol/2 h per 5×10^7 cells, are the mean ± SE of 3 or 4 experiments. In the absence of cofactors there was no appreciable generation of ethane. *Inset*, TBARS from 22:6ω3-enriched L1210 cells treated with 20 μM ET-18-OCH_3, Fe^{2+} (20 μM), and ascorbic acid (100 μM). TBARS, in nmol/2 h per 5×10^7 cells, are the mean ± SE of three separate experiments. In the absence of cofactors there was no appreciable generation of TBARS. *Lower*, short term cell survival, measured by trypan blue dye exclusion, of 22:6ω3-enriched L1210 cells treated with ET-18-OCH_3, Fe^{2+}, and ascorbic acid. Experimental values of total viable cells are the mean ± SE of 3 separate experiments.

drug-induced ethane generation in the presence of these cofactors. ET-18-OCH_3 increased lipid peroxidation as measured by ethane generation (Fig. 3, *upper*). The ethane was about 9-fold higher than controls with Fe^{2+} and ascorbic acid alone. ET-18-OCH_3 in the absence of iron and ascorbate resulted in no ethane release, partially due to the fact that Fe^{2+} is required for the conversion of lipid hydroperoxides to ethane (41). Fig. 3 (*inset*) also shows the results for TBARS release, an alternative measure of lipid peroxidation. The addition of ET-18-OCH_3 in the presence of Fe^{2+} and ascorbate resulted in an increase in TBARS levels that was similar to that of ethane but proportionally smaller. There was no TBARS generation by ET-18-OCH_3 (data not shown) in the absence of cofactors, and this confirms that Fe^{2+} and ascorbate are essential to generate TBARS. Therefore, the generation of both lipid peroxidation products by ET-18-OCH_3 is time dependent and requires Fe^{2+} and ascorbate as cofactors. The generation of TBARS reached a plateau earlier (60 min), compared to ethane. This difference in kinetics suggests that the complementary methods are measuring somewhat different peroxidative pathways.

The effect of the ether lipid on immediate cell survival as measured by cell counts and trypan blue dye exclusion is shown in Fig. 3 (*lower*). Under these conditions, there was a rapid fall in the number of viable cells in cultures containing ET-18-OCH_3, with or without the addition of the cofactors Fe^{2+} and ascorbate.

In an attempt to understand the relationship of cell death to peroxidation, we recalculated the time courses of peroxidation

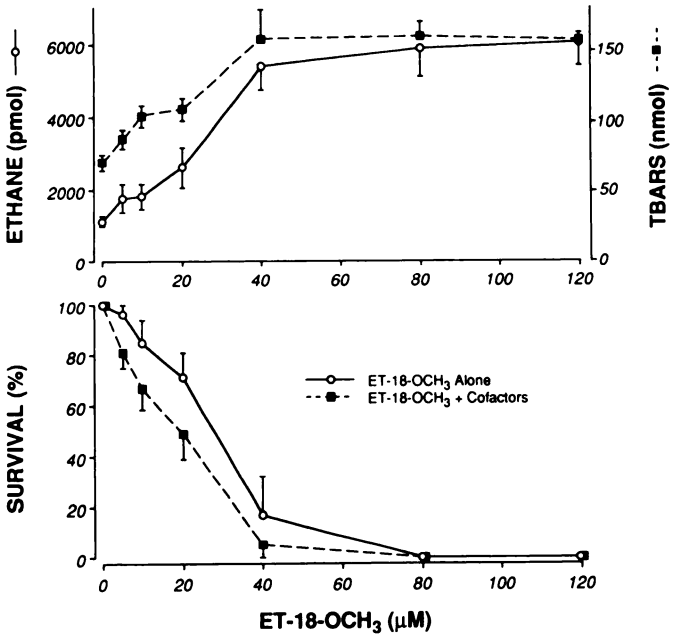


Fig. 4. Concentration dependence of peroxidation and cytotoxicity induced by ET-18-OCH_3. *Upper*, effect of ET-18-OCH_3 concentration on ethane and TBARS generation from 22:6ω3-enriched L1210 cells treated for 120 min in the presence of the cofactors Fe^{2+} (20 μM) and ascorbic acid (100 μM). There was no ethane generation in the absence of cofactors. Ethane values, in pmol/2 h per 5×10^7 cells, and TBARS, in nmol/2 h per 5×10^7 cells, are the mean ± SE of 6 or 7 experiments. *Lower*, concentration dependence of the 2-h cytotoxicity of ET-18-OCH_3 with and without Fe^{2+} (20 μM) and ascorbic acid (100 μM). Total viable L1210 cells were determined using trypan blue dye exclusion. Values were normalized to 100% at time zero. Experimental values of total viable cells are the mean ± SE of 6 or 7 separate experiments. The lines are not statistically different at $P < 0.05$.

