Ethacrynic Acid Butyl-Ester Induces Apoptosis in Leukemia Cells through a Hydrogen Peroxide–Mediated Pathway Independent of Glutathione S-Transferase P1-1 Inhibition

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Abstract

Ethacrynic acid (EA), a glutathione S-transferase inhibitor and diuretic agent, inhibits cell growth and induces apoptosis in cancer cells. To improve the activities, the structure of EA has been modified, and it has been shown that EA esters had an increased cell growth inhibitory ability compared with nonesterified analogue. EA butyl-ester (EABE) was synthesized, and its apoptosis induction ability was studied. The efficacy of EABE was compared with that of EA, and the mechanisms of action were studied in HL-60 leukemia cells. EABE exhibited greater cell growth inhibitory and apoptosis induction abilities than did EA. EABE-induced apoptosis in HL-60 cells correlated with increased levels of reactive oxygen species, the death receptor 5 (DR5), and caspase activation and decreased levels of the mitochondrial membrane potential. Pretreatment with antioxidants, either N-acetylcysteine or catalase, completely blocked EABE-induced apoptosis, H2O2 accumulation, and up-regulation of DR5 levels. RG19, a subclone of Raji cells stably transfected with a GSTπ expression vector, and K562 cells with high endogenous GSTP1-1 activity were less sensitive to EABE-induced apoptosis. EABE was more rapidly taken up than EA by HL-60 cells as determined by high-performance liquid chromatography (HPLC) measurements of intracellular concentrations. These results suggest that (a) H2O2 production is a mediator of EABE and EA-induced apoptosis; (b) GSTP1-1 plays a negative role in EABE and EA-induced apoptosis; and (c) the activity of EABE is greater than EA due to its more rapid entry into cells. [Cancer Res 2007;67(16):7856–64]

Introduction

Ethacrynic acid (EA), a diuretic drug, has been found to be a glutathione S-transferase (GST) inhibitor (1–5). Among GSTs, GSTP1-1 catalyzes the conjugation of reduced glutathione with a wide range of substrates, including chemotherapeutic agents, and acts as a detoxification enzyme (6). GSTP1-1 is overexpressed in a variety of cancer cells, and its levels are further increased in chemotherapeutic-resistant cancer cells (7–9). GSTP1-1 also has been found to play a regulatory role in the mitogen-activated protein kinase (MAPK) pathway and to participate in cellular survival and death signals via protein-protein interactions with c-jun-NH2-kinase 1 (JNK1) and apoptosis signal-regulating kinase 1 (6, 10, 11).

Therefore, GSTP1-1 inhibitors could have therapeutic potential in cancer by reversing drug resistance, by sensitizing chemotherapeutic agents, and/or by inducing cancer cell death directly. EA forms a conjugate with reduced glutathione (GSH) spontaneously and/or due to GST activity; however, the conjugate of EA with GSH exhibits a greater inhibitory effect on GST activity than EA (12–14). EA has been shown to potentiate the cytotoxic effect of chemotherapeutic agents such as cisplatin, chlorambucil, melphalan, mitomycin C, and Adriamycin in vitro (15–17) and has been put into a phase I clinical trial in combination with thiopeta for advanced cancer treatment (18).

EA inhibits cell growth and induces apoptosis in tumor cells at high concentrations by an unknown mechanism (19, 20). Several studies have shown that the depletion of GSH by EA results in an oxidant stress that causes cell death (21, 22). In addition, it has been suggested that activation of the MAPK pathway may also participate in the cell death process induced by EA (19). The structure of EA has been modified, and we found that EA esters retained GSTP1-1 inhibition ability and also had an increased ability to inhibit leukemia cell growth (23). In this communication, we report that EA butyl-ester (EABE) inhibits cell growth and induces apoptosis more potently than EA. EABE induces apoptosis via a reactive oxygen species (ROS)–dependent pathway following the depletion of GSH, decreases of the mitochondrial membrane potential (MMP), and up-regulation of the death receptor 5 (DR5). The greater activity of EABE than EA was found to be due, at least in part, to faster uptake of EABE based on measurements of the intracellular concentrations of EABE and EA.

