Ethacrynic Acid and a Derivative Enhance Apoptosis in Arsenic Trioxide–Treated Myeloid Leukemia and Lymphoma Cells: The Role of Glutathione S-Transferase P1-1

Rui Wang, Changda Liu, Lijuan Xia, Guisen Zhao, Janice Gabrilove, Samuel Waxman, and Yongkui Jing

Abstract

Purpose: Arsenic trioxide (ATO) as a single agent is used for treatment of acute promyelocytic leukemia (APL) with minimal toxicity, but therapeutic effect of ATO in other types of malignancies has not been achieved. We tested whether a combination with ethacrynic acid (EA), a glutathione S-transferase P1-1 (GSTP1-1) inhibitor, and a reactive oxygen species (ROS) inducer will extend the therapeutic effect of ATO beyond APL.

Experimental Design: The combined apoptotic effects of ATO plus ethacrynic acid were tested in non-APL leukemia and lymphoma cell lines. The role of ROS, GSTP1-1, glutathione (GSH), and Mcl-1 in apoptosis was determined. The selective response to this combination of cells with and without GSTP1-1 expression was compared.

Results: ATO/EA combination synergistically induced apoptosis in myeloid leukemia and lymphoma cells. This treatment produced high ROS levels, activated c-Jun-NH2-kinase (JNK), and reduced Mcl-1 protein. This led to the decrease of mitochondrial transmembrane potential, release of cytochrome c, and subsequently, to activation of caspase-3 and -9. Induction of apoptosis in leukemia and lymphoma cells expressing GSTP1-1 required high ethacrynic acid concentrations to be combined with ATO. Silencing of GSTP1 in leukemia cells sensitized them to ATO/EA–induced apoptosis. In a subgroup of B-cell lymphoma, which does not express GSTP1-1, lower concentrations of ethacrynic acid and its more potent derivative, ethacrynic acid butyl-ester (EABE), decreased intracellular GSH levels and synergistically induced apoptosis when combined with ATO.

Conclusion: B-cell lymphoma cells lacking GSTP1-1 are more sensitive than myeloid leukemia cells to ATO/EA–induced apoptosis. Clin Cancer Res; 18(24); 6690–701. ©2012 AACR.

Introduction

Arsenic trioxide (ATO) treatment induces complete remission in patients with acute promyelocytic leukemia (APL). It has been found that ATO could induce both apoptosis and partial differentiation in APL cells at therapeutic concentrations (1–2 µmol/L; refs. 1, 2). Although ATO-mediated degradation of a leukemia-specific fusion protein PML-RARα in APL cells leads to differentiation (3), we and other groups found that ATO-induced APL-cell apoptosis is independent of PML-RARα degradation (4–7). Therefore, it is possible to use ATO to achieve therapeutic results in malignant cells based on apoptosis induction.

ATO-induced apoptosis in APL cells is partially mediated through H2O2 accumulation (6, 8), which is followed by a change in mitochondrial transmembrane potential (MTP), cytochrome c release, and caspase activation (6). We showed that the remarkable sensitivity to ATO-induced apoptosis and H2O2 accumulation of APL-NB4 cells, compared with other types of myeloid leukemia, such as HL-60 and K562 cells, was partly due to lower activity of glutathione S-transferase P1-1 (GSTP1-1; ref. 6). GSTP1-1 is a detoxification enzyme, which can determine cell sensitivity to ATO (9, 10). It catalyzes the conjugation of ATO with glutathione (GSH) causing its efflux from cells (11), it also scavenges intracellular reactive oxygen species (ROS) and detoxifies other products of oxidative damage. Furthermore, GSTP1-1 was found to regulate several signaling pathways, including that of c-Jun-NH2-kinase 1 (JNK1) inhibition via direct protein–protein interactions (12, 13). We previously found that overexpression of GSTP1-1 reduces the intracellular level of free ATO and diminishes ROS-induced apoptosis (9), suggesting that agents that inhibit GSTP1-1 activity might extend the use of ATO for
Arsenic trioxide (ATO) is effective for treatment of acute promyelocytic leukemia (APL) with minimal toxicity. Although B-cell lymphoma cell lines are sensitive to ATO-induced apoptosis in culture, the effect in patients is minimal. We found that a novel combination of ATO with low doses of ethacrynic acid (EA) and a more potent derivative, ethacrynic acid butyl-ester (EABE), can synergistically and selectively induce apoptosis in B-cell lymphoma cell lines lacking glutathione S-transferase P1-1 (GSTP1-1) expression. Both ethacrynic acid and EABE decrease glutathione (GSH) levels leading to enhanced ATO-induced reactive oxygen species (ROS) production and Mcl-1 reduction. This effect is blocked by forced expression of GSTP1-1. Because, with the exception of mantle cell lymphoma, the majority of B-cell lymphomas have low or undetectable levels of GSTP1-1, the combination of ATO with EA/EABE has the potential to be translated into a novel therapy for patients with B-cell lymphoma stratified without GSTP1-1 expression.