and cytotoxicity as rates during defined intervals (data not shown). Peroxidation rates measured by either method were maximal early, at either the 0–30- or 30–60-min interval, and decreased linearly thereafter. The rate of decrease of total viable cells induced by the ether lipid and cofactors was similar in general to that of ethane and TBARS. This would suggest that cell death occurs synchronously with peroxidation. However, cell destruction and loss of viability were induced by ET-18-OCH_3 in the absence of oxidative cofactors, and the drug alone did not result in detectable lipid peroxidation as measured by either ethane or TBARS generation. In addition, Fe^{2+} plus ascorbate without ether lipid led to measurable levels of lipid peroxidation but did not kill cells at an equivalent rate. This could be explained by the lower rate of lipid peroxidation induced by Fe^{2+} plus ascorbate and the lack of a burst of increased peroxidative flux at early time points (data not shown), when contrasted with the pattern seen in the presence of ether lipid. Although Fe^{2+}- and ascorbic acid-induced lipid peroxidation was augmented by the addition of ET-18-OCH_3, these compounds did not lead to decreased short term survival rates. Therefore, these examples of discordance of lipid peroxidation levels and short term survival, some of which cannot be resolved, suggest that lipid peroxidation is apparently not a full explanation for the cytotoxicity of the drug.

Effect of Concentration of ET-18-OCH_3 on Lipid Peroxidation and Cytotoxicity. Fig. 4 (*upper*) shows the concentration dependence of ether lipid-enhanced peroxidation in the presence of Fe^{2+} and ascorbic acid during the same experiments as the peroxidation measurements. There was a linear increase from 0 to 40 μM and then a plateau between 40 and 120 μM. The concentration dependence of TBARS generation (Fig. 4, *upper*) was similar in contour to that of ethane.

Fig. 4 (*lower*) shows the concentration dependence of ET-18-OCH_3 short term cytotoxicity. ET-18-OCH_3 in the presence of cofactors, the conditions that generate ethane and TBARS, resulted in a linear decline in viability between 0 and 40 μM drug, without a shoulder on the survival curve; in the absence of cofactors, there was a similar contour for the effect. Cell death in the presence of cofactors was significantly greater only at 5 μM. The 50% inhibitory dose for ether lipid alone was 26 μM and for ether lipid plus cofactors was 20 μM.

Fig. 5 shows the effect of individual cofactors alone. Ascorbic acid alone resulted in considerable ethane generation, but increasing doses of ether lipid did not increase ethane generation when ascorbic acid was the only cofactor. In fact, there was a decline in ethane generation as the concentration of ET-18-OCH_3 increased; this suggests that ascorbate serves better as an antioxidant than as a prooxidant and, furthermore, that this effect intensifies as the membrane permeability increases with higher concentrations of ether lipid. The substitution of Fe^{2+} for ascorbic acid significantly increased the level of peroxidation at every concentration of ether lipid on this logarithmic scale. However, the maximum peroxidation was achieved with both oxidative cofactors; only with both was there an incremental increase in peroxidation as the dose of ether lipid was increased. There was no ethane generated even at the highest concentration in the absence of cofactors. The effect of individual cofactors on TBARS generation was similar to their effect on ethane production; however, the augmenting effect of the cofactors alone or together was considerably less than with ethane generation. TBARS generation was similar to ethane production in that there was none detected with ether lipid, with or without cofactors, when unmodified or 18:1ω9-enriched cells were utilized.

Effects of Vitamin E on ET-18-OCH_3- and Fe^{2+}/Ascorbic Acid-induced Peroxidation. To study the effects of a lipid-soluble chain-breaking antioxidant on ET-18-OCH_3-enhanced peroxidation, we supplemented 22:6ω3-modified L1210 cells for 24 h in culture with various concentrations of vitamin E acetate (Fig. 6). Vitamin E inhibited ET-18-OCH_3-enhanced ethane generation and TBARS formation. Vitamin E showed maximal inhibition at ≥200 μM. At 200 μM there was no ethane

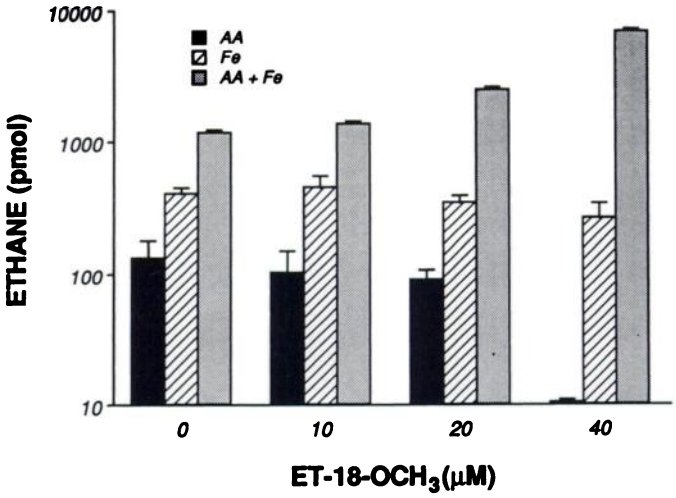


Fig. 5. Effect of individual oxidative cofactors ascorbic acid (*AA*) (100 μM) or Fe^{2+} (*Fe*) (20 μM), or both, on ET-18-OCH_3-enhanced ethane production. 22:6ω3-enriched cells were incubated for 2 h with four different concentrations of drug. The ethane values are the means ± SE of 3–7 determinations. Note that the *vertical scale* is logarithmic.

