

# Pro-oxidant and Antioxidant Mechanisms of Etoposide in HL-60 Cells: Role of Myeloperoxidase<sup>1</sup>

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## ABSTRACT

Etoposide is an effective anticancer agent whose antitumor activity is associated with its phenolic E-ring, which can participate in intracellular redox cycling reactions. Myeloperoxidase (MPO)-catalyzed one-electron oxidation of the etoposide phenolic ring and/or interaction of this phenolic moiety with reactive radicals yields its phenoxyl radical, whose reactivity may determine the pro- or antioxidant effects of this molecule in cells. Using MPO-rich HL-60 cells, we directly demonstrated that both anti- and pro-oxidant activities of etoposide are realized in cells. Etoposide acted as an effective radical scavenger and antioxidant protector of phosphatidylethanolamine, phosphatidylcholine, and other intracellular phospholipids against H<sub>2</sub>O<sub>2</sub>-induced oxidation in HL-60 cells with constitutively high MPO activity and in HL-60 cells depleted of MPO by an inhibitor of heme synthesis, succinyl acetone. MPO-catalyzed production of etoposide phenoxyl radicals observed directly in HL-60 cells by electron paramagnetic resonance (EPR) did not result in oxidation of these membrane phospholipids, suggesting that the radicals were not reactive enough to trigger lipid oxidation. MPO-dependent pro-oxidant activity of etoposide was directly demonstrated by (a) the ability of intracellular reduced glutathione (GSH) to eliminate EPR-detectable etoposide phenoxyl radicals, (b) the ability of etoposide phenoxyl radicals to oxidize GSH and protein thiols (after preliminary depletion of intracellular GSH with a maleimide reagent, ThioGlo-1), and (c) the disappearance of these effects after depletion of MPO by pretreatment of cells with succinyl acetone. In addition, titration of intracellular GSH (in intact cells) using the maleimide reagent ThioGlo-1 resulted in remarkably augmented EPR-detectable etoposide phenoxyl radicals and enhanced etoposide-induced topoisomerase II-DNA covalent complexes. In conclusion, the phenolic moiety of etoposide acts as an effective free radical scavenger, accounting for its antioxidant action. Whereas one-electron oxidation of etoposide by free radical scavenging and/or by MPO results in a phenoxyl radical with low reactivity toward lipids, its high reactivity toward thiols is a determinant of its pro-oxidant effects in HL-60 cells.

## INTRODUCTION

Recent studies have clearly demonstrated that chemotherapy-induced apoptosis and subsequent phagocytosis of cancer cells depend on the redox status and the intracellular balance between pro- and antioxidants (1, 2). Thus, the effectiveness of antitumor chemotherapy may be manipulated by including certain antioxidant agents in treatment protocols (1, 2). This may be particularly important because

commonly used chemotherapy regimens have been reported to deplete antioxidants in the plasma of patients (3–5). Because many antitumor drugs are cytotoxic due to their ability to generate free radicals, characterizing specific metabolic pathways that may regulate the pro-oxidant action of antitumor drugs may be essential for optimizing clinical efficacy. Similarly, an understanding of the antioxidant properties of some anticancer agents may reveal important information concerning metabolic pathways that may limit clinical efficacy.

The DNA topo<sup>3</sup> II poison etoposide (VP-16), a widely used anticancer agent, contains a hindered phenolic ring, a critical structural prerequisite for its antitumor activity (6, 7). As a hindered phenol, etoposide can act as an effective donor of electrons for scavenging reactive (peroxyl) radicals, *i.e.*, it can act as an antioxidant as indicated below.



The protective antioxidant effects of etoposide against lipid peroxidation have been demonstrated in model biochemical systems (8, 9). In contrast, etoposide-induced formation of lipid peroxyl radicals and increased lipid peroxidation have been reported in different clones derived from Chinese hamster ovary cells (10).

Reactivity of the phenoxyl radical formed from etoposide (etoposide-O<sup>•</sup>) in the course of Reaction 1 toward different biomolecules (Reaction 2) is essential for determining whether net antioxidant or pro-oxidant effects of etoposide will be realized in cells.



Thus, etoposide may act as either a pro- or antioxidant toward different intracellular constituents. In particular, MPO-catalyzed one-electron oxidation of etoposide and the secondary reactions of the resultant phenoxyl radical cause oxidative stress (thiol oxidation) in cell-free model systems, cell homogenates (9), and myeloblastic leukemia cells (11).

It has been suggested that oxidative activation of etoposide by cytochrome P450 monooxygenases, MPO, prostaglandin synthetase, and tyrosinase may contribute to its cytotoxicity (12–15). Whereas etoposide is highly efficacious as an antitumor drug, its use is also associated with an increased risk of secondary AML (16). This has prompted its withdrawal from some treatment regimens, potentially compromising efficacy against the original tumor (17). Based on the results of our experiments in cell-free systems and cell homogenates, we have hypothesized that etoposide genotoxicity and carcinogenicity are due to MPO-catalyzed production of etoposide phenoxyl radicals (etoposide-O<sup>•</sup>) in myeloid progenitors and their subsequent pro-oxidant effects (9).

<sup>3</sup> The abbreviations used are: topo, topoisomerase; EPR, electron paramagnetic resonance; GSH, reduced glutathione; MPO, myeloperoxidase; AML, acute myeloid leukemia; SA, succinyl acetone (4,6-dioxoheptanoic acid); 3-AT, 3-amino-1,2,4-triazole; FBS, fetal bovine serum; SH, sulfhydryl; PnA, *cis*-parinaric acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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In the present work, we present direct EPR evidence for the MPO-catalyzed generation of etoposide-O<sup>•</sup> radicals in intact HL-60 cells, their interactions with intracellular thiols, and the resulting modulation of etoposide-induced covalent complex formation between DNA and topo II. In addition, using a sensitive procedure for assay of oxidative stress in different classes of membrane phospholipids, we demonstrate that etoposide-O<sup>•</sup> phenoxyl radicals are not involved in pro-oxidant reactions toward lipids but rather act as antioxidants against H<sub>2</sub>O<sub>2</sub>-induced phospholipid peroxidation.

## MATERIALS AND METHODS

**Materials.** Etoposide (VP-16), hydrogen peroxide, SA, guaiacol, 3-AT, phenylmethylsulfonyl fluoride, glucose, cetyltrimethylammonium bromide, HEPES, fatty acid-free human serum albumin, malachite green base, sodium molybdate, sodium chloride, magnesium chloride, sodium phosphate, GSH, ethanol, lauryl sulfate, sodium salt (SDS), and FBS were purchased from Sigma Chemical Co. (St. Louis, MO). Iscove's medium was from Life Technologies, Inc. (Grand Island, NY). Triton X-100 (*t*-octylphenoxypolyethoxyethanol) was from Bio-Rad Laboratories (Richmond, CA). 2-Propanol, methanol, hexane, and acetonitrile were from Aldrich Chemical Co. (Milwaukee, WI). ThioGlo-1 was from Covalent Associates, Inc. (Woburn, MA). [*methyl*-<sup>3</sup>H]Thymidine and [<sup>14</sup>C]leucine were from DuPont/New England Nuclear (Wilmington, DE). DMSO was from Fisher Scientific Company (Pittsburgh, PA). All stock solutions were kept at -20°C. All other reagents were of the highest grade available.

