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Sensitivity of K562 and HL-60 Cells to Edelfosine, an Ether Lipid Drug, Correlates with Production of Reactive Oxygen Species[1]

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ABSTRACT

Edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; ET-18-OCH_3), a membrane-targeting anticancer ether lipid drug has been shown previously *in vitro* to be capable of initiating oxidative processes in cells. Here we study two human leukemia cell lines (HL-60 and K562) that have different sensitivities to edelfosine; HL-60 cells are more sensitive than K562 cells. To determine whether edelfosine alters the sensitivity of these lines to an oxidative stress, cells were subjected to the oxidative stress of iron(II) plus ascorbate and then monitored for free radical formation, membrane integrity, and cytotoxicity. The HL-60 cell was sensitive to the ether lipid drug in clonogenic and dye exclusion assays; a lipid-derived free radical was generated by this sensitive cell in the presence of small amounts of Fe^{2+} and ascorbate as detected by electron paramagnetic resonance and the spin trap α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone. There was also simultaneous generation of an ascorbate-free radical, which has been shown to estimate cellular oxidative flux. In contrast, the K562 cell was resistant to edelfosine cytotoxicity in all assays and did not generate either lipid-derived or ascorbate-free radicals. Subcellular homogenates of the HL-60 cell generated both radicals when exposed to the drug, but homogenates of K562 did not generate either, suggesting that differential drug uptake or intracellular drug localization is not the cause of the difference in oxidation. Trypan blue uptake by the HL-60, but not the K562 cells, measured under the same conditions as the oxidation experiments, demonstrated a loss of membrane impermeability with similar time and concentration dependence, suggesting a causal relationship of membrane damage and radical generation. Complementary studies of HL-60 cell membrane integrity with propidium iodide impermeability and light scatter using the flow cytometer showed a concentration dependence that was similar to radical generation. Biochemical studies of the fatty acids of the HL-60 cell revealed more highly polyunsaturated lipids in the cells. Cellular antioxidant enzymes and vitamin E contents of the two cell lines were similar. We conclude that there is a time- and concentration-dependent generation of important oxidations by the sensitive HL-60 cells exposed to the membrane-targeted ether lipid, but the resistant K562 cells are oxidatively silent. This may be due in part to the differences in fatty acid polyunsaturation of the cellular membranes. The difference in oxidative susceptibility could be the basis for drug resistance to this membrane-specific anticancer agent.

INTRODUCTION

It is well known that the HL-60 and K562 human myelocytic leukemia cell lines differ in their sensitivity to ether lipid anticancer drugs (1, 2). This difference has been used in trying to establish the mechanism of action of this membrane-targeted class of drugs (3–7). However, the metabolic basis for the difference in sensitivity of the two cell lines to the ether lipids *in vitro* has never been established. Such information could be helpful in the understanding of the mechanism of ether lipid cytotoxicity.

The difference in cytotoxicity between the two cell lines is not due to drug metabolism (8). We have demonstrated previously that the ether lipids are capable of enhancing lipid peroxidative damage (9) and free radical events in cells. In L1210 cells, ether lipids augment generation of $L_d^{\cdot}$[3] (10, 11). The generation of $L_d^{\cdot}$ under various oxidative conditions correlates directly with oxygen consumption (11, 12), extent of cellular polyunsaturation (10, 11, 13), and depletion of cellular tocopherol (14). It requires Fe^{2+} (10) and is decreased by vitamin E (12).

Because of these observations, we postulated an oxidative basis for the resistance of the K562 cell. In the present study, we have studied free radical generation, membrane integrity, clonogenic survival, fatty acid composition, and cellular antioxidants of the sensitive/resistant cell line pair. We have found a major difference between the two cell lines in the production of $L_d^{\cdot}$ and the cellular oxidative state as estimated by $Asc^{\cdot-}$ generation. There was a temporal correlation of these free radical events with a loss of membrane integrity and drug cytotoxicity. These data suggest that oxidative events may play an important role in the mechanism of action of ether lipids, and oxidizability may contribute to cellular drug sensitivity.

MATERIALS AND METHODS

Cell Culture. HL-60 and K562 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 10% FBS (Life Technologies, Inc.) and supplemented with 2 mM L-glutamine. Cells used in experiments were obtained from 48-h cultures in the log phase of growth. They were harvested by pelleting at 300 × *g* and washed twice with 0.9% NaCl. Cells were counted with a Coulter Model Z_f cell counter (Coulter, Inc., Hialeah, FL), and the density was adjusted to 10×10^6/ml in 0.9% NaCl unless otherwise noted.

Preparation of Cell Homogenates. Cellular homogenates of K562 and HL-60 cells were prepared by placing cells at 10×10^6/ml 0.9% NaCl in a Parr model 4635 cell disruption bomb (Parr Instrument Corp., Moline, IL). Cells were pressurized at 1000–1300 PSI with nitrogen and then decompressed 10 min later. The efficiency of disruption was determined by microscopic examination of homogenates; apparent complete disruption of cells occurred, with cell fragments and partially damaged nuclei evident.

Fatty Acid Analysis. Cells from 48-h cultures were centrifuged and washed three times using 0.9% NaCl. Total cellular lipids were extracted with chloroform:methanol (2:1, v/v) (15), dried, transesterified, and separated using gas chromatography as described below. Neutral lipids and phospholipids were separated from total lipid extracts, which were taken up in 1:1 (v/v) chloroform:heptane and applied to silicic acid columns preconditioned with heptane. Neutral lipids were eluted with 100:2 (v/v) chloroform:methanol, and phospholipids eluted with 100:2 (v/v) methanol:water. Neutral lipids and phospholipids were then dried separately under nitrogen.

