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Original Article

Potentiation of arsenic trioxide-induced apoptosis by 8-bromo-7-methoxychrysin in human leukemia cells involves depletion of intracellular reduced glutathione

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The novel chrysin analog 8-bromo-7-methoxychrysin (BrMC) has been reported to induce apoptosis of various cancer cell lines. Arsenic trioxide (ATO) treatment induces clinical remission in acute promyelocytic leukemia patients. The combination of ATO with other agents has been shown to improve therapeutic effectiveness *in vitro* and *in vivo*. In this report, the mechanism of apoptosis induced by treatment with ATO alone or in combination with BrMC was studied in U937, HL-60, and Jurkat cells. Our results demonstrated that BrMC cooperated with ATO to induce apoptosis in human leukemia cells. This co-treatment caused mitochondrial transmembrane potential dissipation and stimulated the mitochondrial apoptotic pathway, as evidenced by cytochrome *c* release, down-regulation of X-linked inhibitor of apoptosis (XIAP) and Bcl-XL, and up-regulation of Bax. BrMC alone or in combination with ATO, decreased Akt phosphorylation as well as intracellular reduced glutathione (GSH) content. The thiol antioxidant *N*-acetylcysteine and exogenous GSH restored GSH content and attenuated apoptosis induced by co-treatment with ATO plus BrMC. In contrast, the non-thiol antioxidant butylated hydroxyanisole and mannitol failed to do so. These findings suggest that GSH depletion explains at least in part the potentiation of ATO-induced apoptosis by BrMC.

Keywords arsenic trioxide; chrysin; 8-bromo-7-methoxychrysin; apoptosis; GSH; leukemia cells

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Introduction

Arsenic trioxide (ATO) is an effective therapeutic agent for the treatment of relapsed and refractory acute promyelocytic leukemia (APL) [1]. The administration of low, physiologically tolerable concentrations of ATO (<2 μM in plasma) inhibited the proliferation of APL cells by inducing terminal differentiation and/or apoptosis [2]. Although this response was originally explained by the ability of the drug to disrupt the promyelocytic leukemia/retinoic acid receptor alpha (PML/RARα) fusion protein characteristic of most APLs, ATO induces other biochemical effects that are also important for apoptosis. These included direct binding to the adenosine nucleotide translocator (ANT) and induction of mitochondrial pore permeabilization [3], as well as inhibition of mitochondrial respiration and generation of reactive oxygen species (ROS) [4]. Because of this, ATO also induces apoptosis in tumor cell types other than APL, a response that opens the possibility of extending the agent's therapeutic applications [5]. Nevertheless, the relatively low sensitivity of most tumor cell types to ATO may require the use of sensitizing strategies or appropriate drug combination to increase the efficacy of ATO and to reduce its dosage to clinically achievable concentrations.

Dietary flavonoids display diverse biological activities, including prevention of cancer initiation and inhibition of cancer progression [6]. Chrysin (5,7-dihydroxy-2-phenyl-4H-chromen-4-one) is a natural flavonoid currently under investigation for its anti-cancer properties [7]. Chrysin has been shown to inhibit proliferation and induce apoptosis, and it is more potent than other tested flavonoids in leukemia cells where it is likely to act through activation of caspases and inactivation of Akt signaling [8,9]. Moreover, chrysin has been reported to potentiate apoptosis induction and/or cell growth inhibition by typical anti-tumor drugs such as mitoxantrone and epirubicin [10,11]. Chrysin and its analogs directly interacted with mitochondria, causing respiratory chain inhibition [12] and might also affect the redox state by altering the intracellular glutathione content [13,14]. This property is particularly important for the potentiation of ATO toxicity, because the glutathione (GSH)-based redox system is a strict determinant of ATO sensitivity. Thus, the apoptotic action of ATO inversely correlated with the endogenous level of GSH in different leukemia cell types [15], and treatments that experimentally depleted the GSH content enhanced ATO toxicity [16]. Nevertheless, poor oral bioavailability has been a major limitation for the successful use of dietary flavonoids as cancer

chemotherapeutic agents [17]. The effect of 8-bromo-7-methoxychrysin (BrMC) to inhibit proliferation and induce apoptosis in colon cancer cell line HT-29 and gastric cancer cell line SGC-7901 was stronger than that of chrysin [18]. BrMC has also been shown to induce apoptosis of human hepatocellular carcinoma cells, at least partly because it promotes the generation of ROS and ROS-dependent sustained activation of jun N-terminal kinase (JNK) [19]. The study by Ramos and Aller showed that quercetin decreased intracellular GSH content and potentiated the apoptotic action of the anti-leukemic drug ATO in human leukemia cell lines [20]. Therefore, we here explored whether co-treatment with BrMC could improve the apoptotic action of ATO. Our data indicated that BrMC cooperated with ATO in inducing apoptosis in human myeloid leukemia cells. The potentiation of ATO toxicity by BrMC correlates to, and may be explained at least in part by, the ability of the flavonoid analog to down-regulate Akt phosphorylation and to decrease the intracellular GSH content in the leukemia cell model.

Materials and Methods

Chemicals

All materials used for cell culture were obtained from Invitrogen (Carlsbad, USA). BrMC was a gift from Prof Jianguo Cao (Laboratory of Medicine Engineering, Medical College, Hunan Normal University, Changsha). Chrysin, ATO, ethidium bromide (EB), acridine orange (AO), propidium iodide (PI), *N*-acetylcysteine (NAC), GSH, butylated hydroxyanisole (BHA), mannitol, and LY294002 were obtained from Sigma Chemical Co. (St Louis, USA). The caspase-3 activity detection kit, caspase-8 colorimetric activity assay kit, and caspase-9 colorimetric activity assay kit were from Millipore (Billerica, USA). Caspase inhibitors, such as z-VAD-fmk, z-IETD-fmk, and z-LEHD-fmk, were purchased from R&D Systems (Minneapolis, USA). Rhodamine (RDM) 123 and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were from Molecular Probes (Eugene, USA). Rabbit anti-human Akt, phospho-Akt (Ser^{473}) and horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Cell Signaling Technology (Beverly, USA). Polyclonal antibodies against procaspase-3, and procaspase-9 and monoclonal antibodies against Bcl-2, Bax, X-linked inhibitor of apoptosis (XIAP), Bcl-XL, Bid, cytochrome *c*, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, USA).