**Translational Relevance**

ATO solution was obtained from the pharmacy of our school hospital. Ethacrynic acid, acridine orange, ethidium bromide, N-acetylcysteine (NAC), Trolox, and INK inhibitor SP600125 were purchased from Sigma Chemical Co.. GSH-conjugated ethacrynic acid (EA-GS) and ethacrynic acid butyl-ester (EABE) were synthesized by Guisen Zhao (18). The general caspase inhibitor Z-VAD-FMK, caspase-8 inhibitor Z-LEHD-FMK, and caspase-9 inhibitor Z-IETD-FMK were obtained from Calbiochem. 3,3-dichlorodihydrofluorescein diacetate (DCFH-DA) were obtained from Molecular Probes. Antibodies to PARP were obtained from Cell Signaling Technology, Inc.; X-linked inhibitor of apoptosis (XIAP), ABL, and Fas ligand from BD Biosciences; Mcl-1, survivin, Bcl-2, GSTP1-1, Nrf2, and β-actin from Santa Cruz Biotechnology, Inc.; X-linked inhibitor of apoptosis (XIAP), ABL, and Fas ligand from BD Biosciences; Fas from Upstate Biotechnology; Bel-Xo, cleaved caspase-9, -3, TRAIL, INK, p-JNK, p-c-Jun (Ser63) II, and cytochrome c from Cell Signaling Technology, Inc.; catalase from Sigma Chemical Co. and voltage dependent anion selective channel protein 1 (VDAC/orin) from Abcam Inc. (Cambridge, MA).

**Materials and Methods**

**Reagents**

ATO solution was obtained from the pharmacy of our school hospital. Ethacrynic acid, acridine orange, ethidium bromide, N-acetylcysteine (NAC), Trolox, and INK inhibitor SP600125 were purchased from Sigma Chemical Co.. GSH-conjugated ethacrynic acid (EA-GS) and ethacrynic acid butyl-ester (EABE) were synthesized by Guisen Zhao (18). The general caspase inhibitor Z-VAD-FMK, caspase-8 inhibitor Z-LEHD-FMK, and caspase-9 inhibitor Z-IETD-FMK were obtained from Calbiochem. 3,3-dichlorodihydrofluorescein diacetate (DCFH-DA) were obtained from Molecular Probes. Antibodies to PARP were obtained from Cell Signaling Technology, Inc.; X-linked inhibitor of apoptosis (XIAP), ABL, and Fas ligand from BD Biosciences; Mcl-1, survivin, Bcl-2, GSTP1-1, Nrf2, and β-actin from Santa Cruz Biotechnology, Inc.; X-linked inhibitor of apoptosis (XIAP), ABL, and Fas ligand from BD Biosciences; Fas from Upstate Biotechnology; Bel-Xo, cleaved caspase-9, -3, TRAIL, INK, p-JNK, p-c-Jun (Ser63) II, and cytochrome c from Cell Signaling Technology, Inc.; catalase from Sigma Chemical Co. and voltage dependent anion selective channel protein 1 (VDAC/orin) from Abcam Inc. (Cambridge, MA).

**Cell lines**

Human acute myeloid leukemia HL-60 cells, chronic myeloid-derived K562 cells, lymphoma Raji, Namalwa, Daudi, and Jurkat cells were obtained from American Type Culture Collection. HP100-1, a H2O2-resistant derivative of HL-60 cell line (19), was obtained from the Japanese Cell Bank. Su-DHL-4 lymphoma cells were obtained from Dr. Ari Melnick (Weill Medical College of Cornell University, New York, NY; ref. 20). All cell lines were not authenticated by us once received in our laboratory. HL-60, HP100-1, and K562 cells were cultured in RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mmol/L L-glutamine, and 10% (v/v) heat-inactivated FBS. Raji, Namalwa, Daudi, Jurkat, and Su-DHL-4 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, 1.5 g/L sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% (v/v) heat-inactivated FBS. RV4 and RG19 cells are clones of human Raji cells transfected with pcDNA3.1 empty vector and pcDNA3.1/GSTP1 plasmid, which we have described previously (9).

**Quantiﬁcation of apoptotic cells**

For morphologic apoptosis quantification, cells were stained with acridine orange and ethidium bromide as described previously (5), and the percentage of apoptotic cells was calculated from 300 cells. Annexin V–FITC Apoptosis Detection Kit (BD Biosciences) was used to quantify apoptosis by ﬂuorescence-activated cell sorting (FACS) analysis. Data were analyzed using CELLQuest (BD Biosciences) software, on 10,000 events (9). Drug interaction was analyzed by Compusyn software (ComboSyn) using a combination at a fixed ratio of 2 agents (21). A CI less than 1 indicates synergism.

**Measurement of mitochondrial transmembrane potential**

MTP was assessed by the retention of DiOC6(3), a membrane-permeable fluorescent cationic dye. The uptake of DiOC6(3) by mitochondria is proportional to the MTP (22). Briefly, cells under different treatments were incubated with 40 nmol/L DiOC6(3) for 15 minutes at 37°C. After washing with ice-cold PBS, the cells were analyzed by FACSscan with excitation and emission wavelength of 484 and 500 nm, respectively.

**Subcellular fractionation**

Cytosolic and mitochondrial fractions were isolated with the Mitochondria Isolation Kit (Pierce/Thermo Fisher...
Scientific) following the manufacturer’s instructions using 2 \times 10^7 cells. The mitochondria-rich and cytosolic fractions were obtained by centrifugation. The mitochondria-rich fraction was washed once with 500 \mu L. Mitochondrial Isolation Reagent C, followed by a final resuspension in lysis buffer [50 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 0.25% sodium deoxycholate, and 1 mmol/L EGTA] with protease inhibitor mixture and stored at −80°C.