The lipids were transesterified to corresponding FAMEs using a modification of the method described by Morrison and Smith (16). Briefly, total lipids and neutral and phospholipid fractions were dried under nitrogen. To the dried

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[3] The abbreviations used are: $L_d^{\cdot}$, lipid-derived free radical; FBS, fetal bovine serum; $Asc^{\cdot-}$, ascorbate free radical; $[Asc^{\cdot-}]_{ss}$, steady-state concentration of $Asc^{\cdot-}$; FAME, fatty acid methyl ester; MBI, methylene bridge index; edelfosine, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; EPR, electron paramagnetic resonance spectroscopy; POBN, [α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone]; SOD, superoxide dismutase.

sample were added 0.5 ml of acetonitrile and 0.5 ml of 12% boron trifluoride in methanol. Samples were heated at 95°C for 45 min, cooled, washed with 3 ml of water, and then extracted two times with *n*-heptane. The pooled *n*-heptane fractions were dried under nitrogen and resuspended in 50 μl of carbon disulfide for gas chromatography analysis. FAMEs were separated on a Hewlett-Packard 5890 gas chromatograph with heated injection onto a 2-mm inside diameter × 6-ft glass column packed with GP 10% SP-2330 100/120 Chromosorb WAW (Supelco) and detected by flame ionization. Nitrogen was used as a carrier gas at 25 ml/min with an oven temperature initially at 170°C for 7 min and increased 2°C/min to 230°C. From gas chromatography analysis of FAME standards, the cell lipid FAME components were identified. The MBI, a measure of the number of methylene *bis*-allylic positions of fatty acids based on mole fractions, was calculated as described by Wagner *et al.* (11).

Clonogenic Assays. HL-60 and K562 cells were washed, counted, and plated at 100,000 cells/ml in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine. Cells were then treated with 0–100 μM edelfosine (ET-18-OCH_3; Medmark Pharma, GmbH, Grünwald, Germany; kindly supplied by Dr. R. Nordström) for 24 h at 37°C in a humidified incubator and subsequently used for clonogenic assay. The clonogenic media contained 0.33% agar (Difco Laboratories, Detroit, MI), 20% FBS, 2 mM L-glutamine, 85 units/ml penicillin, and 85 μg/ml streptomycin in RPMI 1640. Cells were plated and incubated for 7–14 days as required for clonogenic growth (HL-60 cells were generally 14 days, and K562 cells were generally 7 days). Clones were defined as colonies >16 cells. Clonogenic efficiency for HL-60 cells was 3% and for K562 cells, 40%.

Trypan Blue Dye Exclusion. To access membrane damage during short-term exposures of cells to edelfosine, the trypan blue dye exclusion test was used. The assay was used as a measure of membrane integrity of cells and as a general measure of viability. These studies were run in a manner parallel to EPR studies in which 10×10^6 cells/ml in 0.9% NaCl were incubated with 50 mM POBN, 20 μM Fe^{2+}, and 100 μM ascorbic acid with the addition of edelfosine at 5 min. Fe^{2+}, ascorbate, and POBN were added to these experiments. This is especially important because POBN has some antioxidant potential. Because serum proteins bind edelfosine, these studies on trypan blue dye exclusion and the studies of propidium iodide uptake, light scatter, and free radical generation described below were done without added FBS.

Flow Cytometry Measurements of Cell Integrity and Cell Destruction. Washed HL-60 cells (1×10^6/ml) were placed in 0.9% NaCl. Then 1.5 mM propidium iodide in ethanol, spin trap POBN, 100 μM ascorbic acid, and finally 20 μM Fe^{2+} were added. The cells were then immediately added to a specially designed sample-mixing chamber with an electronic event marker that allows continuous monitoring of cells during experiments and event marking with drug injection (built by Justin K. Fishbaugh, The University of Iowa Flow Cytometry Facility). Cells were introduced into the continuous cell flow stream of a Coulter EPICS 753 flow cytometer (Coulter Corp., Hialeah, FL) and monitored for propidium iodide uptake and light scatter at a rate of 1000 cell events/s. An argon laser at 100 mW was used for excitation at 488 nm with a 635-nm band pass filter. Cellular debris was gated out with forward angle and orthogonal light scatter. At 5 min after the start of FACS monitoring, 0–40 μM edelfosine was added to the sample cell using a specially adapted syringe.

Cellular propidium iodide uptake and light scatter were analyzed as 0–1, 1–2, 2–3 min incremental "bins" of data using Coulter Elite 4.0 data analysis software. Propidium iodide data are expressed as percentage of cells excluding dye within 1 min time "bins"; scatter is the average statistic derived from the light scatter for each corresponding 1-min time "bin."

Determination of Free Radical Generation. HL-60 or K562 cells (10×10^6/ml) or their homogenates were suspended in 0.9% NaCl containing 50 mM POBN. An aliquot of the sample was placed in an EPR quartz flat cell and positioned in a TM_{110} cavity of a Bruker ESP-300 EPR spectrometer. One min after the addition of 100 μM ascorbic acid and 20 μM $FeSO_4 \cdot 7H_2O$ (Sigma Chemical Co., St. Louis, MO), EPR scans were initiated at ~60-s intervals (41-s scan with a 17-s wait period between scans and approximately a 2-s reset). At 5 min, edelfosine (0–40 μM) was added to the remaining cell sample and rapidly drawn into the flat cell, displacing the previous sample. The edelfosine was added as the 5-min scan was completed and before the 6-min scan was initiated; scanning continued for 60 min. EPR instrument settings were as follows: 40 mW microwave power at a frequency of 9.78 GHz; modulation frequency of 100 kHz; receiver gain 2.5×10^5; modulation amplitude 0.7 G; and scanning 50 G/42 s with a time constant of 20.5 ms.

EPR lipid radical-POBN adduct (POBN/$L_d^{\cdot}$) peak areas were quantitated using the first peak in the low-field doublet with 3-carboxyproxyl (Aldrich Chemical Co., Milwaukee, WI) as a standard. $Asc^{\cdot-}$ peak heights were measured directly from EPR spectra and are expressed in arbitrary units.

Determination of Antioxidant Enzymes and Vitamin E. Cells were washed and homogenized using sonication. SOD (17, 18), catalase (19), and glutathione peroxidase (20) were determined using published methods. Vitamin E as α-tocopherol was determined by high-performance liquid chromatography as described by Wagner *et al.* (12), except a 98% methanol/2% water mobile phase was used, and dried cell extracts were dissolved in 100% methanol before injection into the high-performance liquid chromatograph.