**Cell Culture.** Human promyelocytic HL-60 cells (from the American Type Culture Collection) were grown in Iscove's medium supplemented with 15% FBS in 95% humidity atmosphere under 5% CO<sub>2</sub> in air at 37°C. Cells from passages 25–40 were used for the experiments. The density of cells at collection time was  $0.5 \times 10^6$  cells/ml. Etoposide was dissolved in DMSO and added to cells (DMSO concentration did not exceed 1%). HL-60 cells ( $1.5 \times 10^5$  cell/ml) were incubated for 48 h with SA (500 μM) dissolved in Iscove's medium. Cell viability was determined microscopically by trypan blue dye exclusion.

**MPO Activity.** HL-60 cells were harvested by centrifugation at 1000 rpm for 5 min. Pellets were washed twice with buffer A containing 25 mM HEPES, 10 mM glucose, 115 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, and 5 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4). The homogenate was prepared by freezing at -77°C and thawing the cells. A spectrophotometric assay (Shimadzu UV 160U spectrophotometer; Kyoto, Japan) of MPO activity was used in which guaiacol oxidation was monitored by changes of absorbance at 470 nm ( $\epsilon = 26.6 \text{ mM}^{-1}/\text{cm}^{-1}$ ; Ref. 1). Cell homogenate ( $0.5 \times 10^6$  cells) was added to 100 mM disodium phosphate buffer containing 0.1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 13 mM guaiacol, 0.02% cetyltrimethylammonium bromide, and 3.75 mM 3-AT (pH 7.0). The reaction was started by the addition of 670 μM H<sub>2</sub>O<sub>2</sub>. Activity of MPO was calculated in nmol tetraguaiacol formed/min/ $10^6$  cells. The data were acquired using Shimadzu PC 160 software version 1.2.

**EPR Spectroscopy of Etoposide Phenoxyl Radicals.** Measurements of etoposide phenoxyl radicals were performed on a JEOL-REIX EPR spectrometer (Tokyo, Japan) at 25°C as described previously (9). Samples (50 μl) contained viable HL-60 cells ( $8 \times 10^4$ ), etoposide (50–400 μM), 3-AT (5 mM), and H<sub>2</sub>O<sub>2</sub> (5–200 μM). Measurements were done in gas-permeable Teflon tubing (internal diameter, 0.8 mm; thickness, 0.013 mm) from Alpha Wire Corp. (Elizabeth, NJ). The tubing filled with mixed sample was folded twice and placed into a 3.0-mm EPR quartz tube. The EPR conditions were as follows: 335.7 mT center field; 2 mT sweep width; 0.04 mT field modulation; 10 mW microwave power; 0.1 s time constant; 4000 receiver gain; and 1 min time scan. The kinetics of etoposide phenoxyl radical formation was measured by repeated scanning of its EPR signal.

**Assay of GSH and Protein SH Groups in HL-60 Cells.** GSH and total protein SH concentration in HL-60 cells was determined using ThioGlo-1, a maleimide reagent that produces a highly fluorescent adduct on its reaction with SH groups (18). GSH content was estimated by an immediate fluorescence response registered on the addition of ThioGlo-1 to the cell homogenate. Protein SHs were determined as an additional increase in fluorescence response after the addition of SDS (4.0 mM) to the same cell homogenate. To demonstrate the initial interaction of ThioGlo-1 mainly with GSH, HL-60 cell

homogenates were treated with glutathione peroxidase to specifically deplete GSH, followed by analysis of fluorescence responses before and after the addition of SDS (*i.e.*, GSH and other low molecular weight thiols or protein thiols, respectively). We found that glutathione peroxidase treatment almost completely eliminated that part of the fluorescence response to ThioGlo-1 obtained in the absence of detergent. For non-detergent-treated cell homogenates, glutathione peroxidase treatment decreased the measured SH level from  $4.98 \pm 1.00$  to  $0.48 \pm 0.14$  nmol/mg protein. At the same time, no effect of glutathione peroxidase treatment on the fluorescence response to ThioGlo-1 was revealed in the presence of detergent ( $19.94 \pm 4.53$  versus  $17.92 \pm 3.32$  nmol SH/mg protein). In HL-60 cells, therefore, the initial fluorescence response elicited by ThioGlo-1 in the absence of detergent was due almost entirely to GSH, whereas the response evoked by the addition of detergent was due mainly to protein SH groups.

For standard assays, homogenate ( $8 \times 10^4$  cells), ThioGlo-1 (10 μM in DMSO), and disodium phosphate buffer [100 mM (pH 7.4)] in a final volume of 1 ml were used. A standard curve was established by the addition of 0.04–4.0 μM GSH to 100 mM phosphate buffer (pH 7.4) containing 10 μM ThioGlo-1 (DMSO solution). A model RF-5301PC spectrofluorophotometer (Shimadzu) was used for the assay of fluorescence using excitation at 388 nm and emission at 500 nm. The data obtained were exported and treated using RF-5301PC Personal Fluorescence Software (Shimadzu). To titrate endogenous GSH, intact HL-60 cells ( $8 \times 10^4$ ) were incubated with different concentrations of ThioGlo-1 (2–10 μM, 10 min) in buffer A (see above). HL-60 cells were then washed twice with buffer A to remove excess non-reacted ThioGlo-1.

**Imaging of GSH in HL-60 Cells Using ThioGlo-1.** HL-60 cells ( $10^6$  cells/75-cm<sup>2</sup> flask) were incubated with 200 μM H<sub>2</sub>O<sub>2</sub>, 400 μM etoposide, and 5 mM 3-AT for 15 min. After incubation, the cells were pelleted, washed twice, and resuspended in PBS. Cells were labeled with 40 μM ThioGlo-1. Cell slides were prepared by spinning cell suspensions at 800 rpm for 5 min using Cytospin 3 (Shandon; Life Sciences International Ltd., Cheshire, United Kingdom). Cells on slides were fixed with 2% paraformaldehyde for 30 min. The glass coverslips (Fisher Scientific) were mounted over slides using Prolong Mounting Medium (Molecular Probes, Eugene, OR). Cells were visualized using an Olympus AX70 photomicroscope (Tokyo, Japan) with a xenon epifluorescence attachment (filter cubes  $\lambda = 360$ –390 nm) and barrier filter ( $\lambda = 515$  nm) to record fluorescence images. Images were captured with a Sony 3CCD color video camera, DXC9000 (Kyoto, Japan) and saved as tif. files using Simple32 software (Compix Inc., Cranberry, PA). Magnification 1000x.

**Determination of Phospholipid Peroxidation in HL-60 Cells.** PnA was incorporated into HL-60 cells by the addition of complex PnA-human serum albumin to yield a final concentration of 2 μg PnA/ $10^6$  cells to serum-free RPMI 1640 without phenol red as described previously (19). PnA-labeled HL-60 cells were treated with 100 μM H<sub>2</sub>O<sub>2</sub> in the presence or absence of 50 μM etoposide for 2 h at 37°C in buffer A (pH 7.4) containing 5 mM 3-AT. H<sub>2</sub>O<sub>2</sub> (25 μM) was added every 30 min. Etoposide was added to cell suspensions 35 min before the addition of H<sub>2</sub>O<sub>2</sub>. At the end of the incubation period, total lipids were extracted using Folch's procedure. The lipid extract was dried under N<sub>2</sub>, dissolved in 0.2 ml of 2-propanol:hexane:water (4:3:0.16, by volume), and separated by normal phase high-performance liquid chromatography using a 5-μm microsil MV column (4.6 × 250 mm; Rainin Instrument Co. Inc.) and an ammonium acetate gradient as described previously (19). The separations were performed using a Shimadzu LC-600 high-performance liquid chromatography system equipped with an in-line configuration of RF-551 fluorescence detector. Fluorescence of PnA was measured at 420 nm emission after excitation at 324 nm. Data were processed and stored in digital form with Shimadzu EZChrom software. Lipid phosphorus was determined using a micromethod (20).