Determination of H2O2 production, Western blot analysis, GSTP1-1 activity, GSH content, and siRNA interference were conducted as we reported earlier (6, 23).

**Statistical analysis**

Data were analyzed for statistical significance using the Student t test (Microsoft Excel, Microsoft Corporation). A P value of less than 0.05 was considered statistically significant.

**Results**

A combination of ATO with ethacrynic acid synergistically induces apoptosis in HL-60 and K562 myeloid leukemia cells

HL-60 and K562 cells were treated with ATO or ethacrynic acid or the combination, and the apoptotic cells were identified by morphology after staining with acridine orange and ethidium bromide. ATO (0.5–4 \mu mol/L) did not induce obvious apoptosis in neither of the 2 cell lines (Fig. 1A and B). Ethacrynic acid alone was ineffective below 40 \mu mol/L and at 80 \mu mol/L it induced apoptosis in 30% of HL-60 cells and in 14% of K562 cells (Fig. 1A and B). Ethacrynic acid at concentrations of 40 to 80 \mu mol/L in HL-60 cells (Fig. 1A) and at concentrations of 60 to 100 \mu mol/L in K562 cells augmented ATO-induced apoptosis (Fig. 1B). Using Compusyn software, the combined apoptotic effects of ATO with ethacrynic acid were analyzed for synergy. CI values were calculated for different dose-effect levels based on parameters derived from median-effect plots of ATO alone, ethacrynic acid alone, or their combinations. As shown in Fig. 1C and D, simultaneous exposure of cells to ATO (1–5 \mu mol/L) and ethacrynic acid (40–80 \mu mol/L) in HL-60 cells and 60–100 \mu mol/L in K562 cells) showed CIs of less than 1, indicating synergistic effects. Although the antagonism seems to be obtained in cells treated with combinations of lower concentrations, it should be considered as ineffectiveness. The apoptosis induction by low concentrations of ethacrynic acid (10–20 \mu mol/L), ATO (0.5 \mu mol/L), and their combinations is minimal (<10%; Fig. 1C and D). Synergistic results were confirmed in HL-60 and K562 cells when annexin V and FACS were used to measure apoptosis. The combination produced approximately 36% apoptosis in each of the cell lines (Supplementary Fig. S1).

ATO/EA induces apoptosis in HL-60 and K562 cells through the mitochondrial pathway

We analyzed HL60 and K562 cells for protein levels of the Bcl-2 family members (mitochondrial pathway) and for death receptors (extrinsic pathway), in control cells and cells treated with ATO (2 \mu mol/L) or ethacrynic acid (40 \mu mol/L) in HL-60 cells and 80 \mu mol/L in K562 cells) alone, or the combination. The antiapoptotic, Bcl-2 family member, Mcl-1 protein was somewhat reduced by
ethacrynic acid alone in both cell lines, which was further reduced by the combination treatment (Fig. 2A). Acute myelogenous leukemia (AML) HL-60 cells express Bcl-2 but not Bcl-xL. The levels of Bcl-2 were not changed by treatment with either agent alone or their combination. Chronic myelogenous leukemia K562 cells contain fusion protein BCR-ABL, which is known to upregulate Bcl-xL (24). K562 cells express Bcl-xL but not Bcl-2. The levels of BCR-ABL were not changed after treatment with each agent and the combination, however, the levels of Bcl-xL were slightly increased by treatments with each agent alone or their combination. Compatible with these changes, cleaved caspase-9 was strongly increased. Of the extrinsic pathway, only a small percentage of cells underwent apoptosis after treatment with ATO/EA combination, as indicated by Annexin V staining and FACS analysis. The ATO/EA combination-induced apoptosis was further increased by silencing of Mcl-1 in K562 cells, while increased Mcl-1 level attenuated ATO/EA-induced apoptosis in HL-60 cells. The combination of ethacrynic acid and ATO induced caspase-3, -8, and -9 activation and downregulated antiapoptotic proteins, which was consistent with the Western blotting results shown in Figure 2A. The relative levels of indicated proteins were determined by Western blotting using specific antibodies. β-Actin served as loading control.