RESULTS

Clonogenic Survival of HL-60 and K562 Human Leukemia Cell Lines. HL-60 cells are more sensitive to edelfosine than K562 cells, as determined in a clonogenic assay (Fig. 1). The LD_{50} for HL-60 cells was 1.5 μM compared to 21 μM for the K562 cell after a 24-h exposure to ether lipid. These results are similar to what has been reported previously (1) and establish that we have an appropriate pair of resistant/sensitive lines.

Trypan Blue Exclusion as a Measure of Membrane Integrity. To estimate early loss of membrane integrity, we used dye exclusion methods. The first of these was trypan blue, which penetrates damaged membrane and stains the cytoplasm. When edelfosine was added to cells in the presence of Fe^{2+}, ascorbate, and POBN, there was only slight membrane leakage of the HL-60 cells at concentrations of edelfosine of 1–10 μM (Fig. 2, *upper panel*). In contrast, higher concentrations of edelfosine, >10 μM, resulted in significant changes in trypan blue dye exclusion of the HL-60 cells that appeared almost

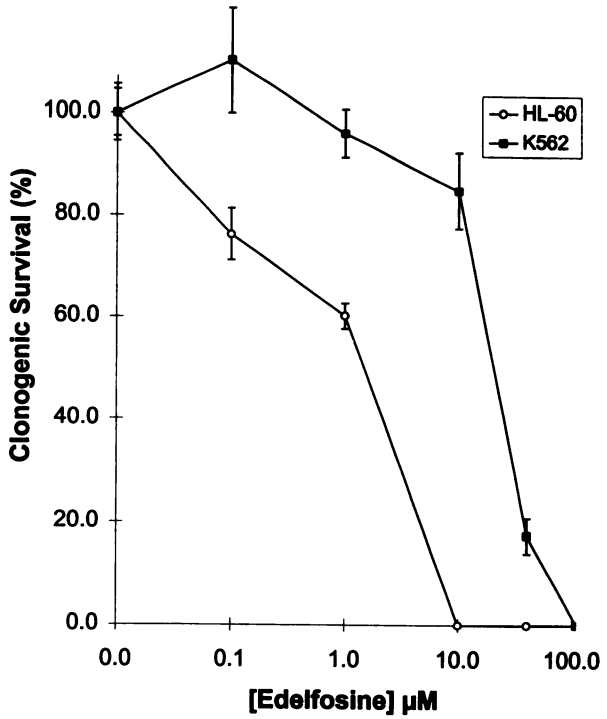

Fig. 1. Clonogenic survival. HL-60 cells are more sensitive to edelfosine than K562 cells after a 24-h exposure to edelfosine. Cells (1×10^6/ml) in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine were exposed to edelfosine at various concentrations for 24 h and subsequently cloned in a soft agar assay. Data represent the mean percentage of survival of cells compared with their respective untreated control cells determined in triplicate; *bars*, SE. The survivals at each edelfosine concentration are significantly different (0.1 μM, $P = 0.04$; 1.0 μM, $P = 0.002$; 10 μM, $P = 0.0003$; and 40 μM, $P = 0.017$).

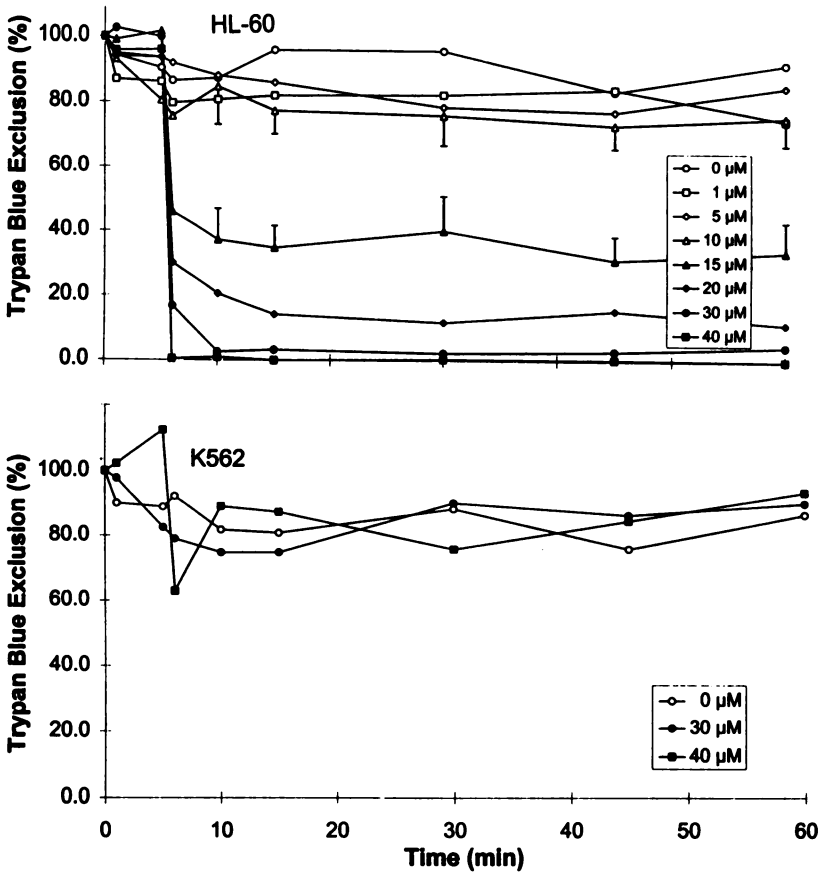

Fig. 2. Trypan blue dye exclusion studies as a measure of cell membrane integrity show that HL-60 (*upper panel*) membranes sustain greater damage than those of K562 cells (*lower panel*) when treated with various concentrations of edelfosine during Fe^{2+} and ascorbic acid-induced lipid peroxidation in the presence of the spin trap POBN. Edelfosine was added at the 5-min time point. Assessments were made at 0, 1, 5, 6, 10, 15, 30, 45, and 60 min. Data represent the mean percentage of change in trypan blue dye-excluding cells of two to four experiments compared with time 0 (untreated control) before addition of Fe^{2+} and ascorbic acid. SE *bars* from triplicates are shown only for 10 and 15 μM to avoid overlay; the other SE *bars* are similar in magnitude. All of the values after the 5-min time point of the top four lines for HL-60 studies (lower concentrations of 0–10 μM) are significantly different from the four lower lines (higher concentrations of 15–40 μM; $P < 0.05$).

immediately after addition of the drug. The changes appear to be concentration dependent from 15 to 40 μM. In contrast, K562 cells treated under similar experimental conditions showed complete lack of response to the drug and appear resistant to the immediate effect of edelfosine on membrane integrity, as compared with HL-60 cells (Fig. 2, *lower panel*).