Cell lines

U937, HL-60, and Jurkat leukemia cells were obtained from the China Center for Type Culture Collection (Wuhan, China) and cultured in RPMI 1640 media supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine and 10% heat-inactivated fetal bovine serum. Cells in logarithmic growth were seeded at 1×10^5/ml for study.

In vitro cytotoxicity assay

Cells were seeded in 96-well plates at a density of 2×10^3 cells/well in 100 μl medium for 24 h to allow adhering. Then cells were treated with various concentrations of BrMC or ATO alone or in combination. After drug exposure for 20 h, the 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide (MTT) solution (5 g/l) was added to the plates. The cells were incubated at 37°C for another 4 h. The formazan was dissolved in 100 μl/well DMSO (dimethyl sulfoxide), and the absorbance was detected at 490 nm using the EL×800 Strip Reader (Bio-Tek, Winooski, USA). All MTT experiments were performed in triplicate and repeated at least 3 times. The percentage of cytotoxicity was calculated as follows: cytotoxicity (%) = $(1-A_{490}$ of experimental well$)/A_{490}$ of control well. The IC_{50} (defined as the drug concentration at which 50% cell growth was inhibited) was assessed from the dose−response curves. The data were analyzed using Calcusyn program to determine the IC_{50} of each drug. The combination index (CI)-isobologram by Chou and Talalay [21] was used to analyze the drug combination: $CI = IC_{50\ (AB)}/(IC_{50\ (A)} + IC_{50\ (B)})$ (A, B represent different drugs). $CI > 1$, $CI = 1$, and $CI < 1$ indicate antagonism, additive effect, or synergism, respectively.

Quantitation of apoptotic cells

Apoptotic cells were examined morphologically after staining with AO and EB [22] for observation of chromatin condensation or cell nuclear fragment. Cells were viewed and counted using a Nikon eclipse TS100 inverted microscope at 400× magnification with excitation filter 480/30 nm, dichromatic mirror cut-on 505 nm wave length and barrier filter 535/40 nm. Tests were done in triplicate, counting a minimum of 200 total cells each. In addition, apoptotic cells were also analyzed using fluorescence-activated cell sorting (FACS) after staining with annexin V-fluorescein isothiocyanate [23] for determination of phosphatidylserine translocation. Viable cells were negative for both PI and Annexin-V; apoptotic cells were positive for Annexin-V and negative for PI, whereas late apoptotic dead cells displayed both high Annexin-V and PI labeling. Necrotic cells were positive for PI and negative for Annexin-V.

Analysis of caspase-3, -8, and -9 activities

The activities of caspase-3, -8, and -9 were evaluated using the caspase-3 activity detection kit, the caspase-8 colorimetric activity assay kit, and the caspase-9 colorimetric activity assay kit, respectively. Briefly, cell lysates were prepared after treatment with the various concentrations of BrMC or ATO alone or in combination. The assays were

performed in 96-well plates by incubating 20 μg of cell lysates in 100 μl of reaction buffer (1% NP-40, 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 10% glycerol) containing 5 μM of caspase-3 substrate Ac-DEVD-*p*NA, caspase-8 substrate Ac-IETD-*p*NA, or caspase-9 substrate Ac-LEHD-*p*NA. Mixtures were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with an enzyme-labeling instrument (ELX-800 type; Bio-Tek). In the caspase inhibitors assay, cells were pretreated with caspase inhibitors (10 μM z-VAD-fmk, z-IETD-fmk, or z-LEHD-fmk) for 1 h prior to the addition of the agents tested.

Determination of ROS

Intracellular ROS accumulation was measured by flow cytometry using the fluorescent probe DCFH-DA [24]. Briefly, cells were incubated with 10 μM of DCFH-DA for 30 min at 37°C in the dark. After incubation, the cells were washed with PBS and analyzed within 30 min using an FACScan (Becton Dickinson, San Jose, USA) equipped with an air-cooled argon laser tuned to 488 nm. The specific fluorescence signals corresponding to DCFH-DA were collected with a 525-nm band-pass filter. As a rule, 10,000 cells were counted in each experiment.

Measurement of mitochondrial transmembrane potential

The mitochondrial transmembrane potential ($\Delta\psi m$) was determined by flow cytometry after cell loading with Rhodamine 123, as described by Troyano *et al*. [25]. Briefly, cells were washed twice with PBS and incubated with 1 μg/ml Rhodamine 123 at 37°C for 30 min. Cells were then washed twice with PBS, and Rhodamine 123 intensity was determined by flow cytometry. Cells with reduced fluorescence (less Rhodamine 123) were counted as having lost some of their $\Delta\psi m$.

Measurement of intracellular GSH

Intracellular GSH contents were measured using a glutathione assay kit (Calbiochem, San Diego, USA). In brief, 5×10^6 cells were homogenized in 5% metaphosphoric acid using a Teflon pestle (Overhead Stirrer; Wheaton Instruments, Millville, USA). Particulate matter was separated by centrifugation at 4000 *g*. The supernatant solution was used for GSH measurement according to the manufacturer's instructions. The GSH content was expressed as nM/10^6 cells.