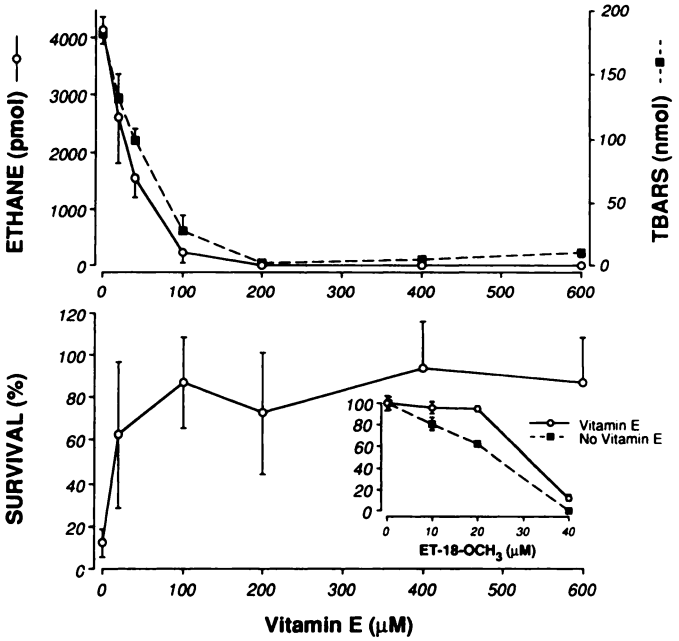


Fig. 6. Effect of vitamin E acetate supplementation on ET-18-OCH_3-enhanced peroxidation and cytotoxicity of 22:6ω3-enriched L1210 cells. *Upper*, ethane and TBARS generation. Cells were incubated for 24 h with the indicated concentrations of vitamin E acetate prior to treatment with 20 μM ET-18-OCH_3 in the presence of the cofactors Fe^{2+} (20 μM) and ascorbic acid (100 μM). Ethane values, in pmol/2 h per 5×10^7 cells, and TBARS values, in nmol/2 h per 5×10^7 cells, are mean ± SE of 5 or 6 replicates. *Lower*, rescue by vitamin E acetate of 22:6ω3-enriched L1210 cells from ET-18-OCH_3-mediated short term cell death, measured as viable cells by trypan blue dye exclusion. Values of viable cells are the mean ± SE of 5 or 6 determinations. *Inset*, effect of vitamin E on short term cell survival at various ET-18-OCH_3 concentrations. L1210 cells enriched with 22:6ω3 by growing for 2 days in growth medium containing 32 μM 22:6ω3 were incubated for 2 h with ET-18-OCH_3 in the presence of the oxidative cofactors ascorbic acid (100 μM) and Fe^{2+} (20 μM), after growth for 24 h in medium containing 200 μM vitamin E or no treatment with vitamin E. Shown are the mean ± SE of 3–5 determinations.

generation even at 40 μM ET-18-OCH_3 in the presence of cofactors. In addition, vitamin E rescued cells from ET-18-OCH_3-plus Fe^{2+}- and ascorbate-induced cytotoxicity with concentration dependence, but optimal inhibition occurred at a much lower concentration of 20–40 μM vitamin E (Fig. 6, *lower*). Fig. 6 (*inset*) shows that 200 μM vitamin E rescued cells from ether lipid toxicity in the presence of oxidative cofactors at higher concentrations of ET-18-OCH_3. Vitamin E failed to rescue cells from the cytotoxic effects of ET-18-OCH_3 in the absence of ascorbic acid or iron except at a vitamin E concentration of 10 μM (data not shown). These experiments demonstrate that vitamin E reverses the effects of ET-18-OCH_3-enhanced peroxidation and cell death in the presence of cofactors in a vitamin E concentration-dependent manner.

Effect of Glutathione Depletion on ET-18-OCH_3-, Fe^{2+}-, and Ascorbic Acid-induced Lipid Peroxidation. To study the effects of conditions that produce intracellular susceptibility to peroxidation, we depleted cells of glutathione. We have previously shown that the addition of 100 μM BSO to L1210 cultures for 24 h depletes glutathione levels by 43% (37). BSO treatments did not affect growth rates or viabilities of L1210 cells grown in culture under these conditions. Table 2 shows the effect of glutathione depletion on ET-18-OCH_3-enhanced lipid peroxidation in the presence of Fe^{2+} and ascorbate. There was a significant rise in TBARS generation at 200 μM BSO but lipid peroxidation of ω-3 fatty acids, as measured by ethane generation, was not increased significantly.