Propidium Iodide Uptake as a Measure of Membrane Integrity and Cellular Disintegration Measured by Light Scatter. Flow cytometry was used to measure changes in cellular permeability to propidium iodide. It enters the cell through damaged plasma membrane and stains nuclear DNA. This is an alternative estimate of membrane integrity (14, 21, 22). Simultaneously, we monitored forward (small-angle) light scatter, which can be used to estimate the refractive index between plasma membrane and suspending medium and is proportional to cell diameter and volume.

When HL-60 cells were exposed to edelfosine, there was no effect on propidium uptake or light scatter at the lower concentrations of 0, 10, 15, and 20 μM drug. Fig. 3 shows the data for 20 μM, but results are the same for the lower ether lipid concentrations. In contrast, at 25 μM edelfosine, there was propidium iodide permeability beginning within 1 min after addition of the drug and continuing until about 8 min when it plateaued (Fig. 3). The results at the higher concentrations of 30 and 40 μM (data not shown) are the same as 25 μM. In these studies of higher concentrations, light scatter declined, indicating a change in cell diameter; this decline in scatter has a mirror image pattern to propidium iodide uptake. It is known that a decrease in forward scatter can be associated with cell death (21, 23).

$L_d^{\cdot}$. To investigate the early oxidative events induced by edelfosine in the mammalian leukemia cells paired for resistance and sensitivity, we used a real-time measurement of lipid peroxidation as estimated by EPR detection of $L_d^{\cdot}$. HL-60 but not K562 cells generated $L_d^{\cdot}$ when exposed to edelfosine. In the sensitive HL-60 cells, edelfosine stimulated free radical production at 20 μM (Fig. 4). This production began at about 3 min after the addition of drug to the cells previously initiated to oxidize with Fe^{2+} and ascorbate; radical adduct increased rapidly thereafter. Additions of 30–40 μM ether lipid increased $L_d^{\cdot}$ levels to an even greater level, and the radical appeared slightly earlier (3–4 min) after the addition of drug. There was some concentration dependence, but it appeared to level off above 20 μM because there was no meaningful difference between 30 and 40 μM. In marked contrast, the K562 cells failed to produce $L_d^{\cdot}$ at all concentrations studied, even up to 40 μM ether lipid (not shown).

$Asc^{\cdot-}$. To further probe the oxidative conditions of the cells, we used $[Asc^{\cdot-}]_{ss}$. The steady state concentration of $Asc^{\cdot-}$ can be used as an estimate of cellular oxidative stress, if conditions are carefully controlled (24). In our experiments, an increase in $[Asc^{\cdot-}]_{ss}$ was detected when HL-60 cells were exposed to higher concentrations of edelfosine but not lower ones. Fig. 5 shows the results of $Asc^{\cdot-}$ generation as peak height *versus* time. $[Asc^{\cdot-}]_{ss}$ increased rapidly with the addition of the ether lipid to the cells previously initiated to oxidize with Fe^{2+} and ascorbate; $[Asc^{\cdot-}]_{ss}$ reached a peak at 10 min (5 min after the addition of drug) and then slowly declined. When exposed to 30 μM edelfosine, HL-60 cells generated a 1.5-fold increase in $[Asc^{\cdot-}]_{ss}$ by 10 min (5 min after the addition of drug) as compared with the baseline at 5 min immediately prior to the addition of drug. There was a similar increase $[Asc^{\cdot-}]_{ss}$ for all concentrations >15 μM but none for 0–15 μM edelfosine (data only for 0 and 30 μM are shown for brevity). At 20 μM edelfosine, the generation of $Asc^{\cdot-}$ did not peak until 20 min. In contrast, there was no increase in $[Asc^{\cdot-}]_{ss}$ when the K562 cells were exposed to the drug (Fig. 5); the only exception was at 40 μM, which resulted in a 1.5-fold increase over baseline but peaked at a

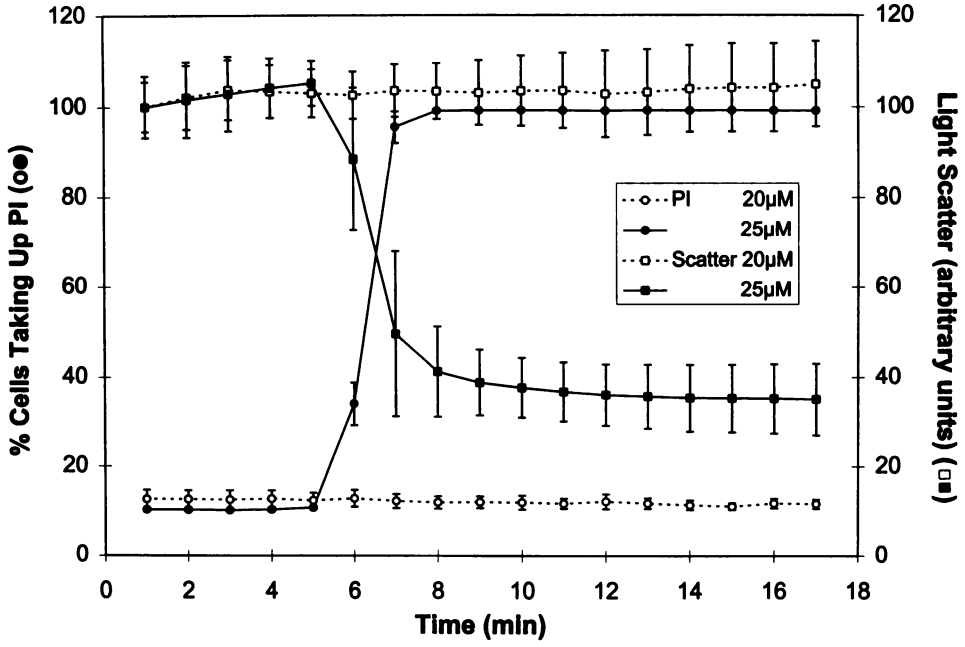

Fig. 3. The effect of edelfosine on HL-60 propidium iodide membrane permeability and light scatter shows a striking change between 20 and 25 μM. Experiments were set up as in Fig. 2, but dye entry and scatter were measured by flow cytometry. Data on the primary *Y*-axis represent the effect of edelfosine on mean dye uptake. On the secondary *Y*-axis is shown the effects of edelfosine on forward (small-angle) light scatter, which is a measure of membrane refractive index and cell diameter. Shown are means of triplicates; *bars*, SE.

value still only one-half the baseline value for HL-60 cells. These data indicate that edelfosine results in a heightened cellular oxidative state in the HL-60 cells, but not the K562 cells, at the concentrations tested, which included edelfosine up to 40 μM. These observations on $Asc^{\cdot-}$ are parallel to those on $L_d^{\cdot}$.