**topo II Covalent Complex Formation.** Mid-log HL-60 cells ( $2.5$ – $3.0 \times 10^5$  cells/ml) were labeled overnight with 0.5 μCi/ml [*methyl*-<sup>3</sup>H]thymidine (0.5 Ci/mmol) and 0.1 μCi/ml [<sup>14</sup>C]leucine (318 mCi/mmol) in Iscove's media containing 15% FBS. Cells were then pelleted by centrifugation, resuspended in fresh DMEM/7.5% calf serum, and incubated for 1 h at 37°C. Cells were pelleted and resuspended in buffer A at 37°C at a final density of  $1.0 \times 10^6$  cells/ml. Cells were then incubated with etoposide (50 μM) for 30 min in the presence or absence of ThioGlo-1 (0.1–10 μM). Cells ( $1.0 \times 10^6$ ) were then pelleted (10 min at  $1700 \times g$ ) after being plunged into

10 ml of ice-cold 10 mM PBS, washed with ice-cold buffer A, pelleted, and lysed. Cellular DNA was sheared, and protein-DNA complexes were precipitated with SDS and KCl as described previously (21). topo II-DNA complexes were quantified by scintillation counting (Beckman LS 6500 multipurpose scintillation counter), and [ $^3\text{H}$ ]DNA was normalized to cell number using coprecipitated [ $^{14}\text{C}$ ]-labeled protein as an internal control.

**Statistical Analysis.** The results are presented as the mean  $\pm$  SE values from at least three experiments, and statistical analysis was performed by either paired/unpaired Student's *t* test or one-way ANOVA. The statistical significance of differences was set at  $P < 0.05$ .

## RESULTS

To determine the anti-/pro-oxidant potential of etoposide, we studied its effects during oxidation of two different types of intracellular biomolecules: (a) membrane phospholipids; and (b) thiols (GSH and proteins SHs). We used MPO-rich HL-60 cells to take advantage of MPO-catalyzed one-electron oxidation of etoposide to its phenoxyl radical, etoposide-O $\cdot$ , and to study interactions of etoposide-O $\cdot$  with phospholipids and thiols in HL-60 cells.

**Effects of Etoposide on Phospholipid Peroxidation.** Using our sensitive and specific procedure to quantitate peroxidation of different classes of membrane phospholipids in intact cells (19), we determined whether etoposide, as a hindered phenolic compound, could act as a radical scavenger. In addition, this technique allowed us to determine whether etoposide-O $\cdot$  radicals were reactive enough to cause direct oxidation of intracellular phospholipids. We prepared HL-60 cells in which the major classes of membrane phospholipids were metabolically labeled with the oxidation-sensitive fluorescent fatty acid PnA (19), and then we exposed cells to etoposide,  $\text{H}_2\text{O}_2$ , or a combination of etoposide plus  $\text{H}_2\text{O}_2$ .

Our data on oxidation of two major phospholipids in HL-60 cells, PC and PE, are shown in Table 1. We found that etoposide alone caused no oxidation of either PC or PE. Consistent with our previous observations (22),  $\text{H}_2\text{O}_2$  produced significant oxidative damage to both PC and PE. When intact cells were exposed to  $\text{H}_2\text{O}_2$  in the presence of etoposide, there was significant protection against PC and PE oxidation, suggesting etoposide-mediated antioxidant effects. We found that neither etoposide nor  $\text{H}_2\text{O}_2$  alone or in combination caused any significant cytotoxicity (trypan blue-positive cells were  $<5\%$ ) during the same 2-h incubation time used in phospholipid peroxidation experiments. Thus, etoposide-induced loss of cell viability is not responsible for the antioxidant effects of etoposide.

In the absence of etoposide,  $\text{H}_2\text{O}_2$ -induced oxidative stress in HL-60 cells has been associated with MPO-catalyzed formation of reactive intermediates such as oxoferryl-derived radical species (23). In this case, etoposide might interfere with the  $\text{H}_2\text{O}_2$ -induced production of these reactive intermediates and prevent oxidative stress by

acting as a MPO substrate (see below). Hence, the "antioxidant" effect of etoposide need not be due to a radical scavenging effect of this phenolic drug. To determine whether etoposide can act as a radical scavenger of MPO-independent  $\text{H}_2\text{O}_2$ -induced oxidative stress in phospholipids, we repeated experiments in HL-60 cells pretreated with an inhibitor of heme synthesis, SA (24). We found that a 48-h incubation of HL-60 cells in the presence of 500  $\mu\text{M}$  SA resulted in a decrease of MPO activity from  $27.8 \pm 1.8$  to  $7.2 \pm 0.8$  nmol tetraguaiacol/min/ $10^6$  cells (mean  $\pm$  SE;  $P < 0.01$ ;  $n = 6$ ). Under these conditions, etoposide was equally effective in protecting PC and PE against  $\text{H}_2\text{O}_2$ -induced oxidation in SA-pretreated and non-SA-pretreated HL-60 cells. In the absence of  $\text{H}_2\text{O}_2$ , etoposide did not cause peroxidation of PC or PE in SA-pretreated cells.  $\text{H}_2\text{O}_2$  produced essentially the same level of phospholipid peroxidation as seen in non-SA-treated cells (Table 1).

Analysis of other minor phospholipid classes (phosphatidylserine, phosphatidylinositol, and sphingomyelin) demonstrated that none were oxidized by etoposide in SA-pretreated or non-SA-pretreated HL-60 cells. On the contrary, etoposide protected these phospholipids against  $\text{H}_2\text{O}_2$ -induced oxidation in both naive and SA-pretreated cells (data not shown). Together, these results indicate that etoposide exerted a pronounced antioxidant effect against  $\text{H}_2\text{O}_2$ -induced phospholipid peroxidation in HL-60 cells. In addition, results show that MPO-catalyzed etoposide-O $\cdot$  radicals do not oxidize membrane phospholipids in HL-60 cells.

**Detection of Etoposide-O $\cdot$  Radicals in Intact HL-60 Cells.** Our previous work has established that in the presence of  $\text{H}_2\text{O}_2$ , MPO in HL-60 cell homogenates catalyzed one-electron oxidation of etoposide, yielding etoposide-O $\cdot$  radicals that were directly detectable by EPR after depletion of thiols (9). We sought to determine whether intact cells were able to metabolize etoposide via the same pathway and whether intracellular thiols were involved in further reactions involving these radical species. We used EPR spectroscopy to monitor the production of etoposide-O $\cdot$  radicals in HL-60 cells. We found that typical EPR spectra of etoposide-O $\cdot$  radicals could be directly recorded from the cells in the presence of etoposide and  $\text{H}_2\text{O}_2$  (Fig. 1A). Importantly, the signal could be recorded only after a lag period that lasted for 16 min under the conditions used. The presence of the MPO cosubstrate,  $\text{H}_2\text{O}_2$ , was essential because no signals were detectable in the absence of the catalase inhibitor, 3-AT.

