Induction of B-Chronic Lymphocytic Leukemia Cell Apoptosis by Arsenic Trioxide Involves Suppression of the Phosphoinositide 3-Kinase/Akt Survival Pathway via c-jun-NH₂ Terminal Kinase Activation and PTEN Upregulation

Javier Redondo-Muñoz, Elizabeth Escobar-Díaz, Mercedes Hernández del Cerro, Atanasio Pandiella, María José Terol, José A. García-Marco, and Angeles García-Pardo

Abstract

Purpose: Arsenic trioxide (ATO) induces B-cell chronic lymphocytic leukemia (B-CLL) cell apoptosis in vitro. We sought to study the mechanism involved in this effect and whether ATO is suitable for combination therapies with protein kinase inhibitors.

Experimental Design: B-CLL cells were isolated from the peripheral blood of 28 patients. Cell viability studies with ATO alone or in combination with kinase inhibitors were done by flow cytometry, Western blotting, and immunofluorescence analyses.

Results: After 48 hours, 3 μmol/L ATO induced apoptosis (average 75%) in all B-CLL samples studied and with minimal effect on normal peripheral blood lymphocytes. Apoptosis entailed Akt and NF-κB inactivation, XIAP downregulation, and PTEN upregulation, thus implying inhibition of the phosphoinositide 3-kinase (PI3K) survival pathway. Indeed, the combination of ATO and PI3K inhibitors increased the apoptotic effect of either agent alone. ATO also induced c-jun-NH₂ terminal kinase (JNK) activation, and this was crucial and required for subsequent apoptotic events, as inhibiting JNK activity by either gene silencing or specific inhibitors prevented Akt and NF-κB inactivation, caspase activation, and mitochondrial damage. Moreover, JNK activation was the earliest response to ATO, preceding and determining reactive oxygen species production.

Conclusions: We identified the mechanism involved in ATO action on B-CLL cells and show that the combination of low doses of ATO and PI3K inhibitors efficiently induces B-CLL cell death. ATO may therefore constitute an efficient treatment for B-CLL, particularly in combined therapies.

B-cell chronic lymphocytic leukemia (B-CLL) is characterized by the accumulation of malignant CD5⁺ B lymphocytes in the peripheral blood, and their progressive infiltration of lymphoid tissues (1, 2). The course of the disease is very heterogeneous, and several molecular markers (high CD38 or/and ZAP-70 expression, del17p13, IgVH unmutated status) seem to be associated with poor response to therapy and worse prognosis (1, 2). Most current treatments for B-CLL consist in the administration of the purine analogue fludarabine, either alone or in combination with other therapeutic drugs (3, 4). Although many patients respond well to these protocols and complete remission can be attained, patients eventually relapse and may die from the disease. It is therefore crucial to continue searching for new compounds that could be useful in the treatment of this type of leukemia, especially in the advanced setting.

Arsenic trioxide (ATO) has been used in traditional Chinese medicine and is currently successfully employed for the treatment of acute promyelocytic leukemia (APL; refs. 5–8). ATO is also being trialed for other hematologic malignancies, including chronic myeloid leukemia, myelodysplastic syndrome, and non-M3 acute myelocytic leukemia and multiple myeloma, either as monotherapy or combined therapy (7, 9). The cytotoxic effect of ATO on these systems involves complex mechanisms that are not fully understood. In many cases, induction of apoptosis
PTN and JNK in ATO-Induced B-CLL Cell Apoptosis

Translational Relevance

B-cell chronic lymphocytic leukemia (B-CLL) remains an incurable disease, making it crucial to continue searching for novel therapies. Arsenic trioxide (ATO) is an efficient treatment for acute promyelocytic leukemia, and it is being trialed for other hematologic malignancies. To explore the potential clinical use of ATO in B-CLL we studied the mechanism by which ATO induces apoptosis in B-CLL cells. We report that JNK and PTEN are crucial molecules and inhibit phosphoinositide 3-kinase (PI3K) survival signaling in response to ATO. Accordingly, PI3K inhibitors efficiently enhanced the apoptotic effect of ATO. These effects were observed at low, nontoxic ATO doses and in all cases studied. Our results strongly suggest that ATO alone or combined with PI3K inhibitors may be considered an alternative therapy for B-CLL.