Subcellular Fractions. To eliminate the possibility that the generation of $L_d^{\cdot}$ and $Asc^{\cdot-}$ by the HL-60 cells was due to a subcellular fraction that was released by disruption of membranes in the sensitive HL-60 cells but not in the resistant K562 cells, we studied cellular homogenates. Cells were homogenized by nitrogen cavitation and then exposed to edelfosine, Fe^{2+}, ascorbate, and the spin trap POBN. HL-60 homogenates produced $L_d^{\cdot}$, even in the absence of edelfosine (Fig. 6). The addition of 40 μM edelfosine augmented the production only slightly. Studies monitoring $[Asc^{\cdot-}]_{ss}$ gave a similar result. The level of $L_d^{\cdot}$ production of HL-60 cellular homogenates was lower than intact cells at some early time points, but the production of $Asc^{\cdot-}$ was similar to intact cells (results not shown). Strikingly, K562 cell homogenates failed to generate $L_d^{\cdot}$ or increase $[Asc^{\cdot-}]_{ss}$. We believe this set of simple experiments helps in eliminating cellular disruption as a likely cause for differences in HL-60 or K562 oxidizability. These experiments also make it unlikely that differences in drug uptake or intracellular drug localization explain the differences in cell oxidizability observed in the free radical experiments.

Fatty Acid Composition. To determine whether there is a structural basis for the differences in cellular oxidation, the fatty acid composition of the total cell lipids of each cell type was determined (Table 1). The HL-60 cells had a higher percentage of polyunsaturated fatty acids and greater mean number of double

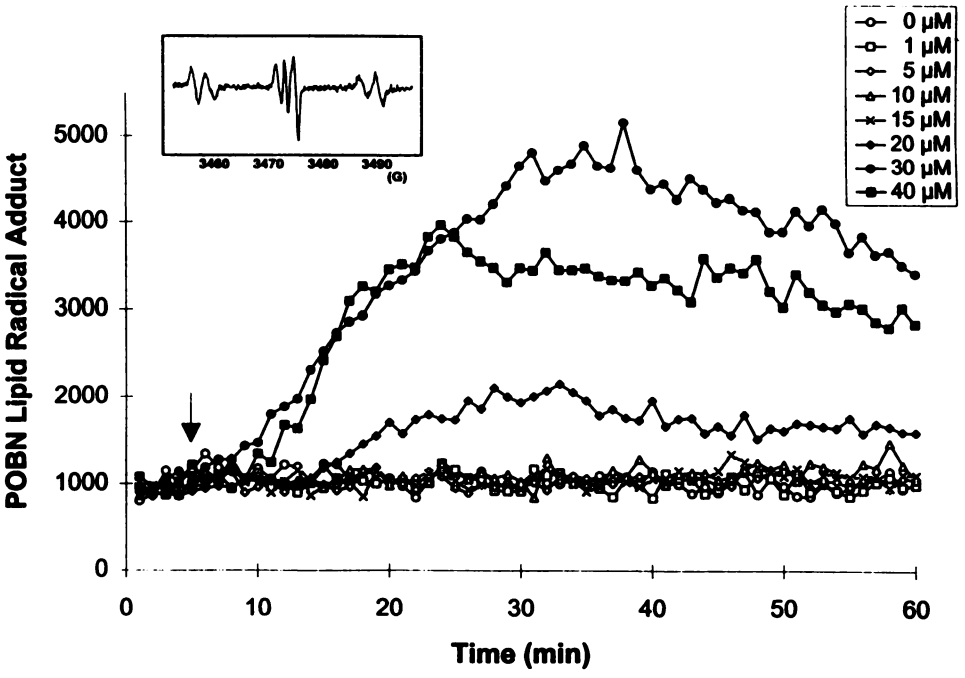

Fig. 4. Effect of edelfosine on lipid peroxidation of HL-60 cells as measured by EPR-detectable POBN/$L_d^{\cdot}$ adduct formation. HL-60 cells (1×10^7/ml) were suspended in 0.9% NaCl containing 50 mM POBN spin trap. Ascorbic acid (100 μM) and 20 μM Fe^{2+} were added at the start of EPR recording. After 5 min (*arrow*), various concentrations of edelfosine were added. Values are the means of two to three experiments for each edelfosine concentration. Estimated POBN/$L_d^{\cdot}$ adduct maximum concentration was 120 nM for the 40 μM ether lipid-treated sample, compared with 27 nM for the studies with 0 μM edelfosine concentration. *Inset*, typical POBN/$L_d^{\cdot}$ EPR spectrum. The ascorbate radical doublet can also be observed.