Materials and Methods

Reagents. EA, N-acetylcysteine (NAC), ethidium bromide (EB), acridine orange (AO), buthionine sulfoximine (BSO), catalase, and hydrogen peroxide were obtained from Sigma Chemical Co. Rhodamine-123 (Rh123) and 5,6-carboxy-2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) were obtained from Molecular Probes. Antibodies to poly-(ADP-ribose)-polymerase (PARP) were obtained from Boehringer Mannheim, to caspase-3 and caspase-8 from BD Biosciences, to DR5 from Alexis Biochemicals, to Bid and β-actin from Santa Cruz Biotechnology, Inc., to Bcl-2 from Roche Diagnostics Corp., and to GSTP1-1 from Calbiochem-Novabiochem Corp.

The synthesis of EABE and EA glutathione conjugate. EABE, [2, 3-dichlorine-4(2-methylene-1-oxo-butyl) phenoxyl] butyl acetate, was synthesized. Briefly, EA (0.65 g, 2.1 mmol) and para-toluensulfonic acid (0.08 g, 0.47 mmol) were dissolved in toluene (10 mL) and n-butanol (0.6 mL, 6.6 mmol). The mixture was heated to 40°C for 5 h. After cooling to room temperature, the mixture was diluted with ether (10 mL) and washed with 5% sodium bicarbonate solution (10 mL × 2) and then with distilled water (10 mL). The ether layer was collected and dried with MgSO4. After filtering, the ether was evaporated in a rotary evaporator at about 60°C, and the target product (colorless oil, 0.71 g, 2.0 mmol, 95.2%) was obtained.

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chemical structure of EABE was confirmed by IR spectroscopy, proton nuclear magnetic resonance spectroscopy, and mass spectroscopy. EA glutathione conjugate (EA-SG) was prepared by Michael addition of the α,β-unsaturated ketone moiety of EA to the cysteinyl-thiol of glutathione in a slightly basic water/ethanol mixture as reported (24).

Cell lines. Human leukemia HL-60 and K562 cells were cultured in RPMI 1640. The media were supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mmol/L L-glutamine, and 10% (v/v) heat-inactivated fetal bovine serum (FBS). BV5 and BG19 cells were subclones of human lymphoma Raji cells which were transfected with a pcDNA3.1 vector and pcDNA3/ GSTP1-cDNA vector, respectively (25). BV5 and BG19 cells were cultured in RPMI 1640 modified to contain 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% (v/v) heat-inactivated FBS.

Cell growth inhibition. Cells were seeded at 1.0 × 10⁵ cells/mL and incubated with various concentrations of EA or EABE for 3 days. The total cell number in each group was determined with the aid of a hemocytometer, and cell viability was estimated by trypan blue exclusion. The drug concentration that inhibited half of the cell-growth (IG₅₀) was calculated.

Quantitation of apoptotic cells. Levels of apoptotic cells were determined by morphologic observation and annexin V assay. For morphologic observation, cells were stained with AO and EB and assessed by fluorescence microscopy as described previously (26). Briefly, 1 μL of a stock solution containing 100 μg/mL of each of AO and EB was added to 25 μL of cell suspension. EB-negative cells with nuclear shrinkage, blebbing, and apoptotic bodies were counted as apoptotic. The percentage of apoptotic cells was calculated after observing a total of 300 cells. Annexin V assay was done according to the manufacturer’s instructions in the annexin V–FITC Apoptosis Detection Kit (BD Biosciences). In general, 10⁶ cells were apoptotic cells were calculated after observing a total of 300 cells. Annexin V fluorescence is displayed on the x-axis. Data were analyzed using CELLQuest (Becton Dickinson) software. For each analysis, 10,000 events were recorded (25).