by ATO involves the release of reactive oxygen species (ROS), and experimental reduction of glutathione peroxidase increases the sensitivity to ATO (10, 11). Blocking the extracellular signal-regulated kinase (ERK)/ERK, phosphoinositide 3-kinase (PI3K)/Akt, or p38–mitogen-activated protein kinase signaling pathways has also been shown to enhance the apoptotic effect of ATO on APL, myeloma, and T-leukemia cells (10, 12–15). Additionally, ATO-induced apoptosis of myeloma and APL cells involves the activation of c-jun-NH2-kinase (JNK; refs. 16, 17).

Very few studies have addressed the effect of ATO on B-CLL cells. In analyses of a small number of B-CLL samples (18) or long exposure times (19), ATO was shown to induce apoptosis of B-CLL cells, concomitant with downregulation of Bcl-2. ATO also induced ROS generation and significantly enhanced the cytotoxic activity of 2-methoxyestradiol and ascorbic acid on B-CLL samples (20, 21). It was recently reported that ATO preferentially induced apoptosis in B-CLL cases with unfavorable prognosis (22). These previous studies strongly suggest that ATO, alone or in combination with other treatments, could be an efficient therapeutic agent for B-CLL. The mechanism by which ATO induces B-CLL cell apoptosis has not been established, and we have addressed this in the present report.

Materials and Methods

Patients, B-CLL cell purification, and normal peripheral blood lymphocytes

Following informed consent, B-CLL cells were purified from the peripheral blood of 28 patients (Table 1) by Ficoll-Hypaque (Nycomed) centrifugation and anti-CD3-conjugated Dynabeads (DynaBiotec ASA). Myeloid cells were not removed by these procedures, but they represent a minor contamination, as the resulting cell population was >95% CD19+, >70% CD5+, considered suitable for subsequent studies. Purified B-CLL cells were either used immediately or frozen and stored in liquid nitrogen.

Peripheral blood lymphocytes (PBL; T plus B lymphocytes) from five adult healthy donors were obtained from buffy coat preparations routinely used for monocytic cell purification. After Ficoll-Hypaque centrifugation, monocytic cells were removed using anti-CD14-conjugated microbeads and MACS separation columns (Miltenyi Biotec). B-CLL and control cells were cultured at 1.5 × 10⁶/mL in RPMI/10% fetal bovine serum, 100 μg/mL gentamicin and penicillin, and 100 U/mL streptomycin (Invitrogen Ltd.).

Approval was obtained from the Consejo Superior de Investigaciones Científicas Bioethics Review Board for these studies.

RNA interference experiments

The small interfering RNA (siRNA) sequence targeting JNK: sense 5′-CAAAAGAUCCCUIGCAAGCAdT-3′ (s11153), and the control siRNA sequence: sense 5′-AUUI-GUAUGCGAUGCAGACTT-3′, were custom-made by Ambion. For transfection of B-CLL cells, siRNAs (500 nmol/L final concentration) in 100 μL RPMI were incubated at room temperature for 10 minutes with 4.5 μL HiPerfect transfection reagent (Qiagen). The mixture was added dropwise to 2 × 10⁶ cells in RPMI/10% FCS, and transfected cells were used after 24 hours. The efficiency of gene silencing was analyzed by Western blotting.

Analysis of apoptosis and mitochondrial membrane potential (Δφm)

B-CLL cells (1.5 × 10⁵) in 100 μL RPMI/10% fetal bovine serum were placed in 96-well plates and treated with various concentrations of ATO or vehicle (PBS). After 24 to 48 hours cells were suspended (10⁶/mL) in 1× binding buffer (Bender Medsystems), containing either 3 μL FITC-Annexin V and 50 μg propidium iodide (PI), or PI alone. After 10 minutes, cells were analyzed by flow cytometry on a Coulter Epics XL. For combined treatments, B-CLL cells were incubated for 30 minutes with either SP600125 (10 μmol/L), SB203580, BisI, UO126, Tricirbine/API-2, LY294002 (all at 5 μmol/L), Z-VAD-FMK, or Z-IE TD-F MK (both at 50 μmol/L) prior to the addition of 2 μmol/L ATO. For Δφm measurements, B-CLL cells were incubated for 24 hours with or without 3 μmol/L ATO and treated for 20 minutes with 20 nmol/L DiOC₆ (Carlcochem) at room temperature in the dark. Cells were washed, resuspended in PBS, and analyzed by flow cytometry.