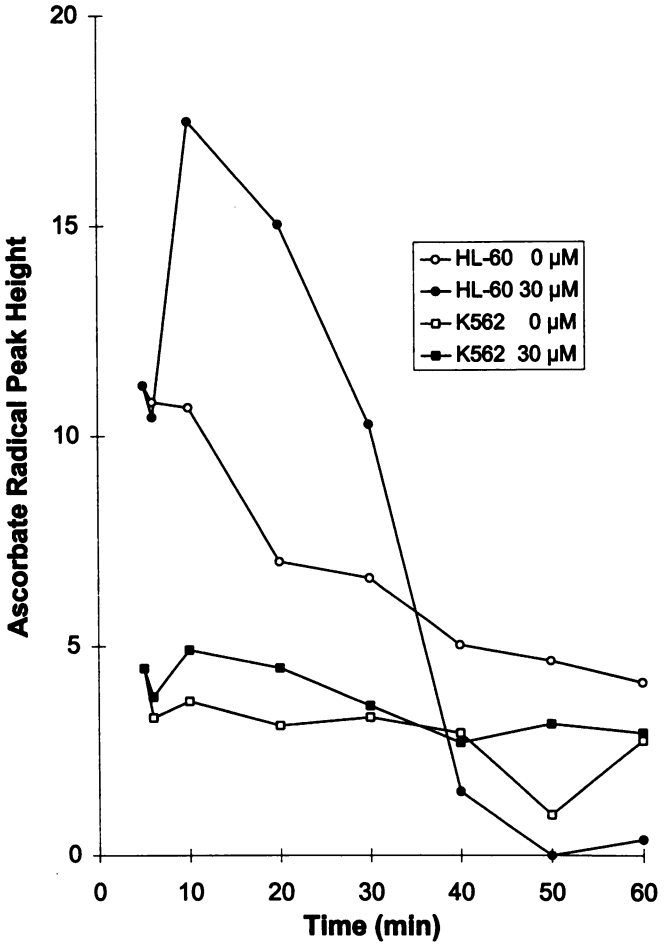

Fig. 5. Edelfosine enhances free radical oxidation in HL-60 cells more than in K562 cells, as measured by EPR-detectable ascorbate radical formation. Data were obtained from the same experiments as Fig. 4. After 5 min, various concentrations of edelfosine were added. Values are the means of two to three experiments at each edelfosine concentration. Estimated $[Asc^{\cdot-}]_{ss}$ maximum concentration was 103 nM for 30 μM ether lipid-treated HL-60 sample.

bonds per fatty acid (double bond index). This was primarily due to a higher percentage of long-chain fatty acids of 20 and 22-carbon length, especially 20:4ω6 and 22:6ω3. Most importantly, the MBI, which is the most accurate estimate of cellular oxidizability (11), was 2-fold greater for the HL-60 cells as compared with the K562 cells. The fatty acid composition of the phospholipids, as a measure of membrane lipids, showed a similar contrast. Similar differences between the HL-60 and K562 cells were also observed in the neutral lipid fatty acid composition, which represent intracellular lipid storage sites.

Antioxidant Enzyme Activity and Vitamin E Content. Antioxidant enzymes are important in the protection against damaging intracellular oxidative processes. We determined the cellular levels of four important enzymes (Table 2). The levels of antioxidant enzymes were not significantly different between the HL-60 and K562 cells at the traditional $P < 0.05$ level.

Likewise, there was no difference in the cellular content of vitamin E, which is the major lipid-soluble, small-molecule antioxidant in cellular membranes, between the HL-60 and K562 cells. For further comparison, the vitamin E content of the K562 cells is only slightly higher than that of the L1210 cell (12). We conclude that increased antioxidant capacity is not likely to explain the drug resistance of the K562 cells to edelfosine.

DISCUSSION

Ether lipids are an innovative class of anticancer drug with a characteristic membrane site of action (25). *In vitro* studies have established that their action is selective for neoplastic cells (26, 27). They have been found to have limited activity in the treatment of lung cancer (28) and for the topical treatment of skin lymphoma (29) and breast cancer (30). However, trials of edelfosine in bone marrow purging have been more promising (31–34).

Ether lipids are the prototype class of drug with membrane-based cytotoxicity. There is compelling evidence that this family of compounds exerts an antitumor action by effects on plasma and intracellular membranes. The compounds have been shown to accumulate at the cell surface (3), increase membrane fluidity of neoplastic cells (35), alter surface nuclear magnetic resonance spectrum (36), and produce dramatic morphological changes in membranes by electron microscopy (37, 38). It is important to recognize that the action of this class of drugs is not a direct detergent-like effect but rather a metabolically or physically based influence on phospholipid bilayer renewal due to their accumulation in membranes, particularly at the surface. There is no evidence that the ether lipids inhibit DNA synthesis (39, 40), and they are additive or synergistic with agents that target DNA or the cytoskeleton (41). Their mechanism of action remains unknown, but there have been many proposals, including that of disturbed phospholipid metabolism (42–44), effect on protein

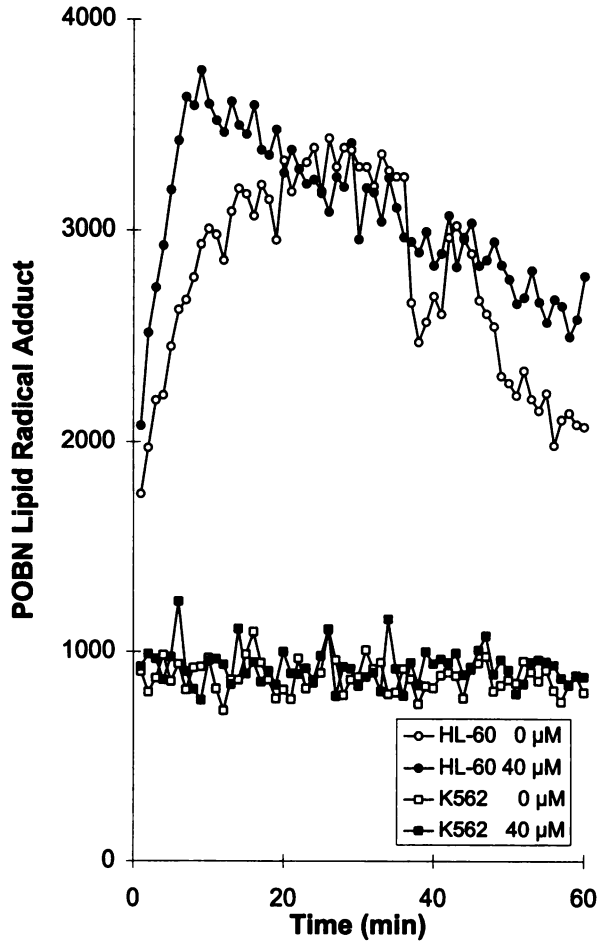

Fig. 6. Lipid radical production by cellular homogenates. Homogenates were prepared from 1×10^7/ml HL-60 or K562 cells in 0.9% NaCl using nitrogen cavitation. Homogenates were then incubated with 100 μM ascorbic acid and 20 μM Fe^{2+} in the presence of 50 mM POBN spin trap. At 5 min, 0 or 40 μM edelfosine were added. The values are the means of three to four experiments.