Because our previous studies demonstrated that etoposide-O $\cdot$  radicals are reactive toward intracellular reductants such as ascorbate and thiols (25), MPO-catalyzed production may be masked due to effective reduction of phenoxyl radicals as shown in Reaction 2 (see "Introduction"). The cell culture medium used did not contain ascorbate; therefore, etoposide-O $\cdot$  will not be reduced by this mechanism. Thus, thiols are expected to act as the primary intracellular reductants for etoposide-O $\cdot$  radicals. Indeed, we found that addition of ThioGlo-1, a maleimide thiol reagent capable of titrating out thiols, was essential for rapid observation of intracellular etoposide-O $\cdot$  (Fig. 1B). In the absence of ThioGlo-1, there was a lag period (Fig. 2B) before typical EPR spectra of etoposide-O $\cdot$  radicals could be observed. Titration out of HL-60 cell thiols with different concentrations of ThioGlo-1 showed that at 1  $\mu\text{M}$ , the cells retained  $\approx 30\%$  of endogenous GSH (Fig. 2A). Increasing ThioGlo-1 concentrations up to 5–10  $\mu\text{M}$  essentially depleted all intracellular GSH. Importantly, at concentrations  $\leq 10$   $\mu\text{M}$ , ThioGlo-1 did not affect the viability of HL-60 cells within 30 min of incubation (cell viability was  $93.3 \pm 0.8\%$  and  $86.1 \pm 3.6\%$  in control samples and after incubation with 10  $\mu\text{M}$  ThioGlo-1, respectively). As ThioGlo-1 concentrations were increased from 0.1–10  $\mu\text{M}$ , the lag period before observation of EPR signals of etoposide-O $\cdot$  radicals was shortened (Fig. 2B), corresponding to the titration out of thiol reductant (Fig. 2A). In fact, at Thio-

Table 1 Effect of etoposide on oxidation of PnA-labeled phospholipids induced by hydrogen peroxide in HL-60 cells (% of control)

PnA-loaded cells were exposed to  $\text{H}_2\text{O}_2$  in the absence and presence of etoposide (50  $\mu\text{M}$ ) for 2 h at  $37^\circ\text{C}$ . Etoposide was added 10 min before the first addition of  $\text{H}_2\text{O}_2$ .  $\text{H}_2\text{O}_2$  (25  $\mu\text{M}$ ) was added every 30 min. All data are the mean  $\pm$  SD. The integration of PnA into PE was estimated as  $116.6 \pm 14.5$  and  $91.3 \pm 7.1$  ng PnA/ $\mu\text{g}$  total lipid phosphorus in control HL-60 cells and HL-60 cells treated with SA, respectively. The integration of PnA into PC was estimated as  $372.9 \pm 24.6$  and  $270.6 \pm 18.9$  ng PnA/ $\mu\text{g}$  total lipid phosphorus in control HL-60 cells and HL-60 cells treated with SA, respectively.

Additions	HL-60 cells		HL-60 cells treated with SA	
	PE	PC	PE	PC
None	100 $\pm$ 12.4	100 $\pm$ 6.6	100 $\pm$ 7.7	100 $\pm$ 6.9
Etoposide	85.6 $\pm$ 3.3	96.3 $\pm$ 2.7	86.0 $\pm$ 8.2	92.7 $\pm$ 7.6
$\text{H}_2\text{O}_2$	23.4 $\pm$ 4.2 <sup>a</sup>	27.9 $\pm$ 3.8 <sup>a</sup>	19.8 $\pm$ 7.1 <sup>a</sup>	27.0 $\pm$ 6.5 <sup>a</sup>
Etoposide + $\text{H}_2\text{O}_2$	62.2 $\pm$ 4.2 <sup>b</sup>	69.1 $\pm$ 6.1 <sup>b</sup>	53.7 $\pm$ 8.4 <sup>b</sup>	61.5 $\pm$ 6.2 <sup>b</sup>

<sup>a</sup>  $P < 0.001$  versus control (none).

<sup>b</sup>  $P < 0.001$  versus  $\text{H}_2\text{O}_2$ .



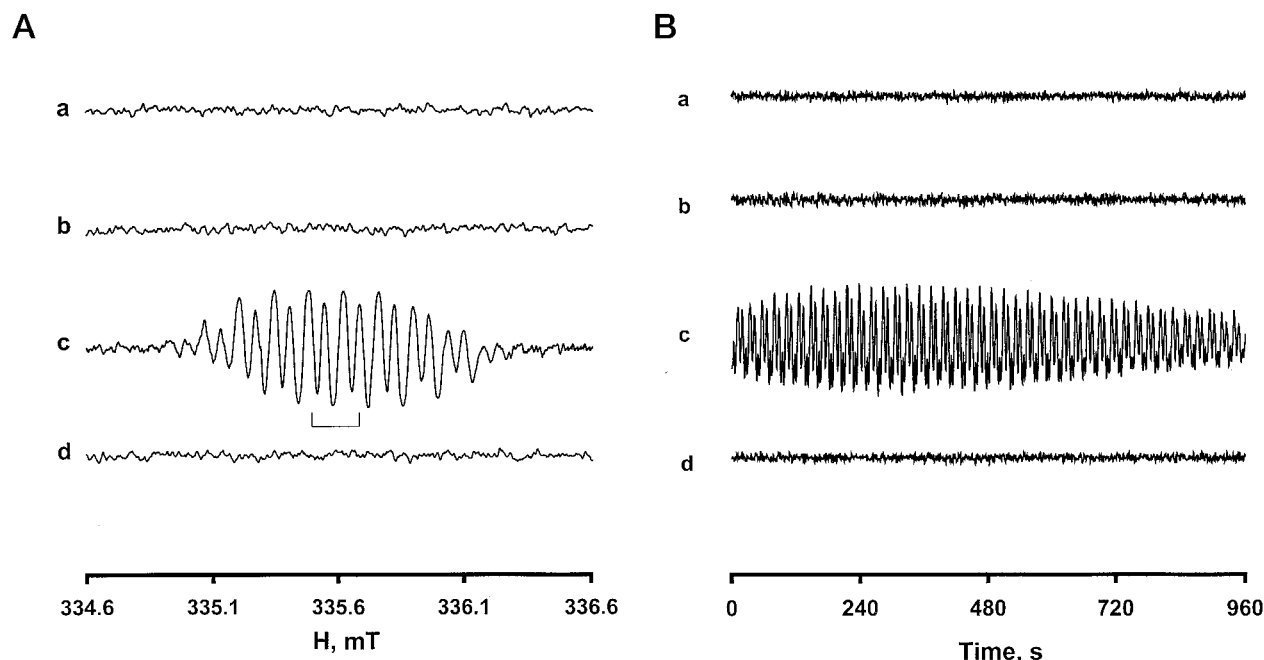


Fig. 1. MPO-catalyzed generation of etoposide phenoxyl radicals in HL-60 cells. *A*, EPR spectra of etoposide phenoxyl radicals generated by MPO in HL-60 cells. *B*, time course of etoposide phenoxyl radical generation by  $\text{H}_2\text{O}_2$ /MPO activity in HL-60 cells. The bar label (*A*, *c*) designates that component of the etoposide phenoxyl radical EPR signal that was used for subsequent repeated scans for recordings of the time course of the radical production shown on *B*. *c*, *a*, cells +  $\text{H}_2\text{O}_2$  (20  $\mu\text{M}$ ) + etoposide (400  $\mu\text{M}$ ); *b*, cells +  $\text{H}_2\text{O}_2$  (20  $\mu\text{M}$ ) + etoposide (400  $\mu\text{M}$ ) + 3-AT (5 mM); *c*, cells treated with ThioGlo-1 (10  $\mu\text{M}$ ) +  $\text{H}_2\text{O}_2$  (20  $\mu\text{M}$ ) + etoposide (400  $\mu\text{M}$ ) + 3-AT (5 mM); *d*, cells treated with ThioGlo-1 (10  $\mu\text{M}$ ) +  $\text{H}_2\text{O}_2$  (20  $\mu\text{M}$ ) + etoposide (400  $\mu\text{M}$ ). HL-60 cells ( $1.5 \times 10^6$  cells/ml) treated or not treated with ThioGlo-1 were incubated in buffer A (pH 7.4) in the presence of etoposide,  $\text{H}_2\text{O}_2$ , and/or 3-AT at 25°C. EPR spectra of etoposide phenoxyl radicals were recorded as described in "Materials and Methods."