**Figure 2.** ATO and ethacrynic acid combination induces activation of caspase-3, -8, and -9 and downregulates antiapoptotic proteins. A, modulation of apoptotic proteins by ATO/EA. HL-60 and K562 cells were treated with ethacrynic acid and ATO at the indicated concentrations for 24 hours. The relative levels of indicated proteins were determined by Western blotting using specific antibodies. β-Actin served as loading control. B, caspase-9 (but not caspase-8) is the major effector of ATO/EA-induced apoptosis. HL-60 cells were pretreated for 4 hours with 50 µmol/L Z-VAD-FMK (a general caspase inhibitor), 50 µmol/L Z-IETD-FMK (a caspase-8 inhibitor), 50 µmol/L Z-LEHD-FMK (a caspase-9 inhibitor), and then incubated with 2 µmol/L ATO and 40 µmol/L ethacrynic acid for another 24 hours. Apoptotic cells were quantified using annexin V-fluorescein isothiocyanate (FITC) staining and FACS analysis. Con, control. C, silencing of Mcl-1 enhances ATO/EA-induced apoptosis in K562 cells. K562 cells were transfected with Mcl-1 siRNA or negative control siRNA and after 18 hours, treated with 2 µmol/L ATO and 60 µmol/L ethacrynic acid for an additional 24 hours. The protein levels were determined by Western blotting and the apoptotic cells were determined by FACS after staining with annexin V–FITC. D, increased Mcl-1 level attenuated ATO/EA-induced apoptosis in HL-60 cells. HL-60 cells transfected with an empty vector (HL-60/V5) and Mcl-1 expression plasmid (HL-60/M15) were treated with 2 µmol/L ATO and 60 µmol/L ethacrynic acid for an additional 24 hours. The Mcl-1 level was determined by Western blotting and the apoptotic cells were determined by FACS after staining with annexin V–FITC.
reduction in c-FLIP (an inhibitor of caspase-8) and elevated cleaved caspase-8 were detected (Fig. 2A). The levels of death receptors and their ligands, such as Fas, Fasl, DR5, and TRAIL, were unchanged, yet there was an increase in cleaved caspase-3 (the target of both caspase-8 and -9) in both cell lines treated with the combination (Fig. 2A). Nrf2, a transcriptional factor, mediates cellular defense against oxidative stress. High levels of Nrf2 are correlated with relative resistance to ATO treatment and Nrf2 is increased in ATO-treated myeloma cells (25, 26). Thus, we determined whether the effectiveness of ATO/EA treatment regulates Nrf2 levels. We found that in either HL60 or K562, the Nrf2 level was not regulated by ethacrynic acid or ATO treatment alone or by their combination (Fig. 2A), suggesting that Nrf2-mediated defense system is not activated in this combination treatment.

To identify which apoptotic pathway is activated, control or ATO/EA–treated HL-60 were incubated with a general caspase inhibitor, Z-VAD-FMK, a caspase-9 inhibitor, Z-LEHD-FMK, and a caspase-8 inhibitor, Z-IETD-FMK. Figure 2B shows that the general caspase inhibitor and caspase-9 inhibitor reduced apoptosis from approximately 38% to approximately 13% and 17%, respectively, whereas inhibition of caspase-8 (extrinsic pathway) had a much lesser effect on apoptosis induction by ATO/EA. Thus, the mitochondrial pathway seems to be the principle mechanism of apoptosis induction by ATO/EA combination.

As shown in Fig. 2A, 3 proteins of the mitochondrial pathway, a Bcl-2 family member, Mcl-1, and 2 inhibitors of caspases, XIAP and survivin, are downregulated by ATO/EA. These proteins were individually knocked down in K562 cells using specific siRNA. The levels of each of the 3 proteins were significantly reduced (Fig. 2C and Supplementary Fig. S2), but only knockdown of Mcl-1 sensitized K562 cells to ATO/EA apoptosis. The Mcl-1 knockdown-K562 cells, treated with ATO/EA, had more cleaved PARP, caspase-3, and -9 (Fig. 2C). The percentage of annexin V–positive cells rose approximately 3.5-fold over the control (intact Mcl-1) treated with 2 μmol/L ATO/60 μmol/L ethacrynic acid, which had only 12% of apoptotic cells (Fig. 2C). To further test the role of Mcl-1 in this combination treatment, subclones of HL-60 cells transfected with the pcDNA3.1 empty vector (HL-60/V5) and the pcDNA3.1/Mcl-1 plasmid (HL-60/M15) were generated. HL-60/M15 cells with much higher levels of Mcl-1 than HL-60/V5 cells were less sensitive to ATO/EA–induced apoptosis (Fig. 2D). To test if Bcl-xl expression has a negative role in ATO/EA–induced apoptosis in K562 cells, Bcl-xl was silenced using siRNA. This intervention did not sensitize K562 cells to ATO/EA-induced apoptosis (Supplementary Fig. S2E and S2F). Taken together, this suggests that the downregulation of Mcl-1, but not of XIAP or survivin, is a crucial step in apoptosis induction by ATO/EA.

**ATO/EA combination decreases MTP, causes release of cytochrome c, and increases ROS**

Following ATO/EA treatment, MTP in HL-60 cells was analyzed using FACS after staining with the cationic dye DOIC_3(3). The combination treatment produced a time-dependent reduction in MTP, which culminated after 18 hours (Fig. 3A, left). When compared with ATO or ethacrynic acid alone, 12 hours treatment with ATO/EA elicited a much more pronounced MTP reduction (Fig. 3A, right). The decrease in MTP was followed by a time-dependent release of cytochrome c from mitochondria into cytosol (Fig. 3B). We previously showed that ATO induces apoptosis in APL via a H2O2-dependent pathway (6), and others showed ROS involvement in ethacrynic acid–induced apoptosis (17). We measured the levels of H2O2 in HL-60 cells using a peroxide-sensitive fluorescent probe, DCFHDA, and showed (Fig. 3C, right) that ATO alone generated only a minimal increase in H2O2, whereas ethacrynic acid produced an increase in H2O2 content similar to that induced by the combination at 6 hours of treatment. Thus, ethacrynic acid is a major contributor to the ROS generation. Moreover, the rapid kinetics (1 hour) of ROS generation by the ATO/EA suggests that ROS is an early response to treatment followed by changes in antiapoptotic protein and activation of caspases.