Cellular fractionation

For assay of release of cytochrome *c*, cells were fractionated into cytosolic and mitochondrial fractions as described by Ling *et al*. [26]. In brief, cells were incubated in a buffer (containing 20 mM HEPES-KOH, pH 7.2, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) at 4°C for 10 min, and then, the cells were homogenized with a Dounce homogenizer for 20 strokes. After addition of a buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM EGTA, and 5 mM Tris-HCl (pH 7.5), the homogenates were centrifuged at 1,000 *g* for 10 min at 4°C. The supernatants were further centrifuged at 15,000 *g* for 30 min at 4°C and collected as the cytosolic fraction. The pellet was further dissolved with lysis buffer containing 1% sodium dodecyl sulfate (SDS) as the mitochondrial fraction.

Western blot analysis

Cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1% (w/v) SDS, 0.5 mM phenylmethanesulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM dithiothreitol. Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, USA). Total protein (50 μg) was electrophoresed in 8%–12% SDS polyacrylamide gels and transferred to polyvinylidene fluoride membrane (Millipore). After incubating with 5% non-fat milk for 1 h, the membranes were incubated with the indicated primary antibody overnight at 4°C, washed with TBS (pH 6.8) and Tween 20 (TBS-T) three times, incubated with the secondary antibody for 1 h at room temperature, and washed with TBS-T three times. The immunocomplex was visualized by enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK).

Statistical analysis

The database was set up with the SPSS 15.0 software package (SPSS Inc, Chicago, USA) for analysis. Data were presented as mean ± SD. The means of multiple groups were compared with one-way analysis of variance, after checking for variance equality, and the two–two comparisons among the means were performed using the least-significant difference method. Statistical comparison was also performed with the two-tailed *t*-test when appropriate. $P < 0.05$ was considered as statistically significant.

Results

Effects of ATO alone or in combination with BrMC on apoptosis in leukemia cells

The cytotoxicity of BrMC and ATO alone or in combination was examined in U937 cells, as determined by MTT assay. The IC_{50} of BrMC and ATO alone were 2.16 and 1.93 μM, respectively. When in combination with BrMC (0.5, 1.0, and 2.0 μM), the IC_{50} of ATO were 0.99 (in the presence of 0.5 μM BrMC), 0.48 (in the presence of 1.0 μM BrMC), and 0.44 (in the presence of 2.0 μM BrMC) μM, respectively. The coefficient of drug interaction (CI) was 0.242 for 0.5 μM BrMC plus 0.5 μM ATO, 0.117 for 1.0 μM BrMC

plus 1.0 μM ATO, and 0.107 for 2.0 μM BrMC plus 2.0 μM ATO, respectively. The similar results were observed for chrysin (ChR) and ATO alone or in combination in U937 cells. The coefficient of drug interaction (CI) was 0.868 for 2.5 μM ChR plus ATO, 0.431 for 5.0 μM ChR plus ATO, and 0.109 for 10.0 μM ChR plus ATO. The data suggest that BrMC or ChR acts synergistically with ATO in cytotoxicity to U937 cells.

The apoptotic induction ability of BrMC and ATO alone or in combination was examined in U937 cells, as determined by chromatin fragmentation. At 24 h of treatment, detectable apoptosis was caused by BrMC at 2 μM, and ATO at 2 μM [**Fig. 1(A)**]. When used together, the synergetic action of BrMC and ATO to induce apoptosis was stronger than all tested concentrations of ATO alone, with maximum efficacy at 2 μM ATO plus 2 μM BrMC [**Fig. 1(A)**]. Using this combination, apoptosis was detected at early 12 h of treatment and increased thereafter [**Fig. 1(B)**]. The cooperation between ATO and BrMC to induce apoptosis was corroborated by measuring the frequency of annexin V-positive cells in flow cytometry assays [**Fig. 1(C)**]. For comparison, determinations were performed using the lead compound chrysin instead of BrMC. It was observed that chrysin at 10 μM cooperated with ATO in inducing apoptosis in U937 cells with efficacy similar to that of BrMC [**Fig. 1(D)**]. Combination of BrMC and ATO was also found to synergentically induce apoptosis in Jurkat and HL-60 cells [**Fig. 1(E,F)**].

Induction of apoptosis by co-treatment with BrMC and ATO correlated with activation of the mitochondrial pathway in leukemia cells

ATO has been characterized as a mitochondria-targeting drug, capable of binding to the ANT [2] and interfering with mitochondrial respiration [3]. Therefore, we investigated the dissipation of $\Delta\psi m$ as a manifestation of mitochondrial dysfunction, as well as the behavior of factors that regulate the mitochondrial death pathway. **Figure 2** indicated that treatment with ATO or BrMC alone had little effect on $\Delta\psi m$, but the combination clearly elicited $\Delta\psi m$ dissipation, as indicated by the decrease in Rhodamine 123-derived fluorescence in flow cytometry assays [**Fig. 2(A)**]. ATO or BrMC alone also had little effect on mitochondrial protein mobilization and XIAP expression, but co-treatment clearly elicited cytochrome *c* release from mitochondria as well as XIAP down-regulation [**Fig. 2(B)**], as determined by immunoblotting using cytosolic and total cellular extracts, respectively. Treatment with ATO plus BrMC did not affect total Bcl-2 expression, but it caused a decrease in Bcl-XL expression and an increase in Bax expression using the total cellular extracts and the mitochondrial fraction, respectively [**Fig. 2(B,C)**]. Pretreatment with 10 mM NAC could block the increase of Bax expression induced by combination with ATO and BrMC [**Fig. 2(C)**], suggesting that Bax protein translocation was associated with intracellular redox state. The co-treatment also caused a decrease in the amount of Bid proform (21 kDa), which represents indirect evidence of protein truncation/activation [**Fig. 2(B)**]. In addition, treatment with ATO plus BrMC induced the cleavage of caspase-3 and -9, as evidenced by reduction of pro-caspase-3 and pro-caspase-9 using western blotting [**Fig. 2(D)**]. However, pretreatment with 10 mM NAC attenuated the activation of caspase-3 and -9, indicating that intracellular redox system was involved in the caspase activation.