Glo-1 concentrations of  $>1 \mu\text{M}$ , the signal was immediately detectable (Fig. 2*B*). Both the magnitude (Fig. 2*B*) and the rate of etoposide- $\text{O}^\bullet$  formation (Fig. 3) were proportional to the concentration of added ThioGlo-1. Repeated scanning of a portion of the etoposide- $\text{O}^\bullet$  EPR spectrum (Fig. 1, *A* and *B*) over time demonstrated that the steady-state concentrations of the radicals were sufficiently high to readily allow its monitoring over 16 min (Fig. 2*B*). The magnitude of the signal was dependent on the concentrations of both etoposide and  $\text{H}_2\text{O}_2$  (Fig. 4), suggesting the involvement of MPO in etoposide- $\text{O}^\bullet$  radical production.

**Formation of Etoposide- $\text{O}^\bullet$  Radical Is MPO Dependent.** We next determined the role that MPO plays in the generation of etoposide phenoxyl radicals. To this end, we used SA, an inhibitor of heme synthesis (24) that is known to decrease MPO activity in HL-60 cells (26). After 48 h of growth in the presence of 50–500  $\mu\text{M}$  SA, HL-60 cell MPO activity decreased to 25% of that seen in control cells (data not shown). We then performed EPR measurements of etoposide- $\text{O}^\bullet$  in control and SA-treated cells incubated with various concentrations of ThioGlo-1 (Fig. 5). As expected, etoposide phenoxyl radical formation increased as ThioGlo-1 concentration increased (Figs. 2*B* and 5). In SA-treated MPO-depleted cells, etoposide- $\text{O}^\bullet$  formation was dramatically decreased even in the presence of ThioGlo-1 concentrations as high as 10  $\mu\text{M}$  (Fig. 5). In separate experiments, we found that short-term (2–6-h) incubation of HL-60 cells with 500  $\mu\text{M}$  SA did not affect either MPO activity or etoposide- $\text{O}^\bullet$  radical generation (data not shown).

**MPO-dependent Metabolism of Etoposide Modulates Its Effects on topo II.** It has been suggested that oxidative activation of etoposide plays a role in its cytotoxicity (12–15). Because cytotoxic and genotoxic effects of etoposide are commonly associated with its poisoning of topo II (27), we next investigated how modulation of MPO activity and intracellular thiols affected etoposide activity. A brief (2-min) preincubation with ThioGlo-1 caused a concentration-dependent biphasic modulation of etoposide activity (Fig. 6). Thio-

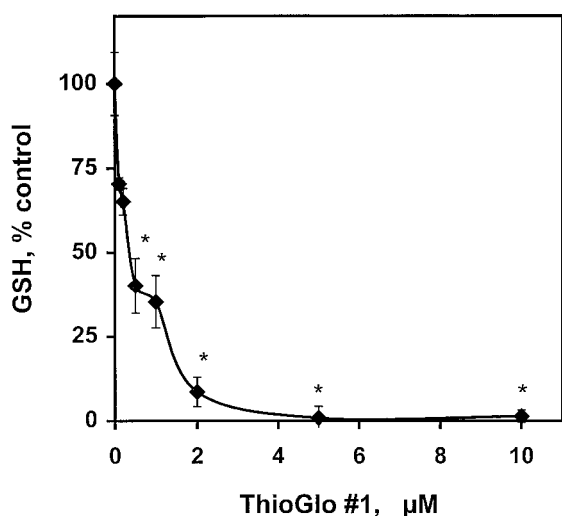
Glo-1 (1  $\mu\text{M}$ ) significantly increased etoposide-induced topo II-DNA covalent complexes ( $>25\%$ ). This result is consistent with previous reports that partial depletion of GSH resulted in increased etoposide-induced DNA damage and cytotoxicity (28). At higher concentrations, ThioGlo-1 progressively inhibited etoposide activity, with essentially complete inhibition observed in the range of 5–10  $\mu\text{M}$  ThioGlo-1.

Furthermore, we found that a portion of cellular etoposide activity was MPO dependent. As shown in Fig. 7, the ThioGlo-1 (1  $\mu\text{M}$ )-mediated enhancement of etoposide-induced topo II-DNA covalent complexes was prevented in cells pretreated for 48 h with 500  $\mu\text{M}$  SA. Similarly, control etoposide activity was reduced by 22% in SA (500  $\mu\text{M}$ )-pretreated HL-60 cells (data not shown).

Together, these observations suggest that etoposide poisoning of topo II in HL-60 cells can be modulated by MPO, likely through the interactions of etoposide- $\text{O}^\bullet$  radicals with intracellular thiols. This conclusion is supported by our experiments in which intracellular GSH conjugates with ThioGlo-1 were visualized in HL-60 cells by fluorescence microscopy. Incubation of HL-60 cells with etoposide in the presence of  $\text{H}_2\text{O}_2$  resulted in decreased fluorescence in cells subsequently treated with ThioGlo-1 (data not shown). This is due to etoposide/ $\text{H}_2\text{O}_2$ -induced oxidation of low molecular weight thiols (GSH; Ref. 9), thus reducing the level of GSH available for ThioGlo-1 binding/fluorescence in HL-60 cells.

We speculate that when concentrations of endogenous GSH are low (either intrinsically or as a result of depletion), MPO-catalyzed etoposide- $\text{O}^\bullet$  formation may allow for direct oxidation of cysteines on topo II that are essential for its function (29, 30). Consistent with this idea, we found that the protein SH groups in HL-60 cells pretreated with 10  $\mu\text{M}$  ThioGlo-1 (a concentration that titrated out all intracellular GSH; Fig. 8*A*) were significantly decreased during a 15-min incubation with etoposide +  $\text{H}_2\text{O}_2$  (Fig. 8*B*). This etoposide/ $\text{H}_2\text{O}_2$ -induced oxidation of protein SH groups was not detectable in cells with substantially higher levels of GSH [*i.e.*, cells not pretreated with