There was a corresponding increase in ether lipid-enhanced cytotoxicity as a result of glutathione depletion (Table 2). Depleted 22:6ω3-enriched cells were 2-fold more sensitive to ET-18-OCH_3 but this was statistically significant only at 100 μM BSO. There was a similar augmentation of sensitivity to Fe^{2+}-plus ascorbate-induced cytotoxicity in the absence of ether lipid.

Lipid Peroxidation Enhanced by BM 41.440. Lipid peroxidation enhanced by ET-18-OCH_3 could be specific for ET-18-OCH_3 rather than a characteristic of this membrane-active group of antineoplastic drugs. Therefore, we examined the effect of the thioether lipid BM 41.440. Fig. 7 (*upper*) shows that the drug increased the generation of ethane and TBARS in the presence of Fe^{2+} and ascorbic acid. There was also a concentration-dependent generation of both that exceeded base line at 20 μM BM 41.440 and plateaued at about 80 μM. Like ET-18-OCH_3, there was no generation of ethane or TBARS by BM 41.440 in the absence of Fe^{2+} and ascorbic acid. Fig. 7 (*lower*) shows the concentration dependence of BM 41.440 immediate cytotoxicity. The kinetics of cell death corresponded inversely

Table 2 *Effect of glutathione depletion on peroxidation and cytotoxicity induced by ET-18-OCH_3 in the presence of cofactors*

22:6ω3-enriched L1210 cells were incubated with BSO for 24 h prior to exposure to 20 μM ET-18-OCH_3. Concentrations of iron and ascorbic acid were 20 and 100 μM, respectively. Values for ethane, in pmol/2 h per 5×10^7 cells, are mean ± SE of 4–8 determinations. Values for TBARS, in nmol/2 h per 5×10^7 cells, are mean ± SE of 3–10 determinations. Values for viable cells are mean ± SE of 3–8 determinations.

	0 μM BSO	100 μM BSO	200 μM BSO
TBARS (nmol)	80 ± 14	100 ± 18	120 ± 9[a]
Ethane (pmol)	1970 ± 290	2810 ± 360	2670 ± 500
Viable cells (%)[b]	47.0 ± 3.8	25.6 ± 6.3[c]	37.1 ± 4.8

[a] Significantly different from 0 μM ($P = 0.03$).
[b] Values are percentages of controls in the presence of BSO at the specified concentration without ET-18-OCH_3 or cofactors.
[c] Significantly different from 0 μM ($P = 0.014$).

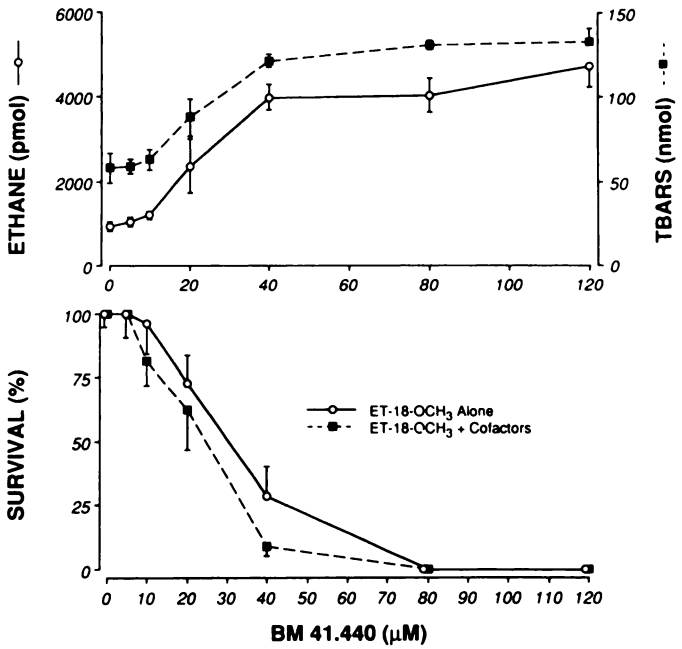


Fig. 7. *Upper*, effect of BM 41.440 on ethane and TBARS generation from 22:6ω3-enriched L1210 cells treated for 120 min in the presence of the cofactors Fe^{2+} (20 μM) and ascorbic acid (100 μM). There was no ethane generation in the absence of cofactors. Values per 5×10^7 cells for 2 h are the mean ± SE of 5 or 6 experiments. *Lower*, short term cell survival, determined by trypan blue dye exclusion, of 22:6ω3-enriched L1210 cells treated with BM 41.440, Fe^{2+} (20 μM), and ascorbic acid (100 μM) or BM 41.440 alone. Experimental values of total viable cells are the mean ± SE of 5 or 6 determinations. The survival curve is a near mirror image of the graph of peroxidation.