The observation that Bid was cleaved in response to ATO plus BrMC led us to investigate the possible activation of caspase-8, because Bid is a substrate of this caspase [27]. We observed that ATO plus BrMC in fact caused caspase-8 activation and that z-VAD-fmk and z-IETD-fmk completely block caspase-8 activation, but z-LEHD-fmk had no effect [**Fig. 2(E)**]. In addition, the co-treatment with ATO and BrMC activated caspase-3 and z-VAD-fmk completely block caspase-3 activation. However, z-IETD-fmk and z-LEHD-fmk partially attenuated caspase-3 activation. Moreover, z-VAD-fmk and z-LEHD-fmk completely blocked and z-IETD-fmk slightly reduced caspase-9 activation induced by the combination with ATO and BrMC. Meanwhile, the efficacy of z-IETD-fmk and z-LEHD-fmk for attenuating apoptosis was lower than the pan-caspase inhibitor z-VAD-fmk in U937 cells [**Fig. 2(F)**].

Induction of apoptosis by co-treatment with BrMC and ATO correlated with the inhibition of Akt phosphorylation

Earlier reports indicated that chrysin and its analog might inhibit Akt phosphorylation [8,9,13]. Therefore, we investigated whether the potentiation of apoptosis induction activity of ATO by BrMC was associated with Akt inactivation. To examine the efficacy of BrMC as an Akt inhibitor in U937 cells, we performed a preliminary assay in which cells were first serum-starved to decrease the basal level of phosphorylated Akt and followed by BrMC treatment in the presence or absence of 20 μM LY294002 for 1 h and then stimulation with 10 μg/ml insulin. The assay corroborated the capacity of the flavonoid analog to inhibit insulin-elicited Akt phosphorylation [**Fig. 3(A)**]. We failed to detect significant alterations in the constitutive levels of phosphorylated Akt upon treatment with BrMC alone, but the combination of both agents decreased Akt phosphorylation on 6 h of treatment [**Fig. 3(B)**]. Notably, the administration of LY294002, a specific inhibitor of PI3K, increased induction of apoptosis by ATO plus BrMC in U937, Jurkat, and HL-60 cells [**Fig. 3(C)**], indicating that apoptosis induction in the cell lines is sensitive to down-regulation of the PI3K/Akt pathway.

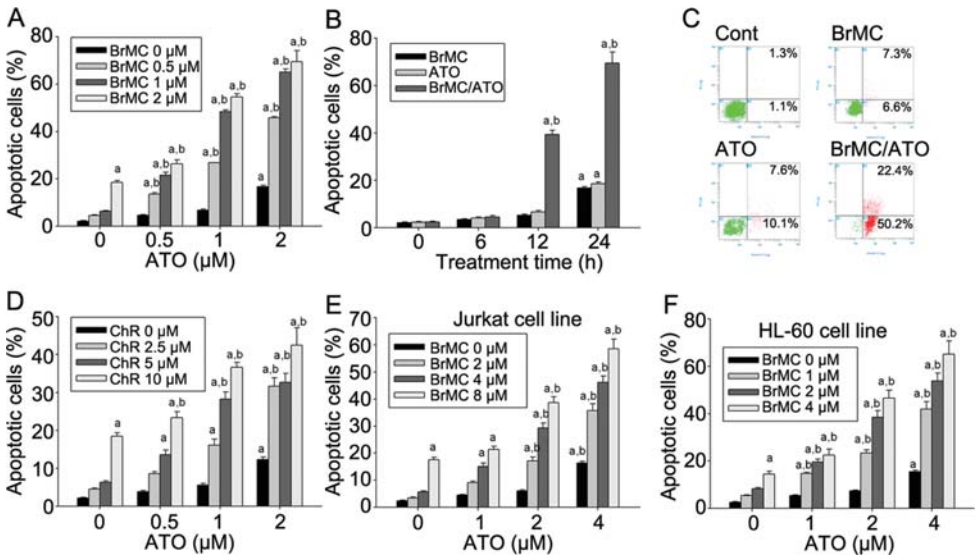

Figure 1 Apoptosis-inducing activity of ATO alone or in combination with BrMC in leukemia cells (A) U937 cells were treated with BrMC or ATO alone or in combination for 24 h at indicated concentrations. Apoptotic cells were examined morphologically after staining with AO and EB. Data are expressed as mean ± SD from three individual experiments. $^{a}P < 0.05$ versus treatment with the solvent (dimethyl sulfoxide, DMSO); $^{b}P < 0.05$ versus treatment with ATO alone. (B) U937 cells were treated with 2 μM BrMC or 2 μM ATO alone or in combination for indicated time. Apoptotic cells were examined morphologically after staining with AO and EB. Data are expressed as mean ± SD from three individual experiments.$^{a}P < 0.05$ versus treatment for 0 h; $^{b}P < 0.05$ versus treatment with ATO alone. (C) U937 cells were treated with 2 μM BrMC or 2 μM ATO alone or in combination for 24 h. Apoptotic cells were examined using fluorescence-activated cell sorting (FACS) after staining with annexin V-FITC. Data are representative of three independent experiments. (D) U937 cells were treated with chrysin (ChR) or ATO alone or in combination for 24 h at indicated concentrations. Apoptotic cells were examined morphologically after staining with AO and EB. Data are expressed as mean ± SD from three individual experiments. $^{a}P < 0.05$ versus treatment with DMSO; $^{b}P < 0.05$ versus treatment with ATO alone. (E) Jurkat cells were treated with BrMC or ATO alone or in combination for 24 h at indicated concentrations. Apoptotic cells were examined morphologically after staining with AO and EB. Data are expressed as mean ± SD from three individual experiments. $^{a}P < 0.05$ versus treatment with DMSO; $^{b}P < 0.05$ versus treatment with ATO alone. (F) HL-60 cells were treated with BrMC or ATO alone or in combination for 24 h at indicated concentrations. Apoptotic cells were examined morphologically after staining with AO and EB. Data are expressed as mean ± SD from three individual experiments. $^{a}P < 0.05$ versus treatment with DMSO; $^{b}P < 0.05$ versus treatment with ATO alone.