To further test the role of ROS in ATO/EA–induced apoptosis, we used an H2O2–resistant subclone of HL-60 cells, HP100-1 (19). ATO (2 μmol/L) combined with ethacrynic acid (40 μmol/L) induced apoptosis in approximately 40% of HL-60 cells but only in less than 10% of HP100-1 cells (results not shown). Likewise, as compared with parental HL-60, PARP cleavage, and caspase-9 and -3 activation in HP100-1 cells were greatly reduced (Fig. 3D) and ROS production was not significantly elevated (Supplementary Fig. S3A). The HP100-1 cells have high catalase activity (27), a finding we confirmed in this cell line (Fig. 3D). Knockdown of catalase in HP100-1 cells produced an increase in cleaved PARP, caspase-9, and -3 and reduced levels of Mcl-1, XIAP, and survivin (Supplementary Fig. S3B). In addition, we found that the antioxidant Trolox attenuated ATO/EA–induced apoptosis in HL-60 cells (Supplementary Fig. S3C). These data suggest that ROS play important role in ATO/EA–induced apoptosis.

**JNK activation is in the pathway of ATO/EA–induced ROS generation**

JNK is activated by ROS, both through activation of upstream kinases and through inactivation of a JNK-specific phosphatase (28). We found that ATO/EA induced a time-dependent increase in the level of p-JNK in both HL-60 and K562 cell lines, followed by an increase in phosphorylated c-Jun, its target (Fig. 4A). To examine whether JNK activation was an integral part of the ATO/EA–induced apoptotic program, cells were pretreated with a JNK inhibitor SP600125 (29) before being treated with ATO/EA. As expected, SP600125 prevented the phosphorylation of JNK, reduced the phosphorylation of c-Jun (Fig. 4B), and suppressed by 50% ATO/EA–induced apoptosis (Supplementary Fig. S4A). Inhibition of JNK activity also blocked the cleavage of PARP and caspase-3 and restored the Mcl-1 level but not the level of XIAP or survivin (Fig. 4B). To determine
the pathways of Mcl-1 reduction by ATO/EA treatment, K562 cells were pretreated with the general caspase inhibitor Z-VAD-FMK and the proteasome inhibitor MG132 followed by ATO/EA treatment. Z-VAD-FMK blocked EA/ATO–induced PARP cleavage without preventing Mcl-1 reduction, suggesting the downregulation of Mcl-1 is not mediated by caspase activation (Supplementary Fig. S4B). MG132 prevented ATO/EA–induced loss of Mcl-1 (Supplementary Fig. S4C).

GSTP1-1 has been shown to inhibit JNK activation through a protein–protein interaction (12, 13). We prepared lysates of K562 cells untreated, or treated with the ATO/EA combination, immunoprecipitated the lysates with an anti-JNK antibody, and determined the amount of JNK-bound GSTP1-1. The treatment did not reduce the GSTP1-1/JNK association (data not shown) suggesting that this was not the mechanism of JNK activation. In contrast, pretreatment of cells with NAC, an antioxidant and glutathione inducer, prevented both ATO/EA–induced JNK phosphorylation (Fig. 4C) in both HL-60 and K562 cells and restored PARP and Mcl-1, proteins, which are considered the hallmark of ATO/EA–induced apoptosis, to nearly normal levels (Fig. 4C). Thus, the state of JNK activation has a profound impact on the apoptotic pathway probably through affecting proteasomal degradation of Mcl-1.
GSTP1-1 and catalase are roadblocks to induction of apoptosis by ATO/EA in K562 cells

To test whether GSTP1-1 was responsible for treatment resistance, it was knocked down with siRNA and the cells were subjected to ATO/EA treatment. Western blot analysis confirmed that the GSTP1-1 knockdown was effective (Fig. 4D). Cells with reduced GSTP1-1 treated with ATO/EA produced much more ROS (data not shown). The expression or activation of other components of the apoptotic pathway, including p-JNK, was enhanced more than that seen in K562 with intact GSTP1-1 treated with ATO/EA (Fig. 4D). Lowering GSTP1-1 levels, increased apoptosis from 14% to 34% in K562 cells treated for 24 hours with 2 μmol/L ATO and 60 μmol/L ethacrynic acid. Together these data suggest that high GSTP1-1 level, by preventing ROS accumulation, made the cells resistant to ATO/EA. Using an additional approach to confirm this conclusion, we targeted catalase with siRNA and found that cells with reduced catalase behave in a way similar to that of K562 cells with knockdown GSTP1-1, that is, displaying enhanced apoptosis, increased levels of p-JNK, and decreased levels of Mcl-1 (Supplementary Fig. S5A and S5B).

Absence of GSTP1-1 expression in lymphoma cells defines a subgroup, which is highly sensitive to ATO/EA induced apoptosis

We showed (Fig. 4D) that knockdown of GSTP1-1 in K562 cells profoundly increased sensitivity to ATO/EA. -
induced apoptosis. We previously found that while Burkitt’s lymphoma (Raji) cell line, which does not express GSTP1-1, was relatively sensitive to ATO alone (14), forced expression of GSTP1-1 caused resistance to ATO (9). We tested whether GSTP1-1 is also a determinant of ATO/EA sensitivity in lymphoma cells by comparing a clone of Raji transfected to express GSTP1-1 (RG19) and a clone transfected with the vector alone (RV4). RV4 and RG19 cells were treated with ATO/EA and the apoptosis was quantified. Remarkably, a high degree of apoptosis (40%, Fig. 5A) was induced in the GSTP1-1–negative RV4 cells with 1 µmol/L ATO and only 15 µmol/L ethacrynic acid, a concentration 3- to 5-fold lower than needed to induce apoptosis in leukemia cells (Supplementary Fig. S1); only 12% of GSTP1-1 expressing, RG19 cells underwent apoptosis (Fig. 5A). Apoptosis was paralleled by a much stronger ROS production (data not shown) and Mcl-1 downregulation in RV4 as compared with RG19 (Fig. 5B). These results opened a possibility that this combination might selectively induce apoptosis in B-cell lymphoma without GSTP1-1 expression. To test this