Determination of H₂O₂ production. Intracellular H₂O₂ production was monitored by flow cytometry using DCFH-DA (27). In the present study, cells in logarithmic growth (1 × 10⁵ cells/mL) were labeled with 5 μmol/L DCFH-DA for 1 h and then treated with or without EABE and EA at 37°C for the indicated periods of time. After washing with PBS, cells were analyzed by FACSscan with excitation and emission wavelengths of 495 and 525 nm, respectively. Cells stimulated with 100 μmol/L H₂O₂ for 1 h were used as a positive control.

Measurement of MMP. MMP was assayed by the retention of Rh123, a membrane-permeable fluorescent cationic dye. The uptake of Rh123 by mitochondria is proportional to the MMP (28). Briefly, cells (1 × 10⁶) were washed twice with PBS and then exposed to annexin V–FITC and propidium iodide (PI) in binding buffer for 15 min in the dark at room temperature. The analysis was done using a FACSscan (Becton Dickinson) emitting an excitation laser light at 488 nm. Fluorescence signals were detected at 518 and 620 nm for FITC and PI detection, respectively. The log of annexin V–FITC fluorescence is displayed on the x-axis, and the log of PI fluorescence is displayed on the y-axis. Data were analyzed using CELLQuest (Becton Dickinson) software. For each analysis, 10,000 events were recorded (25).

Measurement of intracellular glutathione (GSH) content. The levels of intracellular GSH were measured by a monochlorobimane (mBChI) fluorometric method in which mBChI was used as a sensitive and specific probe to analyze GSH in intact cells (30). Briefly, 3 × 10⁶ cells were washed once, resuspended in 1 mL PBS containing 100 μmol/L mBChI, and maintained at 37°C in the dark for 30 min before analysis. The formation of the fluorescent adduct (Gs-mBChI) was monitored with a Fluorescence spectrophotometer (Hitachi 650-60, Japan) using excitation and emission wavelengths of 395 and 482 nm, respectively. The GSH content was calculated as nanomoles per 10⁶ cells based on a GSH standard curve.

GSTP1-1 activity assay. GSTP1-1 activity was determined spectrophotometrically at 25°C using 1-chloro-2,4-dinitrobenzene (CDNB) and GSH as substrates (27). The linear increase in absorption at 340 nm due to the conjugation of GSH (1 mmol/L) with CDNB (1 mmol/L) in HL-60 cell lysates with or without the presence of EABE (40 μmol/L) was determined. An extinction coefficient of 9.6 (mmol/L)⁻¹ cm⁻¹ was used to calculate GSTP1-1 activity and expressed as nanomoles of product per minute per milligram of protein. The activity inhibition rate was calculated as (Ve – Vt)/Ve × 100%. Ve represents GSTP1-1 activity of the control group; and Vt represents GSTP1-1 activity of the treated group.

Quantitation of intracellular EABE and EA content by high-performance liquid chromatography. HL-60 cells (10⁵ cells/mL, 100 mL) were incubated with 300 μmol/L of EA and EABE at 37°C for the indicated periods of time, and more than 95% of cells were viable as determined by trypan-blue exclusion assay. The cells were washed twice with cold PBS, resuspended in 300 μL of cold PBS, sonicated for 10 s on ice, and then centrifuged at 14,000 rpm at 4°C for 15 min. The protein levels of the supernatant fluids were assessed by Bradford protein binding assay. Trichloroacetic acid (TCA) was added to the supernatant fluid fraction at a final concentration of 20% (v/v) to precipitate the proteins in the cell lysates. The mixtures were centrifuged at 14,000 rpm at 4°C for 15 min, and the supernatant fractions and the internal standard (diclofenac) were injected into a chromatographic column. The detection limits of EA and EABE in blank biological samples were 0.45 and 1.90 μg/mL, respectively. The recovery rate was ~55%.