Induction of apoptosis by co-treatment with BrMC and ATO correlated with GSH depletion and ROS generation

A previous study showed that induction of apoptosis by BrMC was due to increased intracellular ROS [19]. In this study, ROS levels were compared in U937 cells after treatment with BrMC and ATO at 2 μM, alone or in combination. ROS levels were increased in U937 cells by co-treatment with BrMC and ATO, but not in U937 cells treated with BrMC or ATO alone [**Fig. 4(A)**]. In addition, the pretreatment of antioxidants, NAC, mannitol, and BHA efficiently blocked the induction of ROS generation by co-treatment with BrMC and ATO [**Fig. 4(A)**].

The antioxidant NAC, which can reduce ROS levels [19], was found to attenuate apoptosis induced by co-treatment with BrMC and ATO in U937 cells [**Fig. 4(B)**], further showing that ROS plays a key role in apoptotic induction by combining with BrMC and ATO.

Earlier studies have demonstrated that ATO toxicity is strictly dependent on intracellular GSH content [16], pharmacologic down-regulation of PI3K/Akt causes GSH depletion in cancer cells [27], and flavonoids may alter the intracellular GSH content [12,13]. Therefore, intracellular GSH in cells treated with ATO and BrMC, alone or in combination, were measured. Determinations at 24 h of treatment showed that 1 or 2 μM ATO did not cause significant alterations, 1 or 2 μM BrMC alone slightly decreased GSH content, and co-treatment with 2 μM ATO exacerbated the decrease of intracellular GSH content produced by 1 or 2 μM BrMC [**Fig. 4(C)**]. In addition, time-course studies indicated that ATO plus BrMC caused a progressive decrease in GSH content from 3 h onwards [**Fig. 4(D)**], which was ahead of the time of apoptosis execution of 12 h [**Fig. 1(C)**]. Importantly, the pan-caspase inhibitor z-VAD-fmk, which successfully inhibited apoptosis, failed to prevent GSH decrease [**Fig. 4(E,F)**]. This excludes the possibility that GSH depletion in cells treated with ATO plus BrMC could be a trivial, secondary consequence of cell death. Chrysin also elicited GSH depletion, which was increased in the presence of ATO [**Fig. 4(C)**].

To shed light on the mechanisms accounting for GSH depletion, as well as on the relationship between GSH depletion and apoptosis potentiation, we determined the effects of thiol antioxidants NAC and exogenous GSH, and