Figure 5. GSTP1-1 levels determine the sensitivity of lymphoma cells to ATO/EA treatment-induced apoptosis. A, RV4 lymphoma cells which lack GSTP1-1 expression are sensitive to ATO/EA–induced apoptosis at low ethacrynic acid concentration. RV4 cells and RG19 (high GSTP1-1 expression) cells were treated with 1 µmol/L ATO/15 µmol/L ethacrynic acid for 24 hours and the apoptotic cells were detected by FACS after staining with annexin V–FITC. B, ATO/EA–induced mitochondrial apoptosis pathway is muted in RG19 cells. RV4 and RG19 cells were treated with 1 µmol/L ATO/15 µmol/L ethacrynic acid for 24 hours and the protein levels of the mitochondrial apoptotic pathways were analyzed by Western blot analysis. C, lymphoma cell lines differ widely in GSTP1-1 protein and activity. The protein levels of GSTP1-1 were determined by Western blotting and the activity was determined biochemically. D, low GSTP1-1 activity lymphoma cells, Raji, Namalwa, and Daudi, are highly sensitive to apoptosis induction by low concentrations of ATO/EA; Jurkat and Su-DHL-4 cells with high GSTP1-1 activity are resistant. Raji, Namalwa, Daudi, Su-DHL-4, and Jurkat cells were treated with 1 µmol/L ATO together with ethacrynic acid at the indicated concentrations for 24 hours. Apoptotic cells were detected by FACS after staining with annexin V–FITC.
possibility, we examined the protein level and activity of GSTP1-1 in 4 B-cell lymphoma lines (Raji, Namalwa, Daudi, and Su-DHL-4) and in 1 T cell lymphoma (Jurkat). Both Jurkat and Su-DHL-4 cells expressed higher levels of GSTP1-1 protein associated with higher GSTP1-1 activities (Fig. 5C). Namalwa cells had much lower levels than Jurkat cells, and Raji and Daudi cells had undetectable levels (Fig. 5C). Individually, ATO, or low concentration of ethacrynic acid (10 μmol/L), was ineffective in apoptosis induction, but a combination of 1 μmol/L ATO with 10 μmol/L ethacrynic acid induced strong apoptosis in the 3 GSTP1-1 low lymphoma cell lines, Raji, Daudi, and Namalwa (Fig. 5D), but not in Jurkat and Su-DHL-4 cells (Fig. 5D). These results show that relatively low levels of ATO/EA can selectively induce apoptosis in malignant cells.

Ethacrynic acid decreases intracellular levels of GSH, which augments ATO-induced apoptosis in non-GSTP1-1 expression cells

The observation that ethacrynic acid enhanced ATO-induced apoptosis only in non-GSTP1-1 expressing cells (Fig. 5D) suggested that ethacrynic acid acts through a pathway, which is independent of GSTP1-1 inhibition. Previously, we also found that the basal levels of GSH control cell sensitivity to ATO-induced apoptosis (5). Using the Raji and Jurkat cells with or without expression of GSTP1-1, we tested the intracellular levels of GSH after treatment with ethacrynic acid, ATO alone and their combination. As shown in Fig. 6A and Supplementary Fig. S6A, ethacrynic acid at 15 μmol/L decreased the levels of GSH equally in both cell lines; ATO did not decrease the levels of GSH and the effect of EA/ATO on GSH was similar to that of ethacrynic acid alone. We synthesized GSH-conjugated ethacrynic acid, EA-GS. EA-GS neither decreased the levels of GSH nor enhanced ATO-induced apoptosis in either of the cell lines (Fig. 6A and Supplementary Fig. S6). EA-GS alone or in combination with ATO failed to increase the levels of ROS (data not shown). Previously, we have modified ethacrynic acid structure and found that EABE was more effective than ethacrynic acid in apoptosis induction in leukemia cells (18). We tested the combined effects of ATO with EABE in Raji cells. EABE at low concentration of 1 and 2 μmol/L decreased the levels of GSH (Fig. 6B) and augmented apoptosis induction by ATO even at as low as 0.5 μmol/L (Fig. 6C). ATO plus EABE at the same concentrations did not induce apoptosis in Jurkat cells, which express GSTP1-1 (data not shown). These data suggest that the ethacrynic acid or EABE-mediated reduction in GSH level plays an important role in increasing the ATO-induced apoptosis.

Discussion

We found that the effective concentrations of ethacrynic acid required to enhance the proapoptotic activity of ATO, which are dependent on the GSTP1-1 level (Fig. 5C and D). To induce apoptosis in AML cells (Fig. 1 and Supplementary Fig. S7A), which have high GSTP1-1 protein (Supplementary Fig. S7B), required several fold greater ethacrynic acid concentration than that required for lymphoma cells, which have either lower, or entirely absent, GSTP1-1 activity. The observation that ethacrynic acid, with a known GSTP1-1 inhibitor, can synergistically enhance ATO-induced apoptosis in non-GSTP1-1 expressing lymphoma cells indicates that ethacrynic acid must have also a GSTP1-1-independent function. If this dependence of ATO/EA effect on GSTP1-1 levels found in cell lines will translate to patient samples, it will provide a novel approach to stratification of patients with lymphoma and selection of those with low or absent GSTP1-1 as best candidates for ATO/EA combination therapy.