The chromatographic system consisted of a LC-10AT vp pump (Shimadzu Corp.) with a LC-10AT vp variable-wavelength UV absorbance detector set at 280 nm. Analysis was done on a Diamonsil C18 reversed-phase column (200 × 4.6 mm internal diameter; 5 μmol/L; Dikma Technologies), operated at 40°C. Diclofenac was used as an internal standard for the high-performance liquid chromatography (HPLC) assay. EA, EABE, and diclofenac have retention times of 6.55, 20.80, and 8.67 min, respectively. Elution was done with a mobile phase [methanol/phosphoric acid (0.2%)/acetonitrile = 40:25:35, v/v/v] at a flow rate of 0.9 mL/min. The sample (10 μL) was injected, and triplicate measurements were done for each time point. Solutions containing 3.0 to 960 μg/mL of EABE, 0.94 to 30.0 μg/mL of EA, or 3.75 μg/mL of diclofenac prepared in PBS were used as standards. Calibration curves were constructed by plotting chromatographic peak ratios of standard area/standard IS area versus concentration of the standard using linear regression. The calibration curves of test drugs were linear over the concentration ranges used, and correlation coefficients (r²) were >0.99.

Statistics. Data were analyzed for statistical significance using the Student’s t test (Microsoft Excel, Microsoft Corporation). Differences were considered significant at P values <0.05.

Results

EABE is a more potent inhibitor of cell growth and inducer of cell death than EA in HL-60 cells. The cytotoxic and cell growth inhibitory effects of EA and EABE in HL-60 cells were investigated. After 3 days of treatment, the IG₅₀ of EA and EABE in HL-60 cells were 40.14 and 5.38 μmol/L, respectively (Fig. 1A). About 60 μmol/L of EA did not affect cell viability, whereas 12 μmol/L EABE decreased viable cells to 15% (Fig. 1A).


Apopotosis Induced by Ethacrynic Acid Butyl-Ester

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To determine whether cytotoxicity-induced by EABE and EA was due to apoptosis, apoptotic cells were determined based on morphologic observations after staining with AO and EB. EABE induced apoptosis in HL-60 cells at a concentration of 6 μmol/L in a time as short as 6 h. About 68% of HL-60 cells underwent apoptosis after treatment with 12 μmol/L EABE for 24 h (Fig. 1B). However, to reach a similar apoptotic effect, EA was required to be at concentrations higher than 100 μmol/L. The apoptotic effect of EABE was confirmed using fluorescence-activated cell sorting (FACS) after staining with annexin V (Fig. 1C). These data suggest that both EABE and EA induce cytotoxicity through apoptosis induction.

Apoptosis induction by EABE and EA correlates with an increase of cellular H₂O₂ and a decrease in MMP in HL-60 cells. ROS accumulation has been proposed to be involved in EA-induced cell death (19, 31, 32). H₂O₂ levels were determined in HL-60 cells after EABE or EA treatment using a peroxide-sensitive fluorescent probe, DCFH-DA. Intracellular H₂O₂ levels were significantly increased after EABE treatment for only 1 h (Fig. 2A). The mean of the oxidized DCF peak, which reflects the H₂O₂ level, shifted from a position 9.6 to 97.0 after treatment with 12 μmol/L EABE for 6 h. As the concentration of EABE was increased from 3 to 12 μmol/L, more H₂O₂ was detected (data not shown). However, although EA treatment increased cellular H₂O₂ levels, it only did so at (high) concentrations that induced apoptosis (Fig. 2A). These data suggest that H₂O₂ production is the cause of EABE- and EA-induced apoptosis in HL-60 cells.