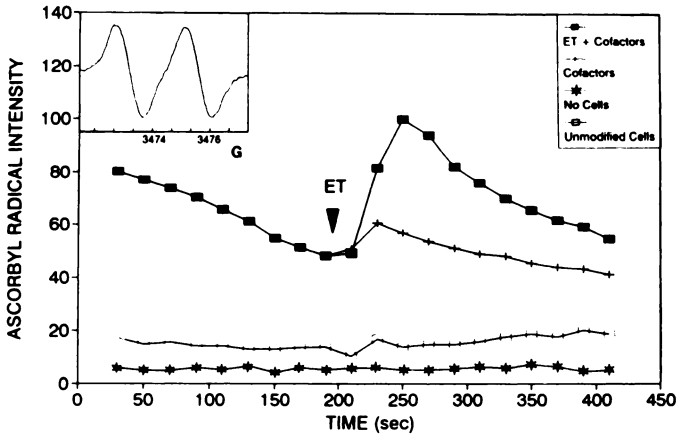


Fig. 8. Effect of ET-18-OCH_3 on ascorbyl free radical concentration generated in 22:6ω3-modified L1210 cells. Ascorbyl radical was monitored by EPR in 22:6ω3-enriched L1210 cells during incubation with Fe^{2+} (20 μM) and ascorbic acid (100 μM) (*ET + Cofactors*). At the point shown, 40 μM ET-18-OCH_3 (*ET + Cofactors*) or diluent without ET-18-OCH_3 (*Cofactors*) was added. Also shown are the radical intensity in unmodified cells (*Unmodified Cells*) and the intensity in the absence of cells (*No Cells*) when medium and cofactors were present and ET-18-OCH_3 was added at *arrow*. Each *point* is the mean of 2 or 3 replicates; the SE (not shown) averaged 2.7% of the means and were never more than 8.1%. The peak ascorbyl radical signal height corresponds to 90 mM. *Inset*, representative ascorbyl radical doublet EPR spectrum (a^H = 1.8 G).

to those of lipid peroxidation. When the concentration dependence of BM 41.440 was studied, the 50% inhibitory dose was 30 μM and in the presence of Fe^{2+} and ascorbic acid was 23 μM.

Electron Paramagnetic Resonance Detection of Ascorbyl Radical. In order to examine the effect of this class of agents on free radical generation, we used EPR techniques. Fig. 8 shows the change in intensity of the ascorbyl radical during an internally controlled experiment in which L1210 cells were incubated in the presence of Fe^{2+} (20 μM) and ascorbic acid (100 μM) for a brief monitoring time prior to the addition of ET-18-OCH_3 at 190 s. The intensity of the ascorbyl radical increased rapidly with the addition of the ether lipid, reached a peak at 60 s thereafter, and then slowly dissipated. When vehicle without ether lipid was injected, the ascorbyl radical intensity increased only slightly. The intensity of the radical and its augmentation by ether lipid was considerably less when unmodified cells were utilized (Fig. 8); the radical intensity from 18:1ω9-enriched cells (data not shown) was even less, which is noteworthy since this type of enrichment resulted in the least sensitivity to the cytotoxic effect of the ether lipid (Fig. 2, *lower*).

DISCUSSION

We conclude that the ether lipids increase cellular susceptibility to lipid peroxidation as measured by three complementary techniques: hydrocarbon generation, TBARS production, and free radical induction. Peroxidation required oxidative cofactors; the presence of Fe^{2+} and ascorbate facilitated peroxidation and allowed the reactions to generate hydrocarbon peroxidation products and TBARS. The comparative kinetics and concentration dependence of cytotoxicity and peroxidation suggested that oxidative reactions could play a role in the mechanism of this class of drugs. However, peroxidation cannot explain fully the cytotoxicity, since there was cytotoxicity without measurable peroxidation when the oxidative cofactors were omitted from the incubation medium.

ET-18-OCH_3 appears to affect ethane generation to a greater extent than TBARS when the two methods for measuring Fe^{2+}- and ascorbate-induced lipid peroxidation are compared. The 9-fold increase in ethane, as opposed to the 2-fold increase in TBARS generation, suggests that these methods measure different aspects of the peroxidative reaction that are differentially affected by ET-18-OCH_3. However, the hydrocarbon generation method is generally a more sensitive measure of membrane lipid peroxidation (36). In addition, the high degree of enrichment of these experimental cells with ω-3 fatty acids and the peroxidative products that are detected differentially by ethane generation may make this method optimal for detecting membrane damage from this class of drug. Further, evidence that the three independent measures of lipid peroxidation are affected differentially by ET-18-OCH_3 during Fe^{2+}- plus ascorbate-induced lipid peroxidation was observed in the differing kinetics. TBARS generation rates were maximal during the first 30 min of the peroxidative challenge; in contrast, ethane generation rates were high for the initial 60 min and ascorbyl radical production occurred in the first few minutes. These differential kinetics may relate to rates of different lipid peroxide species generation or substrate availability and conversion. Alternatively, other unidentified cofactors involved in the production of these compounds could be rate limiting.