Several questions arise from these studies: (i) what is the mechanism of ATO/EA–induced apoptosis in GSTP1-1–expressing cells and what role GSTP1-1 plays in these processes? (ii) How does ethacrynic acid contribute to the synergistic enhancement of the proapoptotic effect of ATO? (iii) What is the GSTP1-1 independent role of ethacrynic acid in enhancing ATO-induced apoptosis in lymphoma cells without GSTP1-1 expression?

GSTP1-1 has been found to oppose ATO-induced apoptosis by detoxification of ATO and ROS (6, 9, 30) and by inhibition of JNK activity through a protein–protein interaction (12, 13). We found that GSTP1-1 knockdown in K562 cells enhanced ATO/EA–induced ROS production, significantly increased apoptosis (data not shown), and substantially activated proteins of the proapoptotic pathway (Fig. 4D). Individually, ATO or ethacrynic acid has been shown to induce both the extrinsic and the intrinsic pathways of apoptosis (17, 18, 31) and ROS were shown to be involved (5, 6, 8, 17, 18), but the combination of ATO/EA on both pathways was not tested. Activation of the intrinsic pathway involves MTP, ROS accumulation, and release of proteins, including cytochrome c, which form the caspase-activating complex, apoptosome (6). As shown in Fig. 3, we found that ATO/EA–induced ROS accumulation represents the crucial and early step in apoptosis induction. Their increase precedes the changes in the proapoptotic protein levels. ROS did not accumulate and apoptosis was not induced by ATO/EA in HP100-1 cells expressing high level of catalase, a resistance that was reversed by knockdown of catalase (Fig. 3D and Supplementary Fig. 53B). Moreover, we found that the antioxidant Trolox blocked ATO/EA–induced apoptosis in HL-60 cells (Supplementary Fig. S3C). These data indicate that ROS play an important role in this combination-induced apoptosis. We found that ROS accumulation was followed by a decrease in MTP, cytochrome c release, and activation of JNK. GSTP1-1 can bind JNK and inhibit its activity (12, 13), but ATO/EA treatment did not affect this interaction (data not shown), suggesting that ROS activation of JNK proceeds through a mechanism that is not due to the JNK dissociation from GSTP1-1. It has been found that JNK phosphorylates Mcl-1 and induces its degradation through proteasome (32). This event contributes to Mcl-1 downregulation (33, 34). We found that Mcl-1 reduction by ATO/EA was blocked by proteasomal inhibitor MG132 but not by the general caspase inhibitor Z-VAD-FMK (Supplementary Fig. S4B and S4C). We show that...
ATO/EA activation of JNK is responsible for the decrease in Mcl-1 level, because inhibition of JNK with a specific inhibitor, SP600125, restores the Mcl-1 level (Fig. 4B) and blocks apoptosis (Supplementary Fig. 4A). Silencing of Mcl-1–sensitized K562 cells to EA/ATO–induced apoptosis (Fig. 2C) and increased levels of Mcl-1 in HL-60 cells attenuated ATO/EA–induced apoptosis (Fig. 2D). These data suggest that JNK activation-mediated Mcl-1 proteasomal degradation contributes to ATO/EA–induced apoptosis. In addition to decreasing Mcl-1, ATO/EA combination abolished XIAP expression and reduced the levels of survivin (Fig. 2A), 2 inhibitors of caspase-9 and -3 (35), but their knockdown did not sensitize cells to ATO/EA–induced apoptosis (Supplementary Fig. S2A and S2C). In cells with high catalase activity (HP100-1) ATO/EA decreased the levels of XIAP and survivin, but not of Mcl-1, and did not induce apoptosis (Supplementary Fig. S3B). JNK inhibition restored the Mcl-1 level, but neither of XIAP nor survivin (Fig. 4B). Thus, XIAP and survivin might aid in ATO/EA–induced apoptosis, but the mechanism of their regulation by ATO/EA, unlike that of Mcl-1, does not require activation of JNK.