The impairment of mitochondrial function has been considered to be a key event in the ROS-mediated apoptotic pathway (27, 33). The MMP in HL-60 cells after EABE or EA treatment were measured using flow cytometry after staining with the cationic dye Rh123. A significant decrease of MMP was observed in HL-60 cells after treatment with 12 μmol/L EABE for 6 h. EA treatment also induced a significant decrease of MMP, but at a higher concentration (120 μmol/L; Fig. 2B). MMP was not influenced by EA at lower concentrations or during treatments for a short time, which were not sufficient to induce H₂O₂ accumulation and apoptosis (data not shown). These data suggest that EABA and EA
may induce apoptosis through a mitochondria-mediated pathway due to H$_2$O$_2$ production.

**EABE-induced apoptosis can be inhibited by the antioxidants of NAC and catalase and is caspase dependent.** To determine whether intracellular H$_2$O$_2$ production is the mediator of EABE-induced apoptosis, two antioxidants, NAC and catalase, were used. Pretreatment with either NAC or catalase effectively prevented apoptosis induction by EABE in HL-60 cells (Fig. 3A). Correlated with apoptosis inhibition, both the EABE-induced H$_2$O$_2$ accumulation and the decrease in MMP were blocked by NAC or catalase (Fig. 3B). Neither NAC nor catalase alone regulated apoptosis, H$_2$O$_2$ production, or MMP in HL-60 cells at the concentrations and treatment times tested (data not shown).

Caspase activation plays a pivotal role in the mitochondria-mediated apoptotic pathway (27, 34). EABE induced a decrease in the level of the procaspase-3 precursor in a dose-dependent manner in HL-60 cells (Fig. 3C). Interestingly, a dose-dependent decrease of the procaspase-8 precursor with the production of the 23-kDa cleavage fragment was observed after EABE treatment (Fig. 3C). EABE treatment also decreased Bid (a substrate of activated caspase-8) levels and increased DR5 levels (Fig. 3C). PARP cleavage, caspase-8 activation, and DR5 up-regulation were all blocked by pretreatment with NAC or catalase. These data suggest that a death receptor-mediated pathway is involved in EABE-induced apoptosis, which follows increased H$_2$O$_2$ production.

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**Figure 2.** EABE and EA induce H$_2$O$_2$ accumulation and decrease MMP in HL-60 cells. A, H$_2$O$_2$ accumulation. HL-60 cells were labeled with 5 μmol/L DCFH-DA fluorescent probe for 1 h and then treated with or without 12 μmol/L EABE or 120 μmol/L EA for the indicated times. The addition of 100 μmol/L H$_2$O$_2$ for 1 h was used as a positive control for H$_2$O$_2$ levels. Oxidized DCF was analyzed using FACScan. Open peaks, untreated cells; shaded peaks, treated cells. The peak shift to the right indicates an increase in H$_2$O$_2$ level. B, MMP. HL-60 cells were treated with 12 μmol/L EABE or 120 μmol/L EA for the indicated times. Disruption of MMP was determined according to changes of fluorescence density upon rhodamine 123 loading as described in Materials and Methods. Open peaks, untreated cells; shaded peaks, treated cells. The peak shift to the left indicates a loss of MMP.
EABE decreases intracellular GSH content. EA can react with –SH residues of GSH and many proteins (35). The intracellular levels of GSH were determined in HL-60 cells after treatment with various concentrations of EABE. There was no significant alteration of GSH content in HL-60 cells treated with 3 or 6 μmol/L EABE for 2 h (Fig. 4A). However, intracellular levels of GSH were significantly decreased following treatment with 9, 12, or 15 μmol/L EABE for 2 h (Fig. 4A). EABE decreased GSH levels after only 5 min of treatment with 12 μmol/L EABE (Fig. 4A).

To further show whether GSH is involved in EABE-induced apoptosis, BSO, a GSH-depleting agent, was used. BSO treatment led to a 40% decrease in GSH content. BSO pretreatment with subsequent EABE exposure resulted in an even greater decrease of GSH levels than those of cells treated with each agent alone. Pretreatment with NAC prevented the decrease of GSH levels due to EABE treatment (Fig. 4B). NAC protected against EABE-induced apoptosis in HL-60 cells, whereas BSO augmented it (Fig. 4C and D). Similarly, EA decreased intracellular GSH levels at concentrations that induced apoptosis (data not shown). These results suggested that the EA- or EABE-induced decrease in intracellular GSH levels may be due to the formation of GSH conjugates. To determine whether the conjugates were active, the conjugate of EA with GSH (EA-SG) was synthesized. Unlike EA, EA-SG did not induce apoptosis at the same concentrations with which EA induced apoptosis (data not shown).

Cells with high levels of GSTP1-1 activity are less sensitive to EABE-induced apoptosis. The inhibitory effect of EABE on GSTP1-1 activity was investigated in cell lysates directly treated with EABE. The activity of GSTP1-1 was inhibited by 34% and 75% after incubation with 2.5 and 10 μmol/L EABE for 30 min, respectively, in a cell-free assay (data not shown). However, GSTP1-1 activity was slightly increased in HL-60 cells treated with EABE at concentrations of 6 to 12 μmol/L for 24 h (Fig. 4A).
To further determine the role of GSTP1-1 in apoptosis induction by EABE, RG19 cells, a subclone of Raji cells transfected with a GSTP1 expression vector which have high levels of GSTP1-1 protein (Fig. 5C) and activity (Fig. 5D), were used to compare the apoptotic effect of EABE with that of RV5, a subclone of Raji transfected with an empty vector. EABE at a concentration of 9 μmol/L induced apoptosis in 63% of RV5, but only induced apoptosis in 24% of RG19 cells after 12 h of treatment (Fig. 5B). K562 cells have higher levels of GSTP1-1 protein (Fig. 5C) and activity (Fig. 5D) than HL-60 cells and were less sensitive than HL-60 cells to EABE-induced apoptosis. EABE at a concentration of 9 μmol/L induced PARP cleavage in HL-60 cells, but not in K562 cells (Fig. 5C). These data suggest that GSTP1-1 may protect against EABE action.

**EABE is taken up faster than EA in HL-60 cells.** The uptake rates of EA and EABE were measured in HL-60 cells using HPLC. A HPLC method using diclofenac as an IS was established to measure EA and EABE uptake in HL-60 cells. EA and EABE (300 μmol/L) were incubated with HL-60 cells for indicated times, and their intracellular levels were determined (Table 1). EA accumulated in HL-60 cells in a time-dependent manner, but its uptake rate was slower than that of EABE (Table 1). Intracellular EA was not detectable at a 30-min incubation, whereas 21.88 nmol/mg protein of EABE was detected at that time. Interestingly, EA was also detected in HL-60 cells after incubation with EABE. The amount of EA increased, and the amount of EABE decreased after longer incubation times with EABE treatment. These data suggest that EABE is not stable in cells and is hydrolyzed to EA. However, the total amount of intracellular EABE (including EABE and its potential metabolite, EA) was about 10-fold higher than that of EA after 60 min when initial concentrations of each were equal (Table 1).

**Discussion**

Although EA has been found to be a potent GSTP1-1 inhibitor in vitro, it inhibits tumor cell growth only at high concentrations (19, 20, 36). To improve its antitumor activity, a group of EA derivatives was synthesized, and some of them have shown increased antiproliferative activity as well as GSTP1-1 inhibition ability (23). Esterification of a compound makes it more lipophilic and increases its cellular uptake. EABE is more rapidly taken up and is a more potent apoptosis inducer than EA in HL-60 cells (Fig. 1A and B, Table 1). Thus, the greater apoptotic effect of EABE is probably due to its higher intracellular concentrations (Table 1).
Because both EABE and EA increase intracellular \( \text{H}_2\text{O}_2 \), decrease MMP, and deplete GSH (Figs. 2–4, and data not shown), EA and EABE probably induce apoptosis by the same or similar mechanisms.