A



B

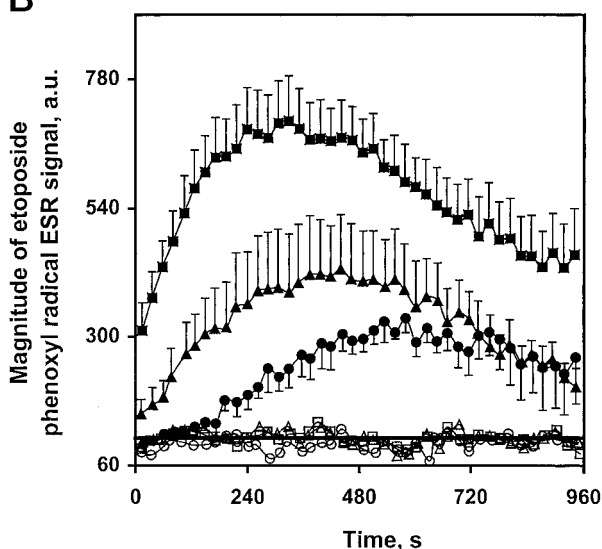


Fig. 2. A, titration of GSH in HL-60 cells by ThioGlo-1. Measurements were performed by reading fluorescence emission from the GSH-ThioGlo-1 adduct as described in "Materials and Methods." HL-60 cells ( $8 \times 10^4$ ) were incubated in buffer A (pH 7.4) in the presence of different concentrations of ThioGlo-1 at 25°C. Data points are presented as the mean  $\pm$  SE from four separate experiments. \*,  $P < 0.05$ , cells treated in the presence versus absence of ThioGlo-1. B, effect of ThioGlo-1 on the formation of etoposide phenoxyl radicals in HL-60 cells. HL-60 cells ( $1.6 \times 10^6$  cells/ml) were pretreated for 10 min with 0.1  $\mu\text{M}$  (● and ○), 1.0  $\mu\text{M}$  (▲ and △), and 10  $\mu\text{M}$  (■ and □) ThioGlo-1, and then the cells were washed free of extracellular drug and incubated in buffer A at 25°C in the presence (closed symbols) or absence (open symbols) of catalase inhibitor 3-AT (5 mM) and etoposide (400  $\mu\text{M}$ ) and  $\text{H}_2\text{O}_2$  (20  $\mu\text{M}$ ). The time course of etoposide phenoxyl radical formation was recorded as described in "Materials and Methods." Data points are presented as the mean  $\pm$  SE from four to six separate experiments. The thick solid line represents the level of the noise signal in the EPR spectra.

ThioGlo-1 or cells pretreated with lower concentrations of ThioGlo-1 (0.1–1.0  $\mu\text{M}$ ; Fig. 8A)].

## DISCUSSION

Etoposide was introduced in 1971 and has since become a widely used anticancer drug. It is frequently used as a first-line drug for treating small cell lung cancer, germ cell tumors, lymphomas, and, more recently, Kaposi's sarcoma associated with AIDS (27). It is also used to treat a variety of leukemias including acute lymphocytic

leukemia. Etoposide is used not only as a single agent but also in combination chemotherapy with other antitumor drugs (7), as well as in concurrent chemoradiotherapy (31).

The cytotoxicity of different antitumor drugs toward neoplastic cells often leads to apoptosis and phagocytosis without induction of an inflammatory response. In contrast, necrotic cells lyse and release their contents into the extracellular space, thus inducing inflammation. Recent results indicate that oxidative stress and antioxidants can modulate chemotherapy-induced cell death pathways (1, 2). Antioxidants may protect tumor cells from apoptosis and phagocytosis,

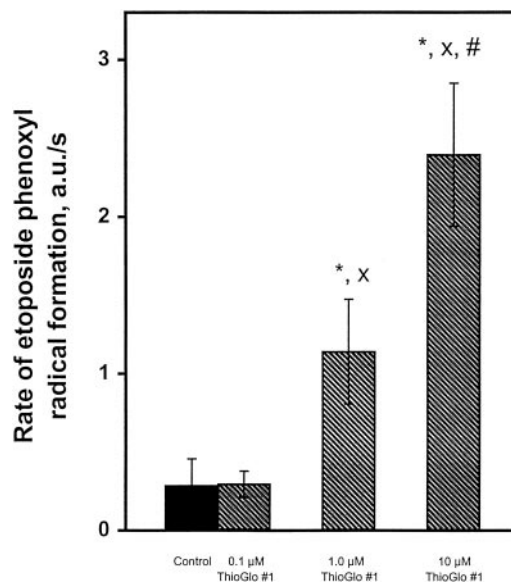


Fig. 3. Effect of ThioGlo-1 on the initial rate of etoposide phenoxyl radical production in HL-60 cells. HL-60 cells were treated with 0.1, 1.0, and 10.0  $\mu\text{M}$  ThioGlo-1 for 10 min at 25°C as described in "Materials and Methods." Time course of etoposide phenoxyl radical formation was recorded as described in "Materials and Methods." Data are the mean  $\pm$  SE;  $n = 4$ . \*,  $P < 0.05$  versus control. X,  $P < 0.05$  versus 0.1  $\mu\text{M}$  ThioGlo-1. #,  $P < 0.05$  versus 1.0  $\mu\text{M}$  ThioGlo-1.

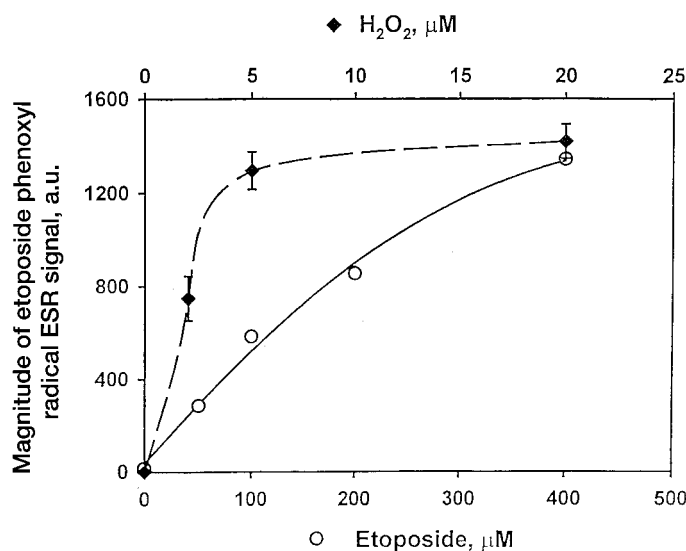


Fig. 4. Dependence of etoposide phenoxyl radical EPR signal on concentration of etoposide and  $\text{H}_2\text{O}_2$ . In experiments with varying concentrations of etoposide, HL-60 cells ( $1.6 \times 10^6$ ) were incubated with etoposide (50, 100, 200, and 400  $\mu\text{M}$ ) and 3-AT (5 mM) in buffer A in the presence of  $\text{H}_2\text{O}_2$  (20  $\mu\text{M}$ ) at 25°C. In experiments with varying concentrations of  $\text{H}_2\text{O}_2$ , HL-60 cells ( $1.6 \times 10^6$  cells/ml) were incubated with etoposide (400  $\mu\text{M}$ ) and 3-AT (5 mM) in buffer A in the presence of different concentrations of  $\text{H}_2\text{O}_2$  (as indicated) at 25°C. EPR spectra of etoposide phenoxyl radicals were recorded as described in "Materials and Methods."

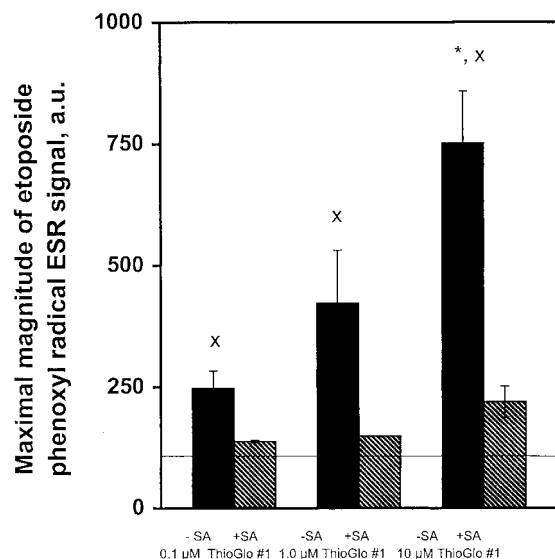


Fig. 5. Effect of SA and ThioGlo-1 on etoposide phenoxyl radical generation in HL-60 cells. HL-60 cells were grown in the presence or absence of SA (500  $\mu$ M, 48 h) and treated with 0.1–10.0  $\mu$ M ThioGlo-1 (10 min at 25°C) as described in “Materials and Methods.” ESR spectra and the time course of etoposide phenoxyl radical generation were measured as described in “Materials and Methods.” Bars represent the mean  $\pm$  SE from five separate experiments, \*,  $P < 0.05$  versus 0.1  $\mu$ M ThioGlo-1 in HL-60 cells without SA (–SA); X,  $P < 0.05$  versus the same ThioGlo-1 concentration in HL-60 cells + SA.