Measurement of intracellular ROS accumulation

B-CLL cells (5 × 10⁵) were treated with or without SP600125 (2-10 μmol/L), NAC (10 μmol/L), or MnTBAP (50 μmol/L) for 30 minutes and cultured in the presence or absence of 3 μmol/L ATO. After 16 hours, 5 μmol/L of H₂DCFDA were added and fluorescence was analyzed after 30 minutes by flow cytometry.
Preparation of nuclear and cytosolic extracts

B-CLL cells \((20 \times 10^6)\) with or without previous incubation (2 hours, 37°C) with 3 \(\mu\)mol/L ATO were harvested, washed, and resuspended in 400 \(\mu\)L of cold buffer A \([10\text{ mmol/L HEPES (pH 7.6), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.75 mmol/L spermidine, 0.75 mmol/L spermine, 1 mmol/L DTT}]\) with 1 mmol/L MoO4Na2, 1 mmol/L NaF, and protease inhibitors (aprotinin, leupeptin, pepstatin, phenylmethylsulfonylfluoride). After 15 minutes on ice, 10% Nonidet-P40 was added and lysates were vortexed for 10 seconds and centrifuged. The supernatant (cytosolic fraction) was removed and 50 \(\mu\)L of cold buffer C \([20 \text{ mmol/L HEPES (pH 7.6), 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, protease inhibitors}]\) were added to the pellet (nuclear fraction) and incubated on a rotary shaker \((4°C, 20\text{ minutes})\). After centrifugation, protein content was determined by the bicinchoninic acid assay (Pierce), and equal amounts from both fractions were analyzed by SDS-PAGE and Western blotting.

Additional information may be found in Supplementary Materials.

Results

ATO induces apoptosis of B-CLL cells but not of normal PBL

To establish the best conditions for studying ATO effect, B-CLL cells (12 patients) were incubated in RPMI/10% fetal bovine serum with several doses of ATO or vehicle, and their viability was measured by flow cytometry using PI. Figure 1A shows that ATO significantly induced apoptosis in a dose-dependent manner, confirming previous reports (19–22). All samples tested were sensitive to ATO, independently of the clinical stage, the ZAP-70/CD38 expression, or whether they had been frozen or were