Table 1 *Fatty acid composition of human leukemia cells (in mol %)*[a]

	Total lipids		Phospholipids		Neutral lipids	
	HL-60	K562	HL-60	K562	HL-60	K562
Individual acids[b]						
14:0	2.0 ± 0.1	1.9 ± 0.4	1.6 ± 0.0	1.0 ± 0.1	3.8 ± 0.8	3.0 ± 0.1
14:1	0.8 ± 0.1	0.3 ± 0.1	5.8 ± 0.3	2.7 ± 0.4	1.9 ± 1.1	1.1 ± 0.1
16:0	23.6 ± 1.6	24.6 ± 1.1	20.1 ± 0.2	22.2 ± 1.1	21.6 ± 1.8	28.2 ± 1.6
16:1	5.2 ± 0.4	5.2 ± 0.2	4.3 ± 0.3	4.2 ± 0.5	8.3 ± 0.7	8.0 ± 0.4
18:0	13.6 ± 0.3	16.9 ± 1.5	12.7 ± 0.3	15.0 ± 0.6	6.9 ± 0.8	12.2 ± 1.1
18:1	20.9 ± 1.4	34.0 ± 1.6	23.8 ± 1.0	35.5 ± 1.6	23.3 ± 1.2	26.0 ± 0.1
18:2ω6	4.3 ± 1.2	2.4 ± 0.1	3.5 ± 0.2	1.9 ± 0.2	6.3 ± 0.2	5.9 ± 0.6
20:1ω9	0.4 ± 0.1	1.3 ± 0.1	0.6 ± 0.1	1.2 ± 0.1	0.8 ± 0.1	0.9 ± 0.3
20:2ω6	0.2 ± 0.2	0.1 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
20:3ω6	4.8 ± 1.7	0.6 ± 0.2	2.8 ± 0.2	0.9 ± 0.2	3.1 ± 0.9	0.8 ± 0.2
20:4ω6	12.1 ± 0.2	6.8 ± 0.8	13.0 ± 0.8	6.6 ± 0.8	5.2 ± 1.2	2.9 ± 0.2
20:5ω3	1.8 ± 0.9	0.4 ± 0.0	1.2 ± 0.1	0.5 ± 0.3	1.1 ± 0.2	0.5 ± 0.2
22:5ω3	3.5 ± 0.2	1.7 ± 0.2	3.3 ± 0.1	2.0 ± 0.4	5.9 ± 1.2	3.1 ± 1.7
22:6ω3	4.4 ± 0.2	2.4 ± 0.2	5.6 ± 0.1	3.0 ± 0.5	6.2 ± 0.8	2.3 ± 0.7
24:0	1.8 ± 0.2	0.1 ± 0.2	0.2 ± 0.0	0.9 ± 0.7	1.0 ± 0.0	2.2 ± 0.1
24:1ω9	0.4 ± 0.1	0.4 ± 0.1	0.9 ± 0.0	1.7 ± 1.0	4.3 ± 0.1	2.5 ± 0.3
Classes of fatty acids						
%PUFA[c]	31.0 ± 1.3	14.5 ± 1.0	29.9 ± 1.3	15.2 ± 1.9	28.1 ± 3.6	15.5 ± 2.6
%MUFA	28.0 ± 1.4	41.4 ± 2.0	35.5 ± 1.3	45.3 ± 0.9	38.5 ± 0.2	38.5 ± 0.5
%SAT	41.0 ± 2.1	44.1 ± 2.0	34.6 ± 0.3	39.4 ± 1.5	33.3 ± 3.4	46.0 ± 2.8
%ω3	9.7 ± 0.9	4.6 ± 0.3	10.1 ± 0.2	5.6 ± 1.1	13.1 ± 1.8	5.8 ± 2.3
%ω6	21.3 ± 1.4	9.9 ± 0.7	19.8 ± 1.1	9.6 ± 1.0	15.0 ± 1.7	9.7 ± 0.7
Indices						
MBI[c]	0.93 ± 0.02	0.45 ± 0.03	0.94 ± 0.04	0.49 ± 0.1	0.87 ± 0.1	0.42 ± 0.1
DBI	1.52 ± 0.04	1.01 ± 0.05	1.6 ± 0.04	1.09 ± 0.1	1.54 ± 0.2	0.96 ± 0.1

[a] HL-60 or K562 human leukemia cells were washed and extracted with $CHCl_3$:CH_3OH, 2:1 (v/v). After alkaline hydrolysis, fatty acids in the saponifiable fraction were methylated, and the methyl esters were separated by gas-liquid chromatography. Mole percentages of various fatty acids were calculated from integrated peak areas on chromatograms and respective molecular weights of FAMEs.

[b] Expressed as mole percentage of total fatty acids. Fatty acids are designated as number of carbon atoms:number of double bonds. Values are the mean ± SE of five independent experimental sample determinations.

[c] PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SAT, saturated fatty acids; MBI, methylene bridge index, which is the mean number of *bis*-allylic positions per fatty acid; DBI, double bond index, which is the mean number of double bonds per fatty acid.

kinase C (4, 45–47), inhibition of phospholipase C (48), influence on calcium flux (49), induction of cellular differentiation (4, 43, 50, 51), or activation of macrophages (44).

In this study, we have demonstrated that the selective cytotoxicity of edelfosine for two human leukemia cell lines is associated with oxidative events. The ether lipid-sensitive HL-60 human leukemia cells, but not the resistant K562 cells, generate $L_d^{\cdot}$ when exposed to pharmacologically relevant concentrations of the drug. The time of onset and concentration dependence of the increased radical production correspond with the onset of early cytotoxicity, suggesting that it may be a seminal event. Measurement of cellular oxidative state during drug exposure using $Asc^{\cdot-}$ generation confirmed the relationship of oxidation and cytotoxicity. Studies of subcellular fractions demonstrated that the results are not due to the release of intracellular components during early membrane damage. However, it should be noted that the onset of radical generation and early evidence of membrane damage are nearly coincident within the sensitivity of our assays. Thus, we cannot rule out the possibility that cellular oxidation is secondary to a prior event leading to a subsequent concatenated sequence of membrane events resulting in cell death.