It would not be anticipated from its chemical structure that ET-18-OCH_3 would generate a free radical directly through its metabolism (4, 9, 42). However, the drug does heighten the peroxidative potential of the cell indirectly, by an effect on membranes. This membrane effect may facilitate the generation of a free radical from unsaturated fatty acids contained in cellular membranes. ET-18-OCH_3 also may affect membrane physical properties that influence the susceptibility to, activity of, or access to phospholipases, especially membrane-bound phospholipase A_2, that are active in the elimination of membrane lipid peroxides (43).

Vitamin E is a hydrophobic lipid chain-breaking antioxidant which localizes in membranes. It is the principal lipid antioxidant. Our experiments demonstrated that supplementation of the medium of 22:6ω3-enriched cells with vitamin E inhibited peroxidation enhanced by the ether lipid. Both measures of peroxidation were affected in a concentration-dependent manner. Most importantly, there was a concomitant reduction in cytotoxicity of ET-18-OCH_3-treated cells as a function of vitamin E concentration. Since vitamin E is localized in membranes, our results provide further evidence that ET-18-OCH_3 enhances lipid peroxidation. Both plasma membranes and intracellular membranes are likely involved. Stimulation of peroxidation, its inhibition by vitamin E, and the effect of vitamin E on survival confirm that ET-18-OCH_3 is membrane targeted.

Glutathione and glutathione reductase constitute a cytosolic system that maintains cell homeostasis via intracellular thiol levels. Glutathione depletion resulted in only equivocal increases in lipid peroxidation and cytotoxicity. The limited effect may result from the fact that ether lipid-enhanced peroxidation is localized in the membrane. Alternatively, glutathione levels may not be as important in maintaining or inhibiting peroxidation if other antioxidants are present at normal levels. There are experimental data to support this. For example, in microsomal peroxidation systems the addition of reduced glutathione lowers the amount of vitamin E needed to inhibit lipid peroxidation, but glutathione alone does not inhibit lipid peroxidation (44). It is possible that glutathione maintains cell viability by a mechanism other than by preventing membrane lipid peroxidation directly.

The effect of the ether lipid on ascorbate radical intensity could be due to increased permeability of the plasma membrane. Ether lipids are known to increase the fluidity of cell membranes (24, 45, 46), and this may increase permeability for

the Fe^{2+} and ascorbate. The increase in ascorbyl radical observed upon introduction of ether lipids suggests that the drug *per se* may bring about heightened conditions for peroxidative reactions. Our observations that 18:1ω9-enriched cells resulted in less augmentation of ascorbate free radical, perhaps because they contain fewer double bonds and are, therefore, not as susceptible to oxidative processes, are consistent with this hypothesis.