Table 1. Clinical characteristics of B-CLL patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex/Age</th>
<th>Stage*</th>
<th>ZAP70/CD38†</th>
<th>Ig status</th>
<th>Genetic alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>M/76</td>
<td>B/II</td>
<td>ND/+</td>
<td>Mutated</td>
<td>del13q14</td>
</tr>
<tr>
<td>P2</td>
<td>F/35</td>
<td>B/II</td>
<td>ND/−</td>
<td>Mutated</td>
<td>del13q14</td>
</tr>
<tr>
<td>P3</td>
<td>M/62</td>
<td>B/II</td>
<td>+/−</td>
<td>Unmutated</td>
<td>del13q14</td>
</tr>
<tr>
<td>P4</td>
<td>F/73</td>
<td>A/0</td>
<td>+/−</td>
<td>Mutated</td>
<td>del13q14, Trisomy 12</td>
</tr>
<tr>
<td>P5</td>
<td>M/56</td>
<td>A/I</td>
<td>+/+</td>
<td>ND</td>
<td>del13q14</td>
</tr>
<tr>
<td>P6</td>
<td>F/73</td>
<td>A/0</td>
<td>+/+</td>
<td>Mutated</td>
<td>Trisomy 12</td>
</tr>
<tr>
<td>P7</td>
<td>M/64</td>
<td>A/0</td>
<td>ND/−</td>
<td>ND</td>
<td>No</td>
</tr>
<tr>
<td>P8</td>
<td>M/42</td>
<td>C/IV</td>
<td>+/−</td>
<td>ND</td>
<td>No</td>
</tr>
<tr>
<td>P9</td>
<td>F/80</td>
<td>B/II</td>
<td>−/−</td>
<td>Mutated</td>
<td>T 14;19</td>
</tr>
<tr>
<td>P10</td>
<td>M/65</td>
<td>B/II</td>
<td>+/−</td>
<td>Mutated</td>
<td>del13q14</td>
</tr>
<tr>
<td>P11</td>
<td>F/63</td>
<td>A/0</td>
<td>−/−</td>
<td>ND</td>
<td>del13q14, del17p13</td>
</tr>
<tr>
<td>P12</td>
<td>F/72</td>
<td>B/II</td>
<td>ND/+</td>
<td>Unmutated</td>
<td>Trisomy 12</td>
</tr>
<tr>
<td>P13</td>
<td>M/47</td>
<td>A/0</td>
<td>−/−</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>P14</td>
<td>M/53</td>
<td>C/III</td>
<td>ND/−</td>
<td>ND</td>
<td>del13q14</td>
</tr>
<tr>
<td>P15</td>
<td>F/78</td>
<td>A/0</td>
<td>−/−</td>
<td>Unmutated</td>
<td>No</td>
</tr>
<tr>
<td>P16</td>
<td>M/63</td>
<td>B/II</td>
<td>+/+</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>P17</td>
<td>F/59</td>
<td>A/0</td>
<td>+/+</td>
<td>Unmutated</td>
<td>No</td>
</tr>
<tr>
<td>P18</td>
<td>M/74</td>
<td>A/0</td>
<td>−/−</td>
<td>ND</td>
<td>del13q14</td>
</tr>
<tr>
<td>P19</td>
<td>F/81</td>
<td>A/0</td>
<td>−/−</td>
<td>Mutated</td>
<td>No</td>
</tr>
<tr>
<td>P20</td>
<td>M/54</td>
<td>A/0</td>
<td>ND/ND</td>
<td>Unmutated</td>
<td>ND</td>
</tr>
<tr>
<td>P21</td>
<td>M/82</td>
<td>A/0</td>
<td>−/−</td>
<td>Unmutated</td>
<td>del17p13</td>
</tr>
<tr>
<td>P22</td>
<td>F/75</td>
<td>A/0</td>
<td>ND/+</td>
<td>Mutated</td>
<td>del13q14</td>
</tr>
<tr>
<td>P23</td>
<td>M/54</td>
<td>B/II</td>
<td>+/+</td>
<td>Unmutated</td>
<td>No</td>
</tr>
<tr>
<td>P24</td>
<td>F/71</td>
<td>A/0</td>
<td>−/−</td>
<td>Unmutated</td>
<td>del13q14</td>
</tr>
<tr>
<td>P25</td>
<td>M/78</td>
<td>B/II</td>
<td>ND/−</td>
<td>Unmutated</td>
<td>Trisomy 12</td>
</tr>
<tr>
<td>P26</td>
<td>M/60</td>
<td>A/0</td>
<td>−/−</td>
<td>Mutated</td>
<td>del13q14</td>
</tr>
<tr>
<td>P27</td>
<td>M/55</td>
<td>A/II</td>
<td>+/+</td>
<td>Unmutated</td>
<td>del13q14</td>
</tr>
<tr>
<td>P28</td>
<td>F/71</td>
<td>B/II</td>
<td>+/+</td>
<td>Mutated</td>
<td>del17p13</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.
*According to Rai et al. (40) and Binet et al. (41).
†The CD38 and ZAP70 expression in B-CLL cells has prognostic value (1, 2). None of the patients had received previous treatment at the time of this study.
freshly isolated. At 3 μmol/L, a concentration within the range used for APL treatment (8, 16), ATO reduced B-CLL cell viability by 50% and 75% after 24 and 48 hours, respectively, compared with untreated cells, which remained highly viable under these culture conditions (Fig. 1A). In contrast, 3 μmol/L ATO had a minor effect (15% cell death after 48 hours) on normal PBL (Fig. 1B). At higher doses, ATO induced 90% B-CLL cell death, but started to produce a significant effect on normal PBL (Fig. 1B). Subsequent experiments were done using 3 μmol/L ATO except when combined with other agents in which 1 to 2 μmol/L ATO was used.