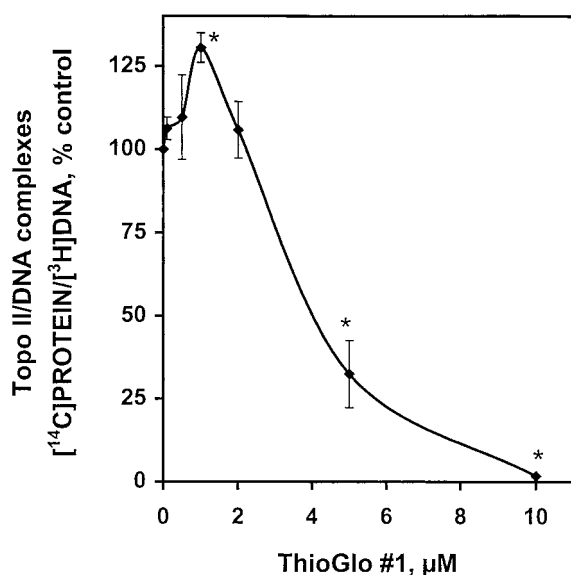


Fig. 6. Effect of ThioGlo-1 on formation of etoposide-induced topo II-DNA covalent complexes in HL-60 cells. Cells were prelabeled with [ $^3$ H]thymidine and [ $^{14}$ C]leucine 24 h before experimentation as described in “Materials and Methods.” Cells were incubated for 2 min in the absence or presence of ThioGlo-1 (0.1–1.0  $\mu$ M), followed by a 30-min incubation at 37°C with etoposide (50  $\mu$ M). SDS-KCl-precipitable topo II-DNA complexes were then isolated and quantified by scintillation counting as described in “Materials and Methods.” Results are presented as a percentage of control topo II-DNA complexes observed in the presence versus absence of ThioGlo-1. Bars represent the mean  $\pm$  SE difference comparing ThioGlo-1-treated versus untreated cells from 4–12 separate experiments. \*, statistically significant difference ( $P < 0.05$ , ThioGlo-1-treated cells versus untreated cells).

whereas pro-oxidants may induce apoptosis, thus affecting the overall effectiveness of antitumor therapy. A significant decline in antioxidant status and elevation of biomarkers of oxidative stress are typical of tissues and plasma of patients in the course of commonly used chemotherapy/radiotherapy protocols. In particular, this relates to treatment regimens that include etoposide (3, 5). Because the toxicity of etoposide and its homologues to tumor cells is mainly dependent on

its ability to induce apoptosis (32–34), its anti-/pro-oxidant effects may modulate apoptotic pathways and hence modulate the overall antitumor activity of the drug.

Over the past 10 years, numerous groups have reported that the same treatment schedules associated with the impressive efficacy of etoposide are also associated with an increased risk of secondary AML (35–37). This has prompted the withdrawal of etoposide from some treatment regimens, potentially compromising efficacy against the original tumor (17). Whereas the causative link between treatment of cancer with etoposide and the development of secondary AML in children and adults has been firmly established, the biochemical mechanisms explaining the extremely high susceptibility of myeloid stem cells to the leukemogenic effects of etoposide have not been elucidated. Recently, we hypothesized that MPO-catalyzed activation of etoposide to a reactive intermediate, etoposide-O $\cdot$ , may trigger oxidative stress and cause cytotoxic and genotoxic effects responsible for leukemogenesis (9). As a direct consequence of this mechanism, we further proposed nutritional antioxidant strategies for preventing etoposide-induced leukemias (9).

Thus, both the clinical efficacy and the leukemogenic action of etoposide may be dependent, at least in part, on the ability of etoposide to modulate the anti-/pro-oxidant status of cells. This prompted us to conduct the present study to investigate both the radical scavenging antioxidant effects of etoposide and its potential pro-oxidant role(s) in MPO-rich HL-60 cells.

Our results demonstrated for the first time that etoposide can act as both an antioxidant and a pro-oxidant toward different intracellular constituents. Using our sensitive and specific assay of phospholipid peroxidation in intact cells (19), we established that etoposide does not cause peroxidation of any of major phospholipids (PC, PE, PI, and PS) in HL-60 cells. In fact, it acts as a potent antioxidant against H $_2$ O $_2$ -induced phospholipid peroxidation. This antioxidant effect of

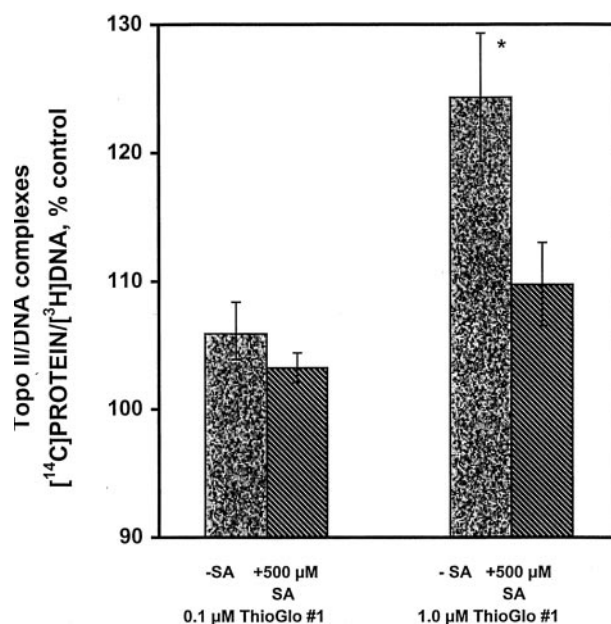


Fig. 7. Effects of SA and ThioGlo-1 on formation of etoposide-induced topo II-DNA covalent complex in HL-60 cells. Cells were prelabeled with [ $^3$ H]thymidine and [ $^{14}$ C]leucine for 24 h and treated in the presence or absence of 500  $\mu$ M SA for 48 h before experimentation. Cells were then incubated for 30 min at 37°C with etoposide (50  $\mu$ M) in the absence or presence of ThioGlo-1 (0.1–1.0  $\mu$ M). SDS-KCl-precipitable topo II-DNA complexes were then isolated and quantified by scintillation counting as described in “Materials and Methods.” Results are presented as a percentage of control topo II-DNA complexes observed in the presence versus absence of ThioGlo-1. Bars represent the mean  $\pm$  SE from four to five separate experiments. \*, statistically significant difference ( $P < 0.05$ ) comparing ThioGlo-1-treated versus untreated cells.

