Preclinical Development

Molecular Cancer Therapeutics

AZ960, a Novel Jak2 Inhibitor, Induces Growth Arrest and Apoptosis in Adult T-Cell Leukemia Cells

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Abstract

Adult T-cell leukemia/lymphoma (ATL) is a highly aggressive disease in which the Jak2/Stat5 pathway is constitutively activated. This study found that AZ960, a novel inhibitor of Jak2 kinase, effectively induced growth arrest and apoptosis of human T-cell lymphotropic virus type 1, HTLV-1–infected T cells (MT-1 and MT-2) in parallel with downregulation of the phosphorylated forms of Jak2 and Bcl-2 family proteins including Bcl-2 and Mcl-1. Interestingly, AZ960 increased levels of Bcl-xL in MT-1 and MT-2 cells in association with accumulation of cAMP response element-binding protein bound to the Bcl-xL promoter as measured by chromatin immunoprecipitation assay. Importantly, genetic inhibition of Bcl-xL by a small interfering RNA potentiated antiproliferative effects of AZ960 in MT-1 cells. Taken together, Jak2 is an attractive molecular target for treatment of ATL. Concomitant blockade of Jak2 and Bcl-xL may be a promising treatment strategy for this lethal disease. *Mol Cancer Ther; 9(12); 3386–95. ©2010 AACR.*

Introduction

Adult T-cell leukemia (ATL) is an aggressive malignancy of $CD4^+$ T lymphocytes, in which the human T-cell lymphotropic virus type 1 (HTLV-1) has been recognized as the etiologic agent (1, 2). Despite the development of intensive combination chemotherapy regimens supported by granulocyte colony-stimulating factor, median survival time of individuals with ATL is less than 13 months (3, 4). Thus, it is urgent to develop new treatment strategies.

The Janus-associated kinase (Jak) family, comprised 4 different protein-tyrosine kinases, Jak1, Jak2, Jak3, and TYK2, plays an important role in cellular survival, proliferation, and differentiation (5). Jak kinases are key mediators of signaling downstream of a variety of cytokine and/or growth factor receptors (6). Each Jak possesses kinase domain, a catalytically inactive pseudokinase, and amini-terminal 4.1, ezrin, radixin, and moesin (FERM). Cytokine receptors bind to FERM domain, leading to activation of Jaks, which create docking sites for members of signal transducers and activators of transcription (Stat) family, including Stat3 and Stat5 (7, 8). On phosphorylation on tyrosine residues, Stats form a dimer and translocate to the nucleus where they bind to DNA and regulate expression of target genes, including Bcl-xL, an antiapoptotic protein (9–11). Constitutive activation of Stat3/5 was shown in HTLV-1–infected T cells (5, 12). Previous studies showed that inhibition of Stats by AG490 induced growth arrest and apoptosis of HTLV-1–infected T cells in association with downregulation of Bcl-2 family members (13).

The cAMP response element-binding protein (CREB) is a well-characterized transcription factor of the basic leucine zipper family (14). In response to various stimuli, such as growth factors, neurotransmitters and stress signals, that elevate intracellular cAMP or Ca^{2+} levels, CREB is phosphorylated at Ser133 and activated in conjunction with recruitment of transducer of regulated CREB activity coactivators (14–17). The activation of CREB turns on the transcription of more than 5,000 target genes that regulate cell growth and apoptosis such as Bcl-xL (11). Previous studies found that CREB had a pathobiologic role in the growth of breast cancer (18), melanoma (19), and hepatocellular carcinoma cells (20). In addition, CREB was shown to act as a protooncogene in acute leukemia (21). Previous studies found that Tax-expressing cells possessed high levels of the phosphorylated forms of CREB at Ser-133. Tax physically interacted with and activated CREB and transducers of regulated CREB activity coactivators, leading to immortalization of HTLV-1–infected T lymphocytes (22–24).

AZ960 is a potent and selective ATP competitive inhibitor of the Jak2 kinase with a K_i of 0.45 nmol/L. Previous studies reported that AZ960 possessed the activity against Jak1, 3, and TYK2, although its effect was less potent than that against Jak2 (25). AZ960 was also shown to be active against other kinases, including TrkA, Aurora-A, and FAK, with IC_{50} of around 0.1 μmol/L (25).

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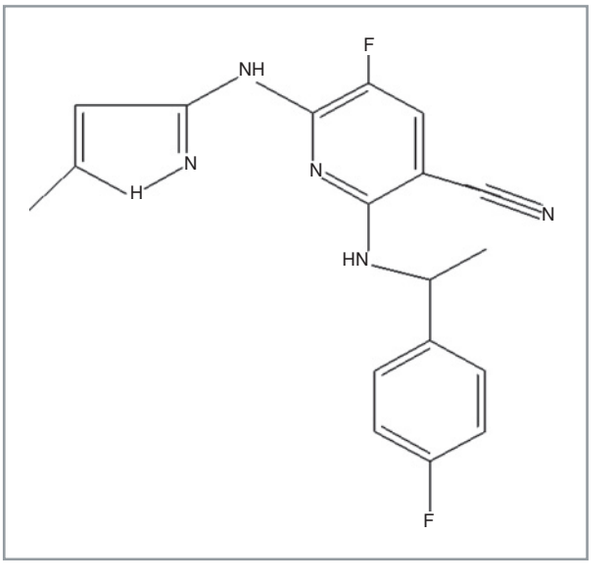

Figure 1. Chemical structure of AZ960 ($C_{18}H_{16}F_2N_6$).

This study demonstrated a new treatment strategy targeting ATL cells by blockade of Jak signaling.

Materials and Methods

Cell culture

HTLV-1–infected T cell lines MT-1 and MT-2 were kind gifts of I Miyoshi (Kochi Medical School). Acute T-lymphoblastic leukemia MOLT-4 cells were purchased from DS Pharma Biomedical Co. Ltd. Cells were suspended in standard PRMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum. The cell lines used in this study have not been recently authenticated. Peripheral blood lymphocytes and ATL cells were freshly isolated from healthy volunteers and patients with ATL, respectively, after obtaining informed consent, as previously described (26).

Reagents

AZ960 (S)-5-fluoro-2-(1-(4-fluorophenyl)ethyl-amino)-6-(5-methyl-1H-pyrazol-3-yl-amino) nicotinonitrile was synthesized by AstraZeneca R&D and its structure is shown in Fig. 1. AZ960 was dissolved in 100% DMSO to a 10^{-2} mol/L. Control siRNA and an siRNA against Bcl-xL were purchased from Santa Cruz Biotechnology and Sigma, respectively.

MTT assays

HTLV-1–infected T cells and MOLT-4 cells (5×10^5 cells/mL) were cultured with various concentrations of AZ960 (0.03–1 μmol/L) for 2 days in 96-well plates (Flow Laboratories). Peripheral blood lymphocytes were activated by phytohemagglutinin (PHA; 5 ng/mL) for 1 hour, then cultured with various concentrations of AZ960 (0.03–1 μmol/L) for 2 days in 96-well plates (Flow Laboratories). After culture, cell number and viability were evaluated by measuring the mitochondrial-dependent conversion of the 3-(4,5-dimethylithiazol-2-yl)-2,5-diphenyl tetrazolium salt (MTT; Sigma), to a colored formazan product.

Thymidine uptake studies

Proliferation of PHA-activated peripheral blood lymphocytes and ATL cells was measured by tritiated thymidine (^{3}H-TdR) uptake (Perkin-Elmer). Cells (5×10^5/mL) were cultured with various concentrations of AZ960 (0.03–1 μmol/L) for 2 days in 96-well plates. Cells were pulsed with ^{3}H-TdR [0.5 ACi (0.185 MBq) per well] during the last 6 hours of a 48-hour culture, harvested onto glass filters with an automatic cell harvester (Cambridge Technology), and counted using the LKB Betaplate scintillation counter (Wallac). All experiments were done in triplicate and repeated at least twice.

Cell-cycle analysis by flow cytometry

Cell-cycle analysis was done on MOLT-4 and HTLV-1–infected T cells incubated with AZ960 (0.3 or 1 μmol/L) for 2 days at 5×10^5 cells/mL in 12-well plates. After incubation, cells were collected, fixed in chilled methanol, and suspended in solution containing RNase A (100 U/mL; Sigma) before staining with 50 μg/mL of propidium iodide. A minimum of 1×10^4 cells were measured by FACSCalibur apparatus (Becton Dickinson) and was analyzed using the CellQuest software package (Becton Dickinson).

Assessment of apoptosis

Cells were plated at a density of 1×10^5 cells/mL and incubated with AZ960 (0.3 or 1 μmol/L) for 2 days in 12-well plates. The ability of AZ960 to induce apoptosis was measured by annexin-V-FITC apoptosis detection kit (Pharmingen, Inc.) according to the manufacturer's instructions.

Western bolt analysis

Immunoblotting was done as previously described (26). Anti-p-Jak1 (Tyr1022/1023; Cell Signaling Technology, #3331s), -Jak1 (Cell Signaling Technology, #3332), -p-Jak2 (Tyr1007/1008; Cell Signaling Technology, #3771s), -Jak2 (Cell Signaling Technology, #3229), -p-Stat5 (Tyr694; Cell Signaling Technology, #9351s), -Stat5 (Santa Cruz Biotechnology, sc-835), -p-Stat3 (Tyr705; Cell Signaling Technology, #9131s), -Stat3 (Cell Signaling Technology, #9132), -p-AKT (Ser473; Cell Signaling Technology, #9271s), -AKT (Cell Signaling Technology, #9272), -p-S6K (Ser235/236; Cell Signaling Technology, #4856s), -S6K (Cell Signaling Technology, #2217), -p-4E-BP1 (Thr37/46; Cell Signaling Technology, #9456s), -4E-BP1 (Cell Signaling Technology, #9452), -PARP (Cell Signaling Technology, #9542), -Bcl-xL (Cell Signaling Technology, #2746), -XIAP (Cell Signaling Technology, #2042), -Bcl-2 (Santa Cruz Biotechnology, sc-509), -Mcl-1 (Santa Cruz Biotechnology, sc-819), and -GAPDH (Abcam) antibodies were used. Band intensities were measured by ImageJ software (Wayne Rasband, NIH).

The levels of p-CREB in nuclei were monitored by Western blot analysis. Cells were suspended in ice-cold

extraction buffer containing 20 mmol/L of HEPES (pH = 7.9), 20% glycerol, 10 mmol/L of NaCl, 0.2 mol/L of EDTA (pH = 8.0), 1.5 mmol/L of $MgCl_2$, 0.1% Triton X-100, 1 mmol/L of DTT, 100 μg/mL of phenylmethylsulfonyl fluoride, 2 μg/mL of aprotinin, 1 μg/mL of pepStatin, and 10 μg/mL of leupeptin. After 10 minutes of incubation on ice, nuclei were collected by a short spin in a microcentrifuge. And the nuclei were resuspended in ice-cold extraction buffer containing 300 mmol/L of NaCl. After 30 minutes of incubation, the supernatant was collected by centrifugation at 15,000 × *g* for 20 minutes at 4°C. Protein concentrations were quantitated using a Bio-Rad assay (Bio-Rad Laboratories). Anti-p-CREB (Cell Signaling Technology, #9196s), and -Histone H1 (Santa Cruz Biotechnology, sc-8030) antibodies were used.

RNA isolation and real-time reverse transcription-polymerase chain reaction

RNA isolation and cDNA preparation were done as described previously (27). We measured expression of 18S for normalization as previously described (27). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was carried out by using Power SyBr Green PCR Master Mix (Applied Biosystems) as described previously (27). Primers for PCR are shown in Table 1. PCR conditions for all genes were as follows: 95°C initial activation for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds, and fluorescence determination at the melting temperature of the product for 20 seconds on an ABI PRISM 7000 (Applied Biosystems).

Transfection

MT-1 cells (1×10^7) were transfected with either control or Bcl-xL siRNA (10 μg) by electroporation (150 V). After 24 hours, cells were seeded in 12-well plates and cultured with various concentrations of AZ960 (0.3 or 1 μmol/L). After 48 hours, cells were harvested, proteins were extracted and subjected to Western blot analyses. The preliminary experiments using the green fluorescence protein-expressing vector found that efficiency of transfection with this program was approximately 51% with cell viability more than 64%, as measured by annexin V staining.

Chromatin immunoprecipitation (ChIP) assay

MT-1 cells (1×10^6/mL) were cultured with AZ960 (0.3 or 1 μmol/L) for 2 days. Formaldehyde was added to the cells to a final concentration of 1% and the cells were incubated at 37°C for 10 minutes. The cells were collected and subjected to ChIP utilizing reagents provided by LPBIO according to the manufacturer's instructions. Anti-CREB (Santa Cruz Biotechnology, sc-186X) antibody or rabbit immunoglobulin G control (IgG) were used for immunoprecipitation. Immunoprecipitated DNA was recovered and used as a template for PCR. CREB-binding site was predicted by TFSEARCH: Searching Transcription Factor Binding Sites (ver 1.3). The proximal primers were designed on the Bcl-xL promoter region, which locates at −400 to −308 bp from the transcription start site of Bcl-xL. The distal primers were located at −2113 to −2019 bp from the transcription start site of Bcl-xL. Primers for PCR are shown in Table 2. PCR for both primer sets was done using the following parameters: 94°C, 10 minutes; 94°C, 30 seconds; 60°C, 40 seconds; and 72°C, 1 minute for 35 cycles followed by a final extension at 72°C, 10 minutes. PCR products were separated by electrophoresis on a 2% agarose gel and visualized with ethidium bromide.

Table 1. RT-PCR primers

Gene	Direction	Primer
BCL2L1	Forward	5′-GATTCAGGCTGCTTGGGATA-3′
	Reverse	5′-GCTTCTGGAGGACATTTGGA-3′
18S	Forward	5′-AAACGGCTACCACATCCAAG-3′
	Reverse	5′-CCTCCAATGGATCCTCGTTA-3′

Table 2. Primers for ChIP assay

Gene	Direction	Primer
Proximal primers	Forward	5′-CCTGGGCTGGT-GCTTAAATA-3′
	Reverse	5′-CCTACTGGGAG-CCAGGAGTA-3′
Distal primers	Forward	5′-CACCACCCTCC-AAAGAAAGA-3′
	Reverse	5′-GGGTAAAAACC-GAAGGCACT-3′

Statistical analysis

All statistical analyses were carried out using the SPSS software (SPSS Japan) and the results were considered to be significant when the *P* value was < 0.05.

Results

Constitutive tyrosine phosphorylation of Jak1/2 and Stat3/5 in HTLV-1–infected T cells

We first examined whether HTLV-1–infected T cells (MT-1 and MT-2) and freshly isolated ATL cells from 2 patients with acute type of ATL expressed the phosphorylated forms of Jak2 and Stat3/5. Both MT-1 and MT-2 cells constitutively expressed the phosphorylated forms of these proteins (Fig. 2), suggesting activation of Jak/Stats signaling in these cells. Freshly isolated ATL cells from case 1 and case 2 also expressed constitutively phosphorylated forms of Jak2 and Stat5. p-Stat3 was slightly expressed in these cells (Fig. 2). On the other hand, neither p-Jak2 nor Stat3/5 was detectable in peripheral blood lymphocytes from healthy volunteer (Fig. 2). Acute T-lymphoblastic leukemia MOLT-4 cells only slightly expressed the phosphorylated forms of Jak2. Neither p-Stat3 nor p-Stat5 was detectable in these cells (Fig. 2).

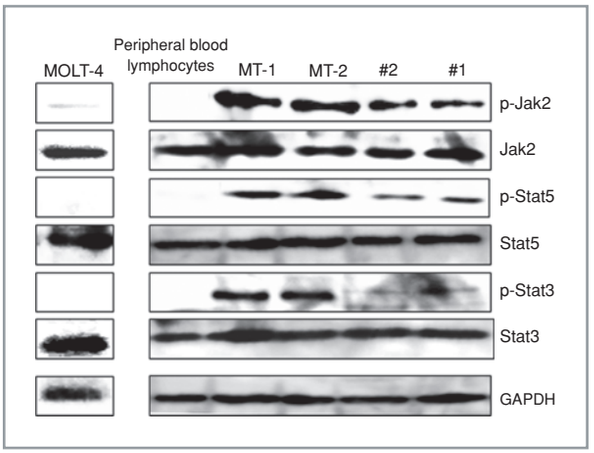

Figure 2. The levels of p-Jak and p-Stats in HTLV-1–infected T cells. Western blot analysis. MOLT-4, MT-1, MT-2 and freshly isolated ATL cells (case #s 1 and 2) and peripheral blood lymphocytes from healthy volunteer were harvested and proteins were extracted and subjected to western blot analyses. The membrane was probed sequentially with the indicated antibodies.

AZ960 inhibits the cell growth of HTLV-1–infected T cells as well as freshly isolated ATL cells from patients

We next examined the effect of AZ960, a novel inhibitor of Jak2, on the proliferation ofHTLV-1–infected T cells as well as peripheral blood lymphocytes from healthy volunteers. AZ960 (0.03–1 μmol/L) suppressed the growth of MT-1 and MT-2 cells with IC_{50} of 0.8 and 1 μmol/L, respectively (Fig. 3A). The antiproliferative effects of AZ960 against freshly isolated ATL cells were measured by thymidine uptake studies. AZ960 inhibited the growth of freshly isolated ATL cells (case #s 1 and 2) in a dose-dependent manner with IC_{50} of 0.3 μmol/L and 0.7 μmol/L, respectively (Fig. 3B). On the other hand, AZ960 did not affect survival of peripheral blood lymphocytes from healthy volunteers. Contrary to our expectation, AZ960 inhibited the growth of MOLT-4 cells in a dose-dependent manner with IC_{50} of 1.2 μmol/L (Fig. 3A), although Jak/Stats signaling was not activated in these cells (Fig. 3C).

AZ960 inhibits Jak/Stat signaling in ATL cells

As expected, exposure of MT-1, MT-2, and freshly isolated ATL cells to AZ960 (0.1–1 μmol/L) effectively downregulated levels of p-Jak2 in parallel with downregulation of p-Stat3/5 (Figs. 3C and D).

Effects of AZ960 on PI3K/AKT signaling pathway

Previous studies found that PI3K/AKT signaling was activated in conjunction with increased Jak2/Stat3 activity in ATL cells (28). We therefore investigated the effects of AZ960 on the PI3K/AKT signal pathway by Western blot analysis. Exposure of MT-1 and MT-2 cells to AZ960 (0.3 or 1 μmol/L, 3 hours) didn't affect the levels of p-AKT, p-S6K, and p-4E-BP1 (figure not shown).

Effect of AZ960 on cell-cycle distribution of HTLV-1–infected T cells

To investigate the mechanisms by which AZ960 inhibited the growth of HTLV-1–infected T cells, we explored the effects of AZ960 on cell-cycle distribution of these cells by flow cytometry. Exposure of MT-1 cells to AZ960 (0.3 or 1 μmol/L, 48 hours) prominently induced accumulation of these cells in the sub-G_1 phase of the cell cycle, a featureof apoptosis (Fig. 3E). Likewise, sub-G_1 population was accumulated in MT-2 cells after exposure to AZ960 (Fig. 3E). In addition, the population of cells in the G_2-M phase of cell cycle was increased in these cells (Fig. 3E). Interestingly, exposure of MOLT-4 cells to AZ960 (1 μmol/L, 48 hours) prominently increased the population of cells with 4N/8N DNA content (Fig. 3E), a feature of diploid cells shown after exposure to an inhibitor of Aurora A kinase (29). AZ960 probably inhibited the proliferation of MLOT-4 cells via inhibition of Aurora A. Furthermore, we found that exposure of MOLT-4, MT-1, and MT-2 cells to AZ960 (0.3 or 1 μmol/L, 48 hours) profoundly increased the population of cells positive for annexin V in a dose-dependent manner, confirming the induction of apoptosis (figure not shown).

Effect of AZ960 on levels of apoptosis-regulating proteins

We next examined whether AZ960 modulated the levels of apoptosis-related proteins in MOLT-4 and HTLV-1–infected T cells. Western blot analysis found that MT-1, MT-2, and ATL cells from patients constitutively expressed antiapoptotic Bcl-xL, Bcl-2, and Mcl-1 proteins (Fig. 4A), which was consistent with the findings of previous studies (26). On the other hand, peripheral blood lymphocytes from healthy volunteer express Bcl-2 but not Bcl-xL and Mcl-1. Exposure of MT-1, MT-2, and ATL cells from patients to AZ960 (0.3 or 1 μM) for 48 hours dramatically decreased levels of Bcl-2 and Mcl-1 proteins in association with increased levels of the cleaved forms of PARP, a feature of apoptosis. Surprisingly, levels of Bcl-xL increased after exposure of these cells to AZ960 (Fig. 4B). Furthermore, real time RT-PCR found that expression of Bcl-xL in MT-1 and MT-2 cells was upregulated at mRNA level after exposure to AZ960 (Fig. 4C).

Downregulation of Bcl-xL by a small interference RNA potentiates apoptosis mediated by AZ960

Previous studies showed the critical role of Bcl-xL in survival of ATL cells (30). We hypothesized that AZ960-stimulated upregulation of Bcl-xL might blunt the effect of AZ960 to induce apoptosis of ATL cells. To confirm our hypothesis, we downregulated levels of Bcl-xL in MT-1 cells by utilizing an siRNA and examined the antiproliferative effects of AZ960. MT-1 cells were transiently transfected with either control or Bcl-xL siRNA. After 24 hours, these cells were exposed to either AZ960 (0.3 or 1 μmol/L) or control dilutant. The siRNA against Bcl-xL almost completely blocked AZ960-induced expression of Bcl-xL in MT-1 cells (Fig. 5A). The clea-

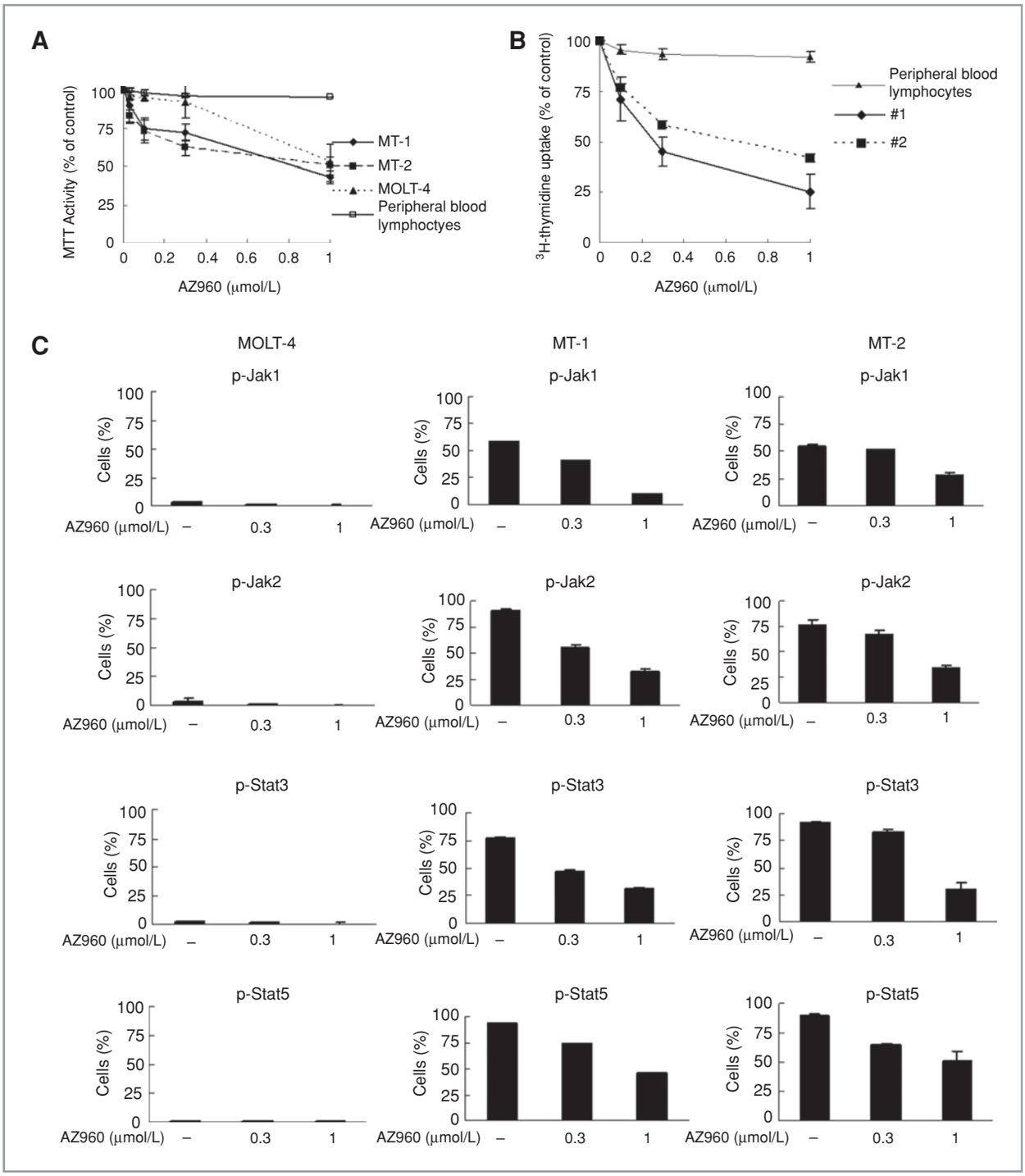

Figure 3. AZ960 inhibits the growth of HTLV-1–infected T cells and freshly isolated ATL cells. A, MTT activity. MOLT-4 and HTLV-1–infected T cells and PHA-activated peripheral blood lymphocytes from healthy volunteers ($n = 3$; 5×10^5cells/mL) were cultured with AZ960 (0.03–1 μmol/L) for 2 days in 96-well plates. After 48 hours, the cells were treated with MTT for 30 minutes, and absorbance was measured. Results represent the mean ± SD of 3 experiments done in triplicate. B, thymidine uptake studies. The effect of AZ960 on the proliferation of ATL cells and PHA-activated peripheral blood lymphocytes from healthy volunteers ($n = 3$; 5×10^5/mL) were measured by tritiated thymidine (^{3}H-TdR) uptake (isotope added 6 hours before harvest). All experiments were done in triplicate and repeated at least twice. Quantification of p-Jak1/2 and -Stats expression in ATL cells. MOLT-4, MT-1, MT-2 (C) and freshly isolated ATL (D) cells were seeded in 12-well plate and cultured with AZ960 (0.3 or 1 μmol/L). After 3 hours, cells were harvested and fixed with 2% formaldehyde. The cells were labeled with the indicated antibodies. The experiments were done by FACScan and data analysis was done with the Cell Quest software. E, effect of AZ960 on the cell-cycle distribution of MOLT-4, MT-1, and MT-2 cells. MOLT-4, MT-1, and MT-2 cells were cultured with AZ960 (0.3 or 1 μmol/L). After 48 hours, cells were stained with propidium iodide and cell-cycle distribution was analyzed by flow cytometry.