Table 2 *Antioxidant enzyme and vitamin E content*[a]

Enzyme	HL-60	K562	*P* (*n*)
Catalase, k/g	1270 ± 114	1120 ± 77	0.34 (6)
SOD, total, units/mg[b]	41.9 ± 11.0	56.1 ± 11.3	0.40 (5)
Cu-ZnSOD, units/mg	26.8 ± 11.1	30.7 ± 8.7	0.79 (5)
MnSOD, units/mg	15.1 ± 5.8	25.4 ± 5.5	0.23 (5)
Glutathione peroxidase, units/g	7.8 ± 2.0	2.4 ± 1.2	0.06 (8)
Vitamin E, ng/mg	11.3 ± 2.1	15.9 ± 3.6	0.3 (10)

[a] HL-60 or K562 cells were washed and homogenized in assay buffer. Enzymes and vitamin E were assayed by the methods referenced in "Materials and Methods." All values are expressed per amount of protein. The protein content of HL-60 and K562 cells is 122 ± 7 μg and 229 ± 7 μg per 1 × 10^6 cells, respectively. Values are means and SE. The last column shows *P* and the number of replicates; none of the differences are statistically significant.

[b] In this assay, 1 unit of SOD activity corresponds to 8 ng of pure bovine Cu-ZnSOD/ml of solution (18).

The present work demonstrates that oxidative events occur early and may be important or even seminal in membrane damage. Because the ether lipid class of drugs does not generate an oxidative intermediate during metabolism (8), our observations suggest that there is a link between this membrane-targeted drug action and intracellular oxidation. The fatty acids of the membrane provide a rich target for the initiation of such an action, and their nature may determine the eventual fate of the cell undergoing oxidation/reduction reactions. In our studies, we found that the sensitive HL-60 cell line had more polyunsaturated fatty acids in membranes than the resistant K562 cell line, thus making it potentially more susceptible to oxidation.

Clonogenic assays are an excellent method for assessing sustained self-renewal, an ultimate measure of viability. However, such assays only provide information on the end result, reproductive death. Clonogenic measures of the ability of the cells to reproduce do not provide information about the early events that led to a loss of clonogenicity. In an attempt to understand these early membrane events, we used three complementary assays, each of which measures an earlier event, membrane damage. The trypan blue assay measures a breach of membrane integrity sufficient to allow entry of the dye into the cell, thereby staining the cytoplasm; it is a frequently used estimate of cellular viability. Propidium iodide is similarly excluded from the cell until membrane damage occurs, at which time it enters the cell and stains the nuclear DNA. Cell size changes reflecting membrane damage are also detected by changes in forward light scatter by flow cytometry. We used all three membrane-related assays in an attempt to define early membrane events in cell destruction. They were confirmatory in defining the temporal and drug concentration relationships of oxidation and early membrane damage.

We used two measures of radical formation and lipid peroxidation

that complement one another. $Asc^{\cdot-}$ and $L_d^{\cdot}$ were detected by EPR when the leukemia cells were subjected to Fe^{2+}-ascorbate-induced oxidative stress. Our EPR technique allows real-time detection of free radical generation from live cells, permitting us to monitor the effect of drug on oxidative events. The $L_d^{\cdot}$ radical has the spectral characteristics of an alkyl radical adduct of POBN, possibly pentyl, ethyl, or pentenyl radical adduct. The radical results from β-scission of lipid alkoxyl radicals formed by the reaction of Fe^{2+} and lipid hydroperoxides (52). We have demonstrated that its generation when exposed to an oxidative stress is a function of cellular lipid *bis*-allylic hydrogen content (11) and that it is inhibited by antioxidants (12). To confirm the relationship of oxidation and membrane damage, we also measured $Asc^{\cdot-}$ generation, which can be used as a measure of oxidative flux (24). The introduction of edelfosine to HL-60 cells, previously incubated with Fe^{2+} and ascorbic acid to initiate peroxidation, caused an increase in the EPR signal of $Asc^{\cdot-}$ at all concentrations above 15 μM, thus paralleling the $L_d^{\cdot}$ observations. In contrast, K562 cells generated no radical after ether lipid exposure.

The ether lipid class of antitumor agents also provide a rich model for the study of inhibition of cell proliferation. Lohmeyer and Workman have previously reported different modes of inhibition, depending on drug concentration and time (53). In their studies of HL-60 and HT29 cells using edelfosine and other ether lipids, concentrations from 5 to 40 μM resulted in a reduction in cell number and decreased clonogenic survival, whereas lower concentrations induced only reversible growth arrest. Above 40 μM, they saw evidence of membrane damage. However, their studies were done in the presence of serum that binds the ether lipid drugs and decreases drug uptake due to binding to high-density lipoprotein and albumin (54). In our studies performed in the absence of serum, we found evidence of membrane damage at concentrations above 10 μM, and this is the critical concentration above which oxidative events were recorded. We think it is likely that the oxidation demonstrated at these higher concentrations corresponds with necrotic cell death because there was: (*a*) an early change in plasma membrane integrity that occurred at the drug concentrations that induced oxidation; (*b*) a decrease in cell number at a time prior to that expected with apoptotic death; and (*c*) an immediate decrease in light scatter. However, we cannot eliminate the possibility that free radical generation corresponds with the initiation of apoptosis because edelfosine induces cell death by apoptosis in the HL-60 cell, but not in the K562 cell, at concentrations of 10–20 μM (55, 56). In addition, apoptosis has been shown to be associated with changes in forward light scatter in the HL-60 cell, such as those we found at $\geq$25 μM edelfosine when exposed to camptothecin, another anticancer drug (21, 23). The physiological and biochemical changes that we recorded are unlikely to be a simple detergent-like effect because of the resistance of the K562 cells to membrane damage and oxidation, even at the higher concentrations of 30 and 40 μM. It is more likely that the oxidation results in cellular demise by the process of necrosis.

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