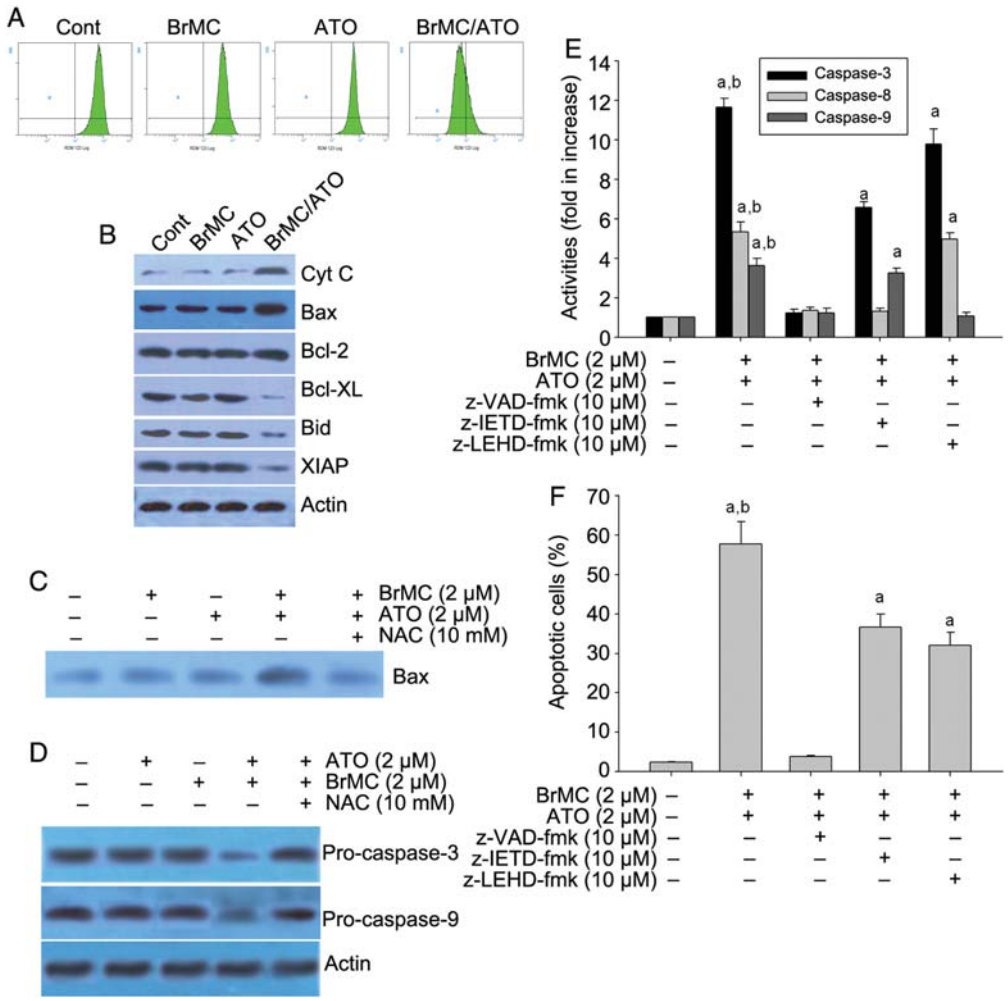

Figure 2 Disruption of mitochondrial transmembrane potential (Δψm) and expression of apoptosis-related proteins by treatment with BrMC and ATO alone or in combination in leukemia cells (A) U937 cell cultures were left untreated (control), or treated with 2 μM ATO alone, 2 μM BrMC alone, or the combination of both drugs. Δψm dissipation was determined by flow cytometry after cell loading with RDM 123. (B) The level of cytochrome *c* in cytosol and expression of XIAP, Bcl-2, Bcl-XL, Bid (21-kDa proform), and Bax in total cellular extracts were determined by immunoblot. (C) U937 cells were left untreated (control), or treated with 2 μM ATO or 2 μM BrMC or both in the presence or absence of 10 mM NAC. The level of Bax was analyzed by western blot in mitochondrial fraction. (D) U937 cells were left untreated (control), or treated with 2 μM ATO or 2 μM BrMC or both in the presence or absence of 10 mM NAC. The expression of pro-caspase-3 and pro-caspase-9 in total cellular extracts was analyzed by western blot. (E) U937 cells were treated with BrMC and ATO alone or in combination for 24 h in the presence or absence of the caspase inhibitors. Enzymatic activities of caspase-3, -8, and -9 were determined by incubation of 20 μg of total protein with 200 μM chromogenic substrate (Ac-DEVD-*p*NA, Ac-IETD-*p*NA, or Ac-LEHD-*p*NA) in 100 μl of assay buffer for 2 h at 37°C. The release of chromophore *p*-nitroanilide (*p*NA) was monitored spectrophotometrically at 405 nm. Data are expressed as mean ± SD from three individual experiments. $^{a}P < 0.05$ versus 0.1% DMSO; $^{b}P < 0.05$ versus pretreatment with the caspase inhibitors. (F) U937 cells were treated with BrMC and ATO alone or in combination for 24 h in the presence or absence of the caspase inhibitors. Apoptotic cells were examined morphologically after staining with AO and EB. Data are expressed as mean ± SD from three individual experiments. $^{a}P < 0.05$ versus treatment with DMSO; $^{b}P < 0.05$ versus pretreatment with the caspase inhibitors.

non-thiol antioxidants BHA and mannitol, on GSH content and apoptotic cell death induced by co-treatment with BrMC and ATO. **Figure 4(E,F)** showed that the thiol antioxidants NAC and exogenous GSH restored GSH content and attenuated apoptosis induced by co-treatment with BrMC and ATO. In contrast, although the pretreatment of mannitol and BHA efficiently blocked induction of ROS generation [**Fig. 4(A)**], they failed to restore GSH content and attenuate apoptosis induced by co-treatment with BrMC and ATO [**Fig. 4(E,F)**]. These results suggest that induction of apoptosis in U937 cells treated with BrMC plus ATO is at least partly dependent on GSH depletion.

Discussion

In the present study, we showed that the novel flavonoid analog BrMC and its lead compound chrysin cooperated with the anti-leukemic agent ATO in inducing apoptosis in human leukemia cells. Although a detailed analysis of the apoptotic machinery was beyond the scope of this work, our data

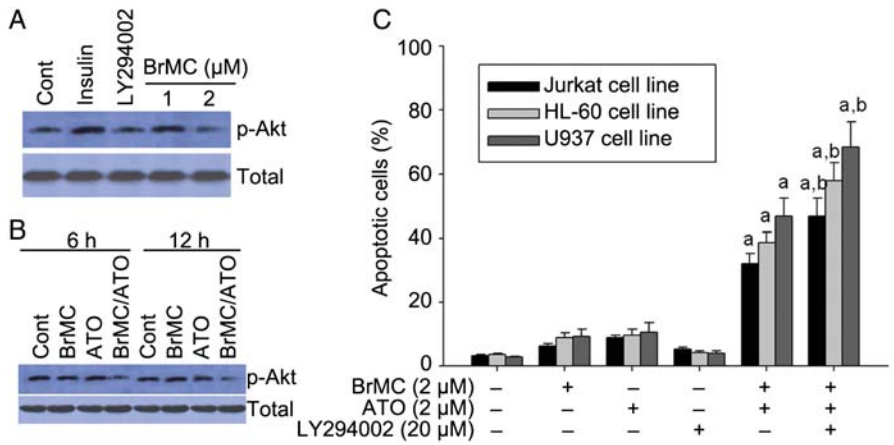

Figure 3 Effects of BrMC and ATO alone or in combination on Akt phosphorylation in leukemia cells (A) Cells were treated with serum-free media for 24 h to decrease the basal level of phosphorylated Akt (as control). Then the cells were treated with indicated concentrations of BrMC in the absence or presence of 20 μM LY294002 for 1 h followed by stimulated with 10 μg/ml insulin The levels of total and phosphorylated (p) Akt were determined by immunoblotting. (B) The levels of total and phosphorylated (p) Akt were determined by immunoblotting in normally growing U937 cells at indicated times of treatment with 2 μM ATO and 2 μM BrMC alone or in combination. (C) Cells were treated with 2 μM BrMC and 2 μM ATO alone or in combination for 24 h in the presence or absence of 20 μM LY294002. Apoptotic cells were examined morphologically after staining with AO and EB. Data are expressed as mean ± SD from three individual experiments. $^{a}P < 0.05$ versus treatment with DMSO; $^{b}P < 0.05$ versus pretreatment with LY294002.