ATO cooperates with PI3K/Akt inhibitors to enhance B-CLL cell apoptosis and inhibits Akt activation by upregulating PTEN

The PI3K/Akt and protein kinase C (PKC) signaling pathways are constitutively activated in many B-CLL cases and contribute to their resistance to apoptosis (23, 24). We studied whether the combination of ATO and PI3K/Akt or PKC inhibitors enhanced the apoptotic effect of either agent alone. When used individually, ATO (2 μmol/L), or the PI3K/Akt inhibitors LY294002 or API-2 produced limited, albeit significant, apoptosis (33-38%, average of 4 samples) after 48 hours, compared with untreated cells. However, when ATO was combined with either inhibitor, apoptosis significantly increased to 68% and 77% for LY294002 and API-2, respectively (Fig. 2A). In contrast, the PKC inhibitor BisI had no effect on B-CLL cell viability and did not increase the apoptotic effect of ATO (Fig. 2A).

To then determine whether ATO-induced apoptosis affected the PI3K signaling pathway, we measured Akt phosphorylation and expression of the Akt targets NF-κB and XIAP (25, 26). As shown in Fig. 2B, for two representative patients Akt was constitutively phosphorylated, and phosphorylation significantly decreased after 3 hours of ATO exposure, being almost undetectable after 24 hours. Phospho-Akt remained unaffected in control cells. In correlation with this, the levels of the NF-κB–associated protein IκB-α clearly increased, indicating that it remained bound to NF-κB in the cytoplasm, likely preventing NF-κB activation. The antiapoptotic protein XIAP also significantly decreased upon 24 hours of ATO exposure (Fig. 2B). Interestingly, and parallel to these observations, expression of the phosphatase PTEN, a well-known PI3K inhibitor (27) significantly increased (4-fold, P ≤ 0.01) after 24 hours of ATO treatment (Fig. 2B). Altogether these results indicated that ATO-induced B-CLL cell apoptosis involved PTEN upregulation and inhibition of the PI3K/Akt/NF-κB survival pathway.

JNK plays a critical role in the induction of B-CLL cell apoptosis by ATO

To determine whether ATO induction of B-CLL cell apoptosis involved JNK activation, we first measured JNK phosphorylation after 24 hours of ATO exposure. Figure 3A shows that ATO clearly induced JNK activation that was prevented by the JNK inhibitor SP600125 in a dose-dependent manner, with complete inhibition at 10 μmol/L. SP600125 had no effect in the absence of ATO (Fig. 3A). Subsequent kinetic studies revealed that JNK was already phosphorylated after 1 hour of ATO treatment, therefore preceding Akt dephosphorylation and PTEN upregulation (Figs. 2B and 3B). Indeed, JNK was responsible for these subsequent events as both were partially inhibited by 5 μmol/L SP600125 (50% and 30% inhibition for Akt and PTEN, respectively; not shown) and completely inhibited by 10 μmol/L SP600125 (Fig. 3B). Further evidence for a crucial role of JNK in ATO-induced B-CLL cell apoptosis was obtained by analyzing NF-κB activation. As shown in Supplementary Fig. S1A, and in agreement with the data shown in Fig. 2B for IκB-α, NF-κB nuclear levels were clearly diminished by ATO but were unaffected by the ATO/SP600125 combination. Moreover, modulation of NF-κB activity in correlation with PTEN expression was apparently specific for JNK because the PI3K inhibitor LY294002, which efficiently induced Akt dephosphorylation and IκB-α upregulation, had no effect on PTEN, either alone or combined with ATO (Supplementary Fig. S1B).

![Fig. 1. Effect of ATO on B-CLL cell viability. A, B-CLL cells were incubated with the indicated concentrations of ATO or vehicle. After 24 or 48 hours, cell viability was determined by flow cytometry using PI. B, PBL from five different healthy individuals were analyzed as above. Determinations were done in duplicate and average values ± SD are shown. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.0001.](https://example.com/fig1.png)
To confirm the above results, we transfected B-CLL cells with JNK siRNA and measured viability, Akt inactivation, and PTEN expression. Figure 3C shows that JNK siRNA reduced JNK levels by 78% resulting in complete inhibition of the ATO effect on Akt and PTEN (Fig. 3C). Transfection with control siRNA, however, did not affect the cell response to ATO (Fig. 3C). In correlation with these results, SP600125 or JNK siRNA transfection inhibited the apoptotic effect of ATO, but had no effect in the absence of this agent (Fig. 3D).