REFERENCES

1. Honma, Y., Kasukabe, T., Okabe-Kado, J., Hozumi, M., Tsushima, S., and Nomura, H. Antileukemic effect of alkyl phospholipids. I. Inhibition of proliferation and induction of differentiation of cultured myeloid leukemia cells by alkyl ethyleneglycophospholipids. Cancer Chemother. Pharmacol., *11:* 73–76, 1983.
2. Berdel, W. E., Korth, R., Reichert, A., Houlihan, W. J., Bicker, U., Nomura, H., Vogler, W. R., Benveniste, J., and Rastetter, J. Lack of correlation between cytotoxicity of agonists and antagonist of platelet activating factor (Paf-acether) in neoplastic cells and modulation of [^{3}H]-Paf-acether binding to platelets from humans *in vitro*. Anticancer Res., *7:* 1181–1188, 1987.
3. Berdel, W. E., Danhauser, S., Schick, H. D., Hong, C., II, West, C. R., Fromm, M., Fink, U., Reichert, A., and Rastetter, J. Antineoplastic activity of conjugates of lipids and 1-β-D-arabinofuranosylcytosine. Lipids, *22:* 943–946, 1987.
4. Mangold, H. K., and Weber, N. Biosynthesis and biotransformation of ether lipids. Lipids, *22:* 789–799, 1987.
5. Modest, E. J., Daniel, L. W., Wykle, R. L., Berens, M. E., Piantadosi, C., Surles, J. R., and Morris-Natschke, S. Novel phospholipid analogs as membrane active antitumor agents. *In:* K. Harrap and T. Connors (eds.), New Avenues in Developmental Cancer Chemotherapy, pp. 387–400. New York: Academic Press, Inc., 1987.
6. Noseda, A., Berens, M. E., Piantadosi, C., and Modest, E. J. Neoplastic cell inhibition with new ether lipid analogs. Lipids, *22:* 878–883, 1987.
7. Storch, J., and Munder, P. G. Increased membrane permeability for an antitumoral alkyl lysophospholipid in sensitive tumor cells. Lipids, *22:* 813–819, 1987.
8. Marx, M. H., Piantadosi, C., Noseda, A., Daniel, L. W., and Modest, E. J. Synthesis and evaluation of neoplastic cell growth inhibition of 1-*N*-alkylamide analogues of glycero-3-phosphocholine. J. Med. Chem., *31:* 858–863, 1988.
9. Munder, P. G., and Westphal, O. Antitumoral and other biomedical activities of synthetic ether lysophospholipids. Chem. Immunol., *49:* 206–235, 1990.
10. Honma, Y., Kasukabe, T., Hozumi, M., Tsushima, S., and Nomura, H. Induction of differentiation of cultured human and mouse myeloid leukemia cells by alkyl-lysophospholipids. Cancer Res., *41:* 3211–3216, 1981.
11. Tidwell, T., Guzman, G., and Vogler, W. R. The effects of alkyl-lysophospholipids on leukemic cell lines. I. Differential action on two human leukemic cell lines, HL60 and K562. Blood, *57:* 794–797, 1981.
12. Berdel, W. E., Fromm, M., Fink, U., Pahlke, W., Bicker, U., Reichert, A., and Rastetter, J. Cytotoxicity of thioether-lysophospholipids in leukemias and tumors of human origin. Cancer Res., *43:* 5538–5543, 1983.
13. Fromm, M., Berdel, W. E., Schick, H. D., Fink, U., Pahlke, W., Bicker, U., Reichert, A., and Rastetter, J. Antineoplastic activity of the thioether lysophospholipid derivative BM 41.440 *in vitro*. Lipids, *22:* 916–918, 1987.
14. Noseda, A., Berens, M. E., White, J. G., and Modest, E. J. *In vitro* antiproliferative activity of combinations of ether lipid analogues and DNA-interactive agents against human tumor cells. Cancer Res., *48:* 1788–1791, 1988.
15. Fujiwara, K., Modest, E. J., Welander, C. E., and Wallen, C. A. Cytotoxic interactions of heat and an ether lipid analogue in human ovarian carcinoma cells. Cancer Res., *49:* 6285–6289, 1989.
16. Berdel, W. E., Fink, U., and Rastetter, J. Clinical phase I pilot study of the alkyl lysophospholipid derivative ET-18-OCH_3. Lipids, *22:* 967–969, 1987.
17. Khanavkar, B., Ulbrich, F., Gatzemeier, U., Meyer-Schwickerath, E., Lorenz, J., Schreml, W., Brugger, R., Schick, H. D., Pawel, J. V., Nordstrom, R., and Drings, P. Treatment of non-small cell lung cancer with the alkyllysophospholipid edelfosine. Intermediate data analysis. Contrib. Oncol., *37:* 224–235, 1989.
18. Herrmann, D. B. J., Neumann, H. A., Berdel, W. E., Heim, M. E., Fromm, M., Boerner, D., and Bicker, U. Phase I trial of the thioether phospholipid analogue BM 41.440 in cancer patients. Lipids, *22:* 962–966, 1987.
19. Berdel, W. E. Membrane-interactive lipids as experimental anticancer drugs. Br. J. Cancer, *64:* 208–211, 1991.
20. Modolell, M., Andreesen, R., Pahlke, W., Brugger, U., and Munder, P. G. Disturbance of phospholipid metabolism during the selective destruction of tumor cells induced by alkyl-lysophospholipids. Cancer Res., *39:* 4681–4686, 1979.
21. Helfman, D. M., Barnes, K. C., Kinkade, J. M., Jr., Vogler, W. R., Shoji, M., and Kuo, J. F. Phospholipid-sensitive Ca^{2+}-dependent protein phosphorylation system in various types of leukemic cells from human patients and in human leukemic cell lines HL60 and K562, and its inhibition by alkyl-lysophospholipid. Cancer Res., *43:* 2955–2961, 1983.
22. McNamara, M. J. C., Schmitt, J. D., Wykle, R. L., and Daniel, L. W. 1-*O*-Hexadecyl-2-acetyl-*sn*-glycerol stimulates differentiation of HL-60 human promyelocytic leukemia cells to macrophage-like cells. Biochem. Biophys. Res. Commun., *122:* 824–830, 1984.