indicated that BrMC and ATO cooperated in activating the mitochondrial executioner pathway; this finding is consistent with the characterization of both agents as mitochondria-targeting drugs [2,3,12]. Two key events in this pathway are (1) the release of cytochrome *c* from mitochondria to the cytosol, which is required for apoptosome assembly and subsequent activation of caspase-9/3 and (2) XIAP down-regulation, which activates caspases by inhibiting XIAP binding to caspases [28]. The potentiation of cytochrome *c* release by ATO plus BrMC was consistent with the decreased expression of Bcl-XL (anti-apoptotic) and the increased expression of Bax (pro-apoptotic). Importantly, our results also demonstrated that the co-treatment of ATO and BrMC translocated Bax protein from cytosol to mitochondria in U937 cells (as an indication of Bax translocation/activation) [29] [**Fig. 2(C)**]. Moreover, inhibitors of apoptosis were degraded by the ubiquitin–proteasome system [30]. A previous study has reported an increase in ubiquitin-conjugated proteins in arsenic-treated cells [31]. Therefore, XIAP down-regulation probably reflects an increase in protein degradation. Notably, in addition to the mitochondrial pathway, co-treatment with BrMC plus ATO also succeeded in activating the caspase-8/Bid pathway. The possibility that caspase-8 activation by ATO is a death receptor-mediated event or, alternatively, represents a secondary event derived from mitochondrial activation, has been analyzed in different leukemia cell models, with non-coincident results [32–34]. Apoptosis induced by co-treatment with BrMC plus ATO was totally abrogated by the pan-caspase inhibitor z-VAD-fmk but only partially reduced by the caspase-8-specific inhibitor z-IETD-fmk, suggesting that caspase-8/Bid activation functions as an amplification loop for the final apoptotic result.

Moreover, the present data indicated that co-treatment with BrMC and ATO down-regulated Akt phosphorylation and decreased the intracellular GSH level in U937 cells, which is consistent with the observations using ATO in combination with typical PI3K/Akt inhibitors [35]. In view of the fact that NAC was able to restore the intracellular GSH level, and on the ground of the well-proven inverse relationship between ATO toxicity and GSH content [15,16], it may be reasonably proposed that GSH depletion explains at least in part the increased toxicity in cells treated with BrMC plus ATO. Because ATO detoxification is largely catalyzed by glutathione S-transferases [36], intracellular GSH depletion may result in the increase in the concentration of intracellular free ATO, and hence in toxicity. Woo *et al.* [9] showed that chrysin induced apoptosis in association with the activation of caspase-3, involving inactivation of Akt signaling and down-regulation of XIAP in U937 cells. Our findings also provided evidence that thiol-containing antioxidants rather than non-thiol antioxidants reduced co-treatment-induced apoptosis. This suppression correlated with their ability to prevent the decrease of GSH content induced by treatment with BrMC plus ATO. Therefore, it is likely that NAC and GSH inhibit the co-treatment-induced apoptosis via their reducing activity. Moreover, our findings showed that administration of LY294002 increased the toxicity of ATO plus BrMC in U937, Jurkat, and HL-60 cells [**Fig. 3(C,D)**], indicating that apoptosis induction is sensitive to down-regulation of the PI3K/Akt pathway. Interestingly, the combination of ATO and BrMC decreased GSH content from 3 h onwards [**Fig. 4(D)**], and before the time of reduction of Akt phosphorylation from 6 h of treatment onward [**Fig. 3(B)**]. The