In agreement with recent reports (21, 22), induction of B-CLL cell apoptosis by ATO was mediated by caspases, as the pan caspase inhibitor Z-VAD-FMK or the caspase-8 inhibitor Z-IETD-FMK nearly completely abrogated the ATO apoptotic effect (samples, n = 5; Supplementary Fig. S2A). Indeed, ATO induced caspase-9, -8, and -3 activation and cleavage of their specific substrates (Supplementary Fig. S2B), indicating that both the extrinsic and intrinsic (mitochondrial) apoptotic pathways were activated by ATO in B-CLL cells. Importantly, all these apoptotic events were completely prevented by inhibiting JNK (Supplementary Fig. S2B).

To further assess the mitochondrial involvement in ATO-induced B-CLL cell apoptosis, we first studied whether ATO induced mitochondrial depolarization. B-CLL cells from two patients were incubated for 24 hours in the absence or presence of 3 μmol/L ATO, treated with DiOC₆, and analyzed by flow cytometry. Figure 4A shows that ATO decreased the mitochondrial membrane potential, increasing the number of DiOC₆-negative, apoptotic cells in both cases. Next, we studied cytochrome c release by confocal microscopy using the mitochondrial protein Hsp60 as reference. Figure 4B shows for a representative sample that after 24 hours, a clear colocalization of

![Graph A](image1)

**Fig. 2.** ATO interferes with the PI3K/Akt survival pathway.
A, B-CLL cells (4 patients) were incubated for 30 minutes with or without 5 μmol/L LY294002, API-2, or BisL, prior to the addition of 2 μmol/L ATO. After 48 hours, cell viability was analyzed by flow cytometry. Const. (constitutive), fresh cell viability. B, B-CLL cells treated or not with 3 μmol/L ATO were lysed at different times, and protein expression was analyzed by Western blotting. Normalized average values are shown. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.0001.
cytochrome c and Hsp60 was observed on control cells but not on ATO-treated cells, confirming the cytochrome c mitochondrial release. As observed for caspase activation (Supplementary Fig. S2B), cytochrome c release was completely prevented by inhibiting JNK signaling (Fig. 4B). Accordingly, active Bax, a proapoptotic factor involved in the mitochondrial pathway, was readily detected on ATO-treated cells, but was absent on control cells or in the presence of the JNK inhibitor (Fig. 4B). Cytochrome c release was not affected by ZVAD-FMK or Z-IETD-FMK, indicating that it preceded caspase activation (data not shown).