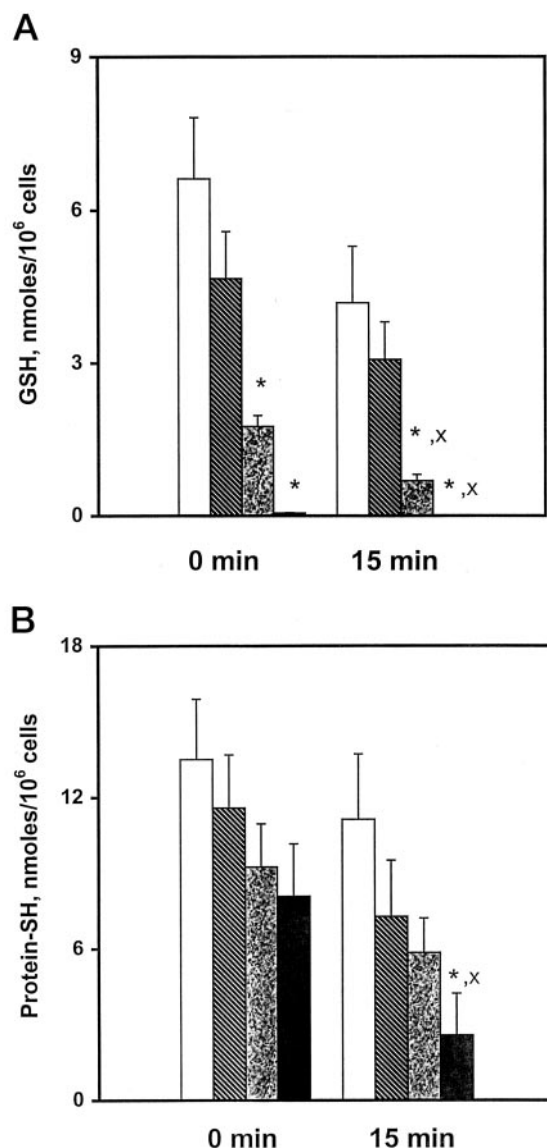


Fig. 8. Effect of etoposide on the content of GSH (A) and protein SHs (B) in HL-60 cells preincubated for 10 min in the absence (□) or presence of 0.1 (▨), 1.0 (speckled bars), or 10  $\mu\text{M}$  (■) ThioGlo-1. HL-60 cells ( $1.6 \times 10^6$ ) were subsequently incubated with  $\text{H}_2\text{O}_2$  (20  $\mu\text{M}$ ), etoposide (400  $\mu\text{M}$ ), and 3-AT (5 mM) in buffer A (pH 7.4) at 25°C. Aliquots of cells were taken at 0 and 15 min, and GSH and protein SHs were determined as described in "Materials and Methods." Bars represent the mean  $\pm$  SE from five separate experiments. \*,  $P < 0.05$  versus 0  $\mu\text{M}$  ThioGlo-1 at the same time point; X,  $P < 0.05$  versus the same ThioGlo-1 concentration at 0 min.

etoposide was completely independent of MPO activity in HL-60 cells. In a recent report, etoposide-induced formation of lipid peroxyl radicals and accumulation of lipid peroxidation products were found in Chinese hamster ovary cells with compromised genomic integrity (10). However, the authors failed to determine whether these pro-oxidant effects of etoposide in lipids were typical of live cells, and they did not establish that the elevated levels of free radical damage to lipids were derived from the fraction of damaged cells dying by apoptotic or necrotic processes. In contrast, our results provide unequivocal direct evidence for the lack of pro-oxidant effects toward lipids and the potent antioxidant effect of etoposide in membrane phospholipids of intact HL-60 cells. Thus, the reactivity of etoposide- $\text{O}^\bullet$  is not sufficient to directly attack intracellular phospholipids and cause their oxidative damage.

Our studies on thiol oxidation, however, established that etoposide can directly oxidize GSH and protein SH groups (after prior depletion

of GSH by pretreatment with ThioGlo-1) in HL-60 cells. This oxidizing activity can be attributed entirely to MPO-catalyzed generation of etoposide- $\text{O}^\bullet$  radicals and their subsequent interactions with thiols. These conclusions are supported by several results observed in the present study. First, our EPR measurements directly detected formation of etoposide- $\text{O}^\bullet$  radicals in HL-60 cells. The EPR signals were observable in cells incubated in the presence of etoposide and  $\text{H}_2\text{O}_2$  only after a lag period, the duration of which was strictly dependent on the intracellular GSH levels. When intracellular GSH was titrated out with ThioGlo-1, a GSH-specific maleimide reagent, the lag period was no longer observed. This suggests that reduction of etoposide- $\text{O}^\bullet$  by GSH was occurring during the lag period. Second, manipulations of MPO activity in HL-60 cells using a heme synthesis inhibitor, SA, demonstrated that the enzyme-catalyzed reaction was absolutely required for both the production of etoposide- $\text{O}^\bullet$  radicals and their interactions with GSH. Finally, etoposide- $\text{O}^\bullet$  radicals were able to attack protein SH groups only after essentially complete depletion of intracellular GSH by ThioGlo-1. Thus, GSH acts as a potent protector of protein SHs against etoposide- $\text{O}^\bullet$  radical oxidation. Our demonstration of etoposide- $\text{O}^\bullet$  radical-induced oxidation of thiols explains previous findings on GSH depletion in cells treated with etoposide (11, 28, 38).

Throughout these experiments, we used several reagents and inhibitors such as 3-AT, SA, and ThioGlo-1 to readily observe etoposide phenoxyl radicals in HL-60 cells. However, in cell-based assays, neither exogenous  $\text{H}_2\text{O}_2$  nor 3-AT was required to observe MPO-dependent activity of etoposide where SA pretreatment alone reduced etoposide activity by 22%. Hence, we have demonstrated that a portion of etoposide activity is correlated with both MPO activity and the production of etoposide phenoxyl radicals. Overall, our results indicate that formation of etoposide phenoxyl radicals occurs in MPO-containing cells and that there are potentially important biological consequences related to etoposide activation.

We have previously put forth the idea (9) that etoposide phenoxyl radicals can result in oxidative stress, causing both genotoxicity and cytotoxicity. Because etoposide phenoxyl radicals can be produced in cells that contain oxidizing enzymes capable of one-electron transfer reactions such as peroxidases and tyrosinase, these metabolites may contribute to (a) the antitumor activity of etoposide in malignant cells capable of producing these phenoxy radicals and (b) the carcinogenic action of this agent in myeloid stem cells that contain MPO.

It is tempting to speculate that oxidation of essential cysteines on topo II by etoposide- $\text{O}^\bullet$  radicals is responsible for both the enhancement and inhibition of etoposide activity, depending on the extent of oxidation. Indeed, we observed a significant oxidation of protein SH groups in HL-60 cells in which GSH was completely depleted with ThioGlo-1. Interestingly, topo II expression is sensitive to thiol-mediated redox regulation at the posttranscriptional level by modulation of the interaction of the 3'-untranslated region of topo II mRNA with redox-sensitive protein complexes (39). However, this important regulatory mechanism is likely to differ from the mechanism(s) involving modulation of etoposide activity after a decrease in GSH levels induced by treatment with ThioGlo-1. Additional studies are necessary to establish whether this oxidative modification of protein cysteines does in fact include those on topo II and whether cysteines are oxidized to any significant extent in the presence of physiologically relevant levels of GSH. It is obvious, however, that modulation of the intracellular redox status by etoposide metabolism can affect drug-induced activity at the level of topo II because moderately decreased levels of GSH correlated with potentiation of etoposide-induced topo II-DNA complexes only when MPO-catalyzed metabolism to etoposide- $\text{O}^\bullet$  radicals was sufficiently high.

In conclusion, etoposide can act as both an antioxidant toward



membrane phospholipids and a pro-oxidant toward intracellular thiols in cells. This redox activity of etoposide may contribute to its antitumor effectiveness and to its genotoxic and carcinogenic potential. Understanding the intracellular redox biochemistry of etoposide may be instrumental for the development of nutritional antioxidant strategies for safer and more efficacious clinical use of etoposide.

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