23. Kiss, Z., Deli, E., Vogler, W. R., and Kuo, J. K. Antileukemic agent alkyllysophospholipid regulates phosphorylation of distinct proteins in HL60 and K562 cells and differentiation of HL60 cells promoted by phorbol ester. Biochem. Biophys. Res. Commun., *142:* 661–666, 1987.
24. van Blitterswijk, W. J., van der Bend, R. L., Kramer, I. M., Verhoeven, A. J., Hilkmann, H., and de Widt, J. A metabolite of an antineoplastic ether phospholipid may inhibit transmembrane signalling via protein kinase C. Cancer Res., *22:* 842–846, 1987.
25. Kosano, H., and Takatani, O. Reduction of epidermal growth factor binding in human breast cancer cell lines by alkyl-lysophospholipid. Cancer Res., *48:* 6033–6036, 1988.
26. Seewald, M. J., Olsen, R. A., Sehgal, I., Melder, D. C., Modest, E. J., and Powis, G. Inhibition of growth factor-dependent inositol phosphate Ca^{2+} signaling by antitumor ether lipid analogues. Cancer Res., *50:* 4458–4463, 1990.
27. Powis, G., Seewald, M. J., Gratas, C., Melder, D., Riebow, J., and Modest, E. J. Selective inhibition of phosphatidylinositol phospholipase C by cytotoxic ether lipid analogues. Cancer Res., *52:* 2835–2840, 1992.
28. Guffy, M. M., Rosenberger, J. A., Ido, S., and Burns, C. P. Effect of fatty acid alteration on hyperthermic sensitivity in cultured L1210 murine leukemia cells. Cancer Res., *42:* 3625–3630, 1982.
29. Burns, C. P., Luttenegger, D. G., Dudley, D. T., Buettner, G. R., and Spector, A. A. Effect of modification of plasma membrane fatty acid composition on fluidity and methotrexate transport in L1210 murine leukemia cells. Cancer Res., *39:* 1726–1732, 1979.
30. Burns, C. P., and North, J. A. Adriamycin transport and sensitivity in fatty acid-modified leukemia cells. Biochim. Biophys. Acta, *888:* 10–17, 1986.
31. Burns, C. P., Haugstad, B. N., Mossman, C. J., North, J. A., and Ingraham, L. M. Membrane lipid alteration: effect on cellular uptake of mitoxantrone. Lipids, *23:* 393–397, 1988.
32. Burns, C. P., Lambert, B. J., Haugstad, B. N., and Guffy, M. M. Influence of rate of heating on thermosensitivity of L1210 leukemia: membrane lipids and M_r 70,000 heat shock protein. Cancer Res., *46:* 1882–1887, 1986.
33. Burns, C. P., Wagner, B. A., and North, J. A. Effect of hyperthermia on selective expression of HL-60 heat shock proteins. Med. Oncol. Tumor Pharmacother., *6:* 245–253, 1989.
34. Burns, C. P., Petersen, E. S., North, J. A., and Ingraham, L. M. Effect of docosahexaenoic acid on rate of differentiation of HL-60 human leukemia. Cancer Res., *49:* 3252–3258, 1989.
35. Burns, C. P., and Petersen, E. S. Effect of butylated hydroxytoluene on bilineage differentiation of the human HL-60 myeloblastic leukemia cell. J. Cell. Physiol., *144:* 36–41, 1990.
36. Burns, C. P., and Wagner, B. A. Heightened susceptibility of fish oil polyunsaturate-enriched neoplastic cells to ethane generation during lipid peroxidation. J. Lipid Res., *32:* 79–87, 1991.
37. Hromas, R. A., Andrews, P. A., Murphy, M. P., and Burns, C. P. Glutathione depletion reverses cisplatin resistance in murine L1210 leukemia cells. Cancer Lett., *34:* 9–13, 1987.
38. Bernheim, F., Bernheim, M. L. C., and Wilbur, K. M. The reaction between thiobarbituric acid and the oxidation products of certain lipides. J. Biol. Chem., *174:* 257–264, 1948.
39. Dahle, L. K., Hill, E. G., and Holman, R. T. The thiobarbituric acid reaction and the autooxidations of polyunsaturated fatty acid methyl esters. Arch. Biochem. Biophys., *98:* 253–261, 1962.
40. Buettner, G. R., and Jurkiewicz, B. A. Ascorbyl free radical as a marker of oxidative stress. Free Radical Biol. Med., in press, 1992.
41. Dillard, C. J., Dumelin, E. E., and Tappel, A. L. Effect of dietary vitamin E on expiration of pentane and ethane by the rat. Lipids, *12:* 109–114, 1977.
42. Weltzien, H. U., and Munder, P. G. Synthetic alkyl analogs of lysophosphatidylcholine: membrane activity, metabolic stability, and effects on immune response and tumor growth. *In:* H. K. Mangold and F. Paltauf (eds.), Ether Lipids: Biochemical and Biomedical Aspects, pp. 277–307. New York: Academic Press, Inc., 1983.
43. Sevanian, A., Muakkassah-Kelly, S. F., and Montestruque, S. The influence of phospholipase A_2 and glutathione peroxidase on the elimination of membrane lipid peroxides. Arch. Biochem. Biophys., *223:* 441–452, 1983.
44. Leedle, R. A., and Aust, S. D. The effect of glutathione on the vitamin E requirement for inhibition of liver microsomal lipid peroxidation. Lipids, *25:* 241–245, 1990.
45. Storme, G. A., Berdel, W. E., van Blitterswijk, W. J., Bruyneel, E. A., de Bruyne, G. K., and Mareel, M. M. Antiinvasive effect of racemic 1-*O*-octadecyl-2-*O*-methylglycero-3-phosphocholine on MO_4 mouse fibrosarcoma cells *in vitro*. Cancer Res., *45:* 351–357, 1985.
46. Noseda, A., Berens, M. E., White, J. G., and Modest, E. J. *In vitro* antiproliferative activiy of combinations of ether lipid analogues and DNA-interactive agents against human tumor cells. Cancer Res., *48:* 1788–1791, 1988.