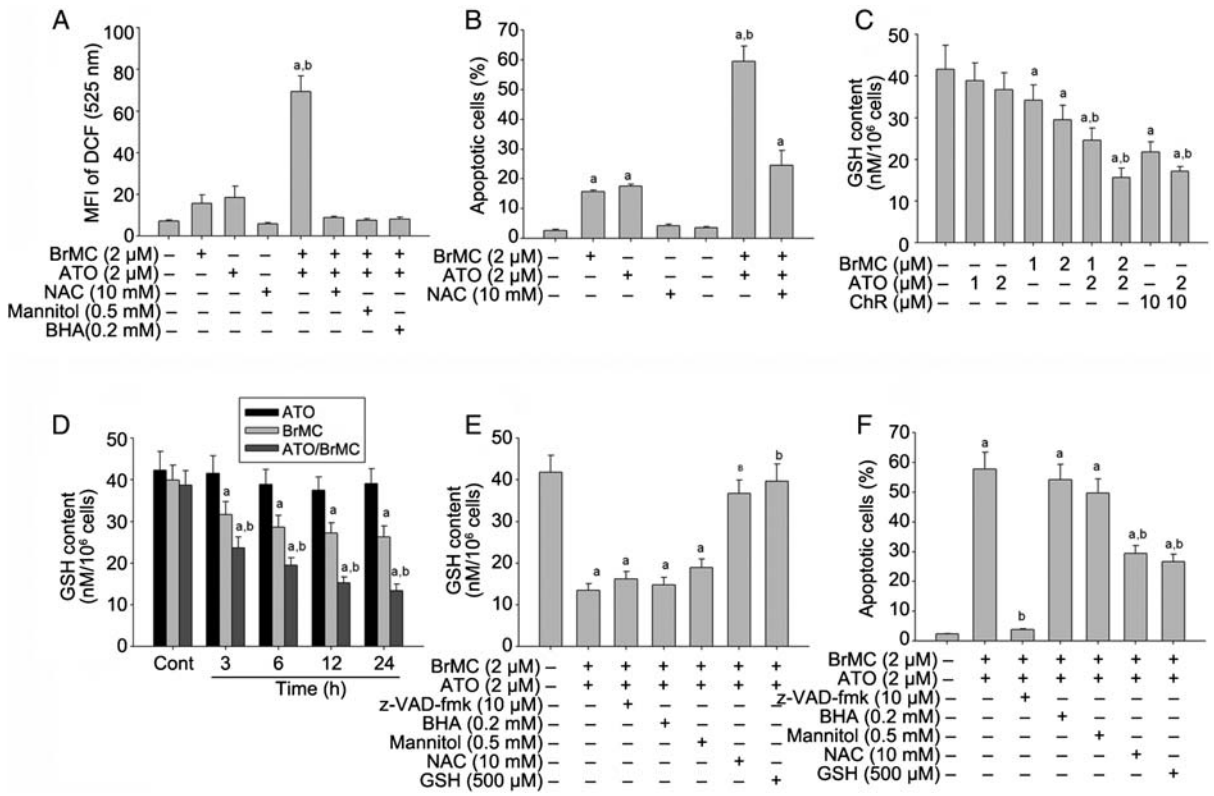

Figure 4 Effects of BrMC and ATO alone or in combination on ROS generation and GSH depletion in U937 cells (A) U937 cells were treated with BrMC and ATO or in combination for 3 h in the presence or absence of 10 mM NAC, 0.5 mM mannitol, or 0.2 mM BHA. The mean fluorescence intensity of DCF was measured by FCM using DCFH-DA fluorescence. Data are expressed as mean ± SD from three individual experiments. $^{a}P < 0.05$ versus treatment with DMSO; $^{b}P < 0.05$ versus pretreatment with NAC, mannitol or BHA. (B) U937 cells were treated with BrMC and ATO alone or in combination for 24 h in the presence or absence of 10 mM NAC. Apoptotic cells were examined morphologically after staining with AO and EB. Data are expressed as mean ± SD from three individual experiments. $^{a}P < 0.05$ versus treatment with DMSO; $^{b}P < 0.05$ versus pretreatment with NAC. (C) U937 cells were treated with BrMC or ChR and ATO alone or in combination at indicated concentration for 24 h. GSH content was measured using a glutathione assay kit. Data are expressed as mean ± SD from three individual experiments. $^{a}P < 0.05$ versus treatment with DMSO; $^{b}P < 0.05$ versus treatment with BrMC or ChR alone. (D) U937 cells were treated with 2 μM ATO and 2 μM BrMC alone or in combination for indicated time. GSH content was measured using a glutathione assay kit. Data are expressed as mean ± SD from three individual experiments. $^{a}P < 0.05$ versus treatment with DMSO; $^{b}P < 0.05$ versus treatment with ATO or BrMC alone. (E) U937 cells were co-treated with BrMC and ATO for 24 h in the presence or absence of thiol antioxidants NAC and GSH, and non-thiol antioxidants butylated hydroxyanisole (BHA) and mannitol. GSH content was measured using a glutathione assay kit. Data are expressed as mean ± SD from three individual experiments. $^{a}P < 0.05$ versus treatment with DMSO; $^{b}P < 0.05$ versus treatment with ATO plus BrMC. (F) U937 cells were co-treated with BrMC and ATO for 24 h in the presence or absence of thiol antioxidants NAC and GSH, and non-thiol antioxidants BHA and mannitol. Apoptotic cells were examined morphologically after staining with AO and EB. Data are expressed as mean ± SD from three individual experiments. $^{a}P < 0.05$ versus treatment with DMSO; $^{b}P < 0.05$ versus treatment with ATO plus BrMC.

findings suggest that the reduction of Akt phosphorylation seems not be the cause of GSH depletion. Indeed, the present results do not exclude the possibility that factors other than the Akt/GSH pathway may participate in regulating the susceptibility to apoptosis. For instance, ATO might directly inhibit the expression or activity of multidrug resistance-associated protein (MRP) drug efflux pumps, thus reducing drug detoxification [36].

In addition, our findings showed that ROS generation was increased by co-treatment with BrMC and ATO and that the antioxidant NAC attenuated apoptosis induced by co-treatment with BrMC and ATO in U937 cells. BrMC has been shown to promote generation of ROS and ROS-dependent sustained activation of JNK [19]. Thus, whether the combination with BrMC and ATO sustained the activation of JNK remains to be explored.

Clinically, resistance to apoptosis is a major obstacle in chemotherapeutic treatments of cancers. The ability of BrMC to sensitize leukemia cells to ATO-induced apoptosis at low concentrations makes BrMC a potentially effective preventative and/or therapeutic agent against leukemia. However, the potential clinical implications of our studies will depend on whether or not BrMC can be safely administered to patients at pharmacologically active doses. Oral flavonoids cannot be utilized in the clinic since they are rapidly metabolized in the liver and intestinal wall. However, Walle *et al.* [37] reported that 5,7-dimethoxyflavone (5,7-DMF) was clearly detected in plasma with a peak concentration of 2.5 ± 0.8 μM (mean ±

SEM) 1 h after gavage (5 mg/kg each) in rats. In that study, the area under the plasma concentration-time curve was 58.8 μg/ml/min. 5,7-DMF was also clearly detected in the liver, lung, and kidney tissues with the concentration in the liver exceeding that in the plasma by as much as seven-folds (16.5 ± 5 μM) 1 h after administration. The liver, which showed the highest accumulation of 5.7-DMF, might be a site where DMF exerted its greatest activity. Therefore, additional *in vivo* studies are needed to evaluate the applicability of BrMC as a chemopreventive and/or therapeutic agent for cancer.

In summary, the present results indicated that BrMC selectively potentiated the apoptotic action of ATO in myeloid leukemia cells, an effect that may be explained at least in part by the capacity of the flavonoid analog to cause Akt down-regulation and GSH depletion. These observations may offer a rationale for the use of the novel flavonoid analog to improve the clinical efficacy of ATO.

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