Generation of ROS mediates ATO-induced apoptosis in many cell types and apparently plays an essential function in the case of B-CLL cells (21, 22). We therefore studied whether JNK activity was required for induction of ROS in response to ATO. B-CLL cells were treated with 3 μmol/L ATO, and ROS levels were measured using the fluorescent probe H2DCFDA. Figure 4C shows that ROS
Fig. 4. JNK activation is critical for the ATO apoptotic effect in B-CLL cells. A, flow cytometric analysis of the loss of mitochondrial membrane potential (ΔΨm) after B-CLL cell incubation with 3 μmol/L ATO for 24 hours. B, B-CLL cells treated or not with 10 μmol/L SP for 30 minutes were cultured with or without 3 μmol/L ATO. After 24 hours, cells were analyzed by confocal microscopy using antibodies for cytochrome c, or active Bax (green) and Hsp60 (red). Colocalization of cytochrome c and Hsp60 is further shown by dot-plot analyses. C, B-CLL cells treated or not with SP or transfected with control (Ctl) or JNK siRNA were incubated with or without ATO for 16 hours. H2DCFDA was added, and ROS production was measured by flow cytometry. Shaded areas, untreated cells; green lines, 10 μmol/L SP-treated cells; blue lines, ATO-treated cells; red lines, SP/ATO-treated cells. Average mean fluorescence intensity values are shown. D, B-CLL cells were treated with 10 mmol/L NAC or 50 μmol/L MnTBAP and cultured with or without 3 μmol/L ATO for 24 hours. H2DCFDA was added, and ROS production was measured by flow cytometry. Shaded areas, untreated cells; green lines, cells treated with NAC or MnTBAP; blue lines, ATO-treated cells; red lines, NAC/ATO- or MnTBAP/ATO-treated cells. Quantitative values are also shown. In parallel, cells were lysed and JNK activation was analyzed by Western blotting. **, P ≤ 0.01.
production was significantly increased \( (P \leq 0.01) \) after 16 hours of ATO exposure, compared with control cells. The JNK inhibitor SP600125 had no effect when added alone, but prevented the ATO-induced ROS production in a dose-dependent manner (Fig. 4C). Accordingly, JNK gene silencing also inhibited ROS production in response to ATO, whereas the control siRNA had no effect (Fig. 4C). To then establish whether ROS induction preceded or followed JNK activation, we used the antioxidants N-acetylcysteine (NAC) and Mn-tetakis-(4-benzoic acid) porphyrin (MnTBAP). Incubation of B-CLL cells with NAC or MnTBAP prior to ATO treatment completely prevented the increased ROS production (Fig. 4D) and the ATO apoptotic effect, yielding 55% and 54% viable cells for NAC and MnTBAP, respectively, compared with 13% (no antioxidant) and 68% (control; not shown). In contrast, NAC or MnTBAP had no effect on the levels of JNK phosphorylation, which remained elevated (Fig. 4D). Similar results were obtained with catalase (not shown). Altogether these results established that JNK activation is an early B-CLL cell response to ATO and is crucial for subsequent events that lead to apoptosis. Figure 5 shows a schematic representation of the ATO-induced apoptotic mechanism in B-CLL cells.

**Discussion**

We studied the mechanism involved in induction of B-CLL cell apoptosis by ATO and whether ATO is suitable for combination therapies with protein kinase inhibitors. We show for the first time that (a) JNK activation is critical for the ATO effect on B-CLL cells, (b) ATO induces PTEN upregulation and PI3K/Akt inactivation, and (c) ATO cooperates with PI3K/Akt inhibitors enhancing B-CLL cell apoptosis.

All 28 patient samples analyzed were very sensitive to the cytotoxic effect of ATO, regardless of their prognostic markers or clinical stage. This included two B-CLL samples carrying a deletion in chromosome 17p13 (which confers drug resistance), in agreement with a previous report (22). Importantly, efficient B-CLL cell apoptosis was attained by concentrations of ATO (3 \( \mu \)mol/L) currently used in the clinical treatment of APL (8, 16), with minimal effect on normal PBL, further emphasizing the potential therapeutic use of ATO in B-CLL.

The PI3K/Akt signaling pathway is constitutively activated in many B-CLL cases, as we also find in our study, and contributes to the typical B-CLL cell antiapoptotic phenotype (23, 24). In this report we show for the first time that suboptimal concentrations of ATO (2 \( \mu \)mol/L) specifically cooperated with pharmacologic inhibitors of the PI3K survival pathway and enhanced B-CLL cell apoptosis. Low doses of ATO may thus prove to be useful in combined therapies with PI3K inhibitors. Our study also shows that induction of B-CLL cell apoptosis by ATO was accompanied by inhibition of the PI3K/Akt signaling pathway, assessed by NF-\( \kappa \)B inactivation and downregulation of the Akt (26) and NF-\( \kappa \)B (28) target XIAP. Modulation of NF-\( \kappa \)B activity by ATO seems to be specific as other antiapoptotic proteins such as Mcl-1, which is mainly regulated by signal transducers and activators of transcription (STAT) family members (29), did not change upon ATO exposure (data not shown). Thus, although ATO was shown to reduce STAT activity in AML (30) and
downregulate Mcl-1 in myeloma cells (31), our results suggest that this is not the case in B-CLL.