EABE- and EA-induced apoptosis correlates with \( \text{H}_2\text{O}_2 \) production, a decrease of MMP, and caspase activation (Figs. 1–3). Mitochondria have been shown to play a critical role in ROS-mediated apoptosis (37–40). Because EABE-induced \( \text{H}_2\text{O}_2 \) production, a decrease of MMP, and the activation of caspase-3 can be inhibited by antioxidants NAC and catalase, it seems that a cascade of \( \text{H}_2\text{O}_2 \) production to decrease MMP to caspase activation occurs during the apoptotic process. However, cleaved fragments of caspase-8 and Bid and up-regulated levels of the DR5 protein were detected in EABE-treated HL-60 cells (Fig. 3C). These data suggest that a death receptor-mediated pathway is also involved in EABE-induced apoptosis. DR5 has been found to be induced by DNA-damaging agents in a p53-dependent fashion (41), and its transcription can be directly transactivated by p53 through an intronic, sequence-specific, p53 binding site (42). The possible role of p53 in EABE-induced expression of DR5 could be excluded because HL-60 cells lack a functional p53 (43). Recently, it has been found that DR5 expression could be induced through a CAAT/enhancer binding protein homologous protein (CHOP)-mediated pathway (44, 45), and that the CHOP-mediated DR5 up-regulation after curcumin treatment was inhibited by the antioxidant NAC (45). EABE-induced up-regulation of DR5 was also inhibited by antioxidants (NAC and catalase; Fig. 3C). These data suggest that increased DR5 levels after EABE treatment may be due to the up-regulation of CHOP following \( \text{H}_2\text{O}_2 \) production.

GSH has been thought to be a primary regulator of intracellular redox (46, 47). It has been found that intracellular GSH was depleted before the onset of apoptosis induced by various agents (48), and that the depletion of intracellular GSH rendered cells more sensitive to apoptotic agents (26). Consistent with these observations, EABE and EA decreased the content of GSH in...
HL-60 cells at concentrations which induced apoptosis (Fig. 4A).

BSO decreased basal levels of GSH (Fig. 4B) and augmented apoptosis induction in HL-60 cells treated with EABE (Fig. 4C and D). Conversely, NAC prevented the decrease of GSH content in HL-60 cells treated with EABE (Fig. 4B) and protected against EABE-induced ROS accumulation and apoptosis (Figs. 3B, 4C and D). These data suggest that the decrease of GSH might be due to the conjugation of EABE or EA with GSH. Because the conjugate of EA with GSH decreases apoptosis induction (data not shown), it seems that GSH binds competitively to EABE/EA and prevents them from binding to proteins containing thiol groups that probably are EABE/EA targets for apoptosis induction.

EA and its GSH conjugate are known GSTP1-1 inhibitors (13, 14). Similar to EA, EABE also inhibits GSTP1-1 activity in a cell-free assay. However, cells with higher GSTP1-1 activity and with overexpressed GSTP1-1 protein were less sensitive to EABE-induced apoptosis (Fig. 5B and C). This suggests that GSTP1-1 may protect against EABE-induced apoptosis by catalyzing the conjugation of EABE with GSH and which then inactivates it. GSTP1-1 has been found to be an inhibitor of JNK activation, and the levels of phosphorylated JNK is increased in EABE-treated cells (Fig. 3D). This suggests that GSTP1-1 activity in cells may be reversibly inhibited in EABE-treated cells. However, it seems that EABE and EA induce apoptosis through a GSTP1-1 inhibition–dependent pathway because cells without GSTP1-1 are more responsive to EABE/EA treatment, and EA-SG with increased GSTP1-1 inhibition ability has less apoptosis induction ability (data not shown).

In summary, the presented data show that EABE and EA induce apoptosis through a ROS-mediated pathway, which is independent of GSTP1-1 inhibition, but that both extrinsic and intrinsic apoptotic pathways participate in EABE-induced apoptosis.

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