Our results showed that sensitivity of lymphoma cells to ATO/EA depends strictly on GSTP1-1 levels and that low GSH content is crucial for synergistic apoptosis induction by a combination of ATO with ethacrynic acid and its derivatives in lymphoma cells without GSTP1-1 expression. A, ethacrynic acid, but not EA-GS, decreases the levels of intracellular GSH. Raji cell were treated with 1 µmol/L ATO, 15 µmol/L ethacrynic acid, and 15 µmol/L EA-GS for 6 hours. B, EABE decreases GSH levels in Raji cells. Raji cells were treated with 0.5 µmol/L ATO, 2 µmol/L EABE, or their combination for 6 and 16 hours. The intracellular GSH levels were determined as described in Materials and Methods. C, EABE enhances ATO apoptosis in Raji cells. Raji cells were treated with 0.5 µmol/L ATO, 1 µmol/L, or 2 µmol/L EABE or their combination for 3 days; medium with fresh drug supplements was changed daily. The apoptotic cells were detected by FACS after staining with annexin V–FITC. D, a working model of ATO/EA–induced apoptosis in lymphoma cells with or without GSTP1-1 expression. ATO or EA/EABE can individually increase ROS through separate pathways. When combined, the 2 drugs strongly increase ROS causing reduction in MTP as well as activation of JNK. Active JNK participates in downregulation of Mcl-1, leading to maximal cytochrome c release, activation of caspase-9, and induction of apoptosis. Ethacrynic acid enhances ATO ability to produce ROS by decreasing intracellular GSH levels. GSTP1-1 detoxifies EA/EABE, ROS, and ATO and blocks apoptosis induction. ATO combined with low concentrations of EA/EABE selectively induces apoptosis in B-cell lymphoma lacking GSTP1-1 expression.
concentration of ethacrynic acid in combination with ATO has the ability to produce more ROS and synergistically induce apoptosis in cells that do not have GSTP1-1, such as Raji, Namalwa, and Daudi cells (Fig. 5). Ethacrynic acid can interact directly with GSH and by forming EA-GS conjugate, reduce the level of GSH. This interaction could also be catalyzed by GSTP1-1 (36). We found that ethacrynic acid decreased GSH levels in both Raji and Jurkat cells suggesting that GSTP1-1 is not absolutely required for ethacrynic acid to interact with GSH (Fig. 6A and Supplementary Fig. S6A). The fact that EA-GS did not decrease GSH levels and did not enhance ATO-induced apoptosis suggests that the decrease in the levels of GSH by ethacrynic acid plays an important role in enhancing ATO-induced apoptosis. Therefore, GSTP1-1 is considered as an enzyme to detoxify ethacrynic acid. Our results revealed an intriguing insight into the mechanism of ATO/EA synergy in lymphoma cells that do not express GSTP1-1 (Fig. 6D). Previously several groups, including our own, showed that buthionine sulfoximine (BSO) decreased the levels of GSH and enhanced ATO-induced apoptosis (37, 38) but in an unselective manner. We find that addition of BSO further enhances ATO/EA-induced apoptosis in AML cells (data not shown). Overall, these findings indicate that the GSTP1-1 blockade of EA/ATO effect resides in the ability of GSTP1-1 to detoxify ROS, ethacrynic acid, and/or ATO. Ethacrynic acid has been included in a clinical trial with thiotepa in patients with advanced cancer (39). When given orally at a tolerable dose the peak plasma concentration of ethacrynic acid is only approximately 3 μmol/L, whereas an intravenous injection of the same dose produces a peak plasma concentration about 30 μmol/L. It is believed that high plasma concentrations can be maintained by intravenous infusion (40) making the ethacrynic acid–enhanced ATO therapy for B-cell lymphoma lacking GSTP1-1 a clinical reality. We have conducted structure modifications of ethacrynic acid and found that ethacrynic acid esters had an increased ability to inhibit leukemia cell growth (41), and that one of these ethacrynic acid derivatives, EABE, was more rapidly taken up by leukemia cells and was a more active apoptosis inducer in leukemia cells (18). EABE at 1 to 2 μmol/L in combination with ATO, even at 0.5 μmol/L, induced apoptosis in Raji cells (Fig. 6C). Thus, EABE could be considered as an alternative enhancer of ATO-induced apoptosis in B-cell lymphomas that do not express GSTP1-1. Recently it has been found that, with the exception of mantle cell lymphoma, the majority of other B-cell lymphomas belonging to different phenotypes, do have low levels, or entirely absent GSTP1-1 (42, 43). In Raji cells, the GSTP1-1 is silenced because of GSTP1 promoter methylation (44). The GSTP1 promoter methylation in lymphomas has been reported (45) and it seems that the gene is silenced in 55.5% of follicular lymphoma, 52.0% Burkitt’s lymphoma, and 50.0% of mucosa-associated lymphoid tissue lymphoma (46). Combined with the results of our studies, these findings indicate that patients whose cells lack GSTP1-1 expression or have methylated GSTP1 promoter, can be selected to benefit from this novel ATO/EA combination therapy without suffering severe side effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: R. Wang, G. Zhao, J. Gabrilove, Y. Jing

Development of methodology: R. Wang, C. Liu, L. Xia, Y. Jing

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Wang, J. Gabrilove

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Wang, C. Liu, Y. Jing

Writing, review, and/or revision of the manuscript: R. Wang, J. Gabrilove, S. Waxman, Y. Jing

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Zhao, Y. Jing

Study supervision: Y. Jing

Acknowledgments

The authors thank Dr. Liliana Ossowski for critical reading of this article.

Grant Support

This work was supported by NIH grant ROI-CA93533 to Y. Jing.

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

Received March 7, 2012; revised October 5, 2012; accepted October 13, 2012; published OnlineFirst October 18, 2012.

References


Apoptosis Induction by Arsenic Trioxide plus Ethacrynic Acid


Ethacrynic Acid and a Derivative Enhance Apoptosis in Arsenic Trioxide–Treated Myeloid Leukemia and Lymphoma Cells: The Role of Glutathione S-Transferase P1-1

Rui Wang, Changda Liu, Lijuan Xia, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-0770

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/10/18/1078-0432.CCR-12-0770.DC1

Cited articles
This article cites 44 articles, 26 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/18/24/6690.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/18/24/6690.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.