Parallel to the observed NF-κB inactivation by ATO, our results also show a significant upregulation of PTEN. NF-κB has been shown to repress PTEN expression, being one of the mechanisms by which NF-κB does its antiapoptotic function in tumors (32). The NF-κB target XIAP was also recently shown to ubiquitinate PTEN and to regulate its content and compartmentalization (33). As PTEN specifically inhibits PI3K signaling through its lipid phosphatase activity (27, 34), our findings indicate that ATO-induced B-CLL cell apoptosis affects the PI3K survival pathway at various levels. They also provide the first evidence for a possible functional role of PTEN in B-CLL cells. PTEN induction also decreased the resistance of HL-60 monocyctic cells to the apoptotic effect of ATO (16, 35), again highlighting that at least in certain cells, suppression of the PI3K/Akt/NF-κB survival pathway is crucial for the ATO cytotoxic action.

NF-κB inactivation, however, may not be sufficient to upregulate PTEN in B-CLL cells, as our results also show that inhibiting the PI3K/Akt/NF-κB pathway in the absence of ATO did not upregulate PTEN or enhance the effect of ATO on PTEN. This suggests that other molecules specifically induced by ATO may also contribute to PTEN upregulation. One such molecule is likely JNK. It is known that JNK activation counteracts the survival effect of NF-κB (36), regulates PTEN expression (37), and may be a target of PTEN in certain cell types (38). JNK is involved in apoptotic signaling in many cell types (39) and has been shown to mediate the cytotoxic effect of ATO in APL (16) and myeloma (17) cell lines. As we show in this report, JNK activation was an early response to ATO treatment and was required for subsequent apoptotic events in B-CLL cells, including PTEN upregulation and ROS production. Thus, although it was suggested that JNK activation upstream of ROS mostly leads to necrosis (35), our results clearly show that in B-CLL cells, JNK activation preceded ROS formation and initiated the apoptotic response to ATO.

Our study therefore establishes the mechanism of ATO action in B-CLL cells, revealing a crucial role for JNK and PTEN. It is also the first to report that when combined with PI3K inhibitors, very low doses of ATO were sufficient to produce efficient malignant cell death. The use of ATO in mixed therapies has been proven very successful in the killing of myeloma, APL, and T-leukemia cells in vitro (5, 7, 10). As we now show, the combination of ATO and PI3K inhibitors may represent a promising and efficient alternative for the clinical treatment of B-CLL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the B-CLL patients who donated blood samples for this research, Dr. Patricio Aller for reviewing the manuscript and providing some reagents, and Dr. Pedro Laster for help with the flow cytometry analyses.

Grant Support

Grants PI060400, RD06/0020/0011, and SAF2009-07035 (A. García-Pardo), RD06/0020/0041 (A. Pandiella), and PI061637 and RD06/0020/0080 (M.L. Terol) from the Ministerio de Ciencia e Innovación, and grant from the Fundación de Investigación Médica Mutua Madrileña (A. García-Pardo). JRM was supported by a fellowship from the Fundación Ramón Areces, Madrid, Spain. 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 01/12/2010; revised 04/29/2010; accepted 05/27/2010; published OnlineFirst 06/09/2010.

References

Clinical Cancer Research

Induction of B-Chronic Lymphocytic Leukemia Cell Apoptosis by Arsenic Trioxide Involves Suppression of the Phosphoinositide 3-Kinase/Akt Survival Pathway via c-jun-NH₂ Terminal Kinase Activation and PTEN Upregulation


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-10-0072</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://clincancerres.aacrjournals.org/content/suppl/2010/08/27/1078-0432.CCR-10-0072.DC1">http://clincancerres.aacrjournals.org/content/suppl/2010/08/27/1078-0432.CCR-10-0072.DC1</a> <a href="http://clincancerres.aacrjournals.org/content/suppl/2010/06/09/1078-0432.CCR-10-0072.DC2">http://clincancerres.aacrjournals.org/content/suppl/2010/06/09/1078-0432.CCR-10-0072.DC2</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 41 articles, 16 of which you can access for free at: <a href="http://clincancerres.aacrjournals.org/content/16/17/4382.full#ref-list-1">http://clincancerres.aacrjournals.org/content/16/17/4382.full#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citing articles</td>
<td>This article has been cited by 6 HighWire-hosted articles. Access the articles at: <a href="http://clincancerres.aacrjournals.org/content/16/17/4382.full#related-urls">http://clincancerres.aacrjournals.org/content/16/17/4382.full#related-urls</a></td>
</tr>
</tbody>
</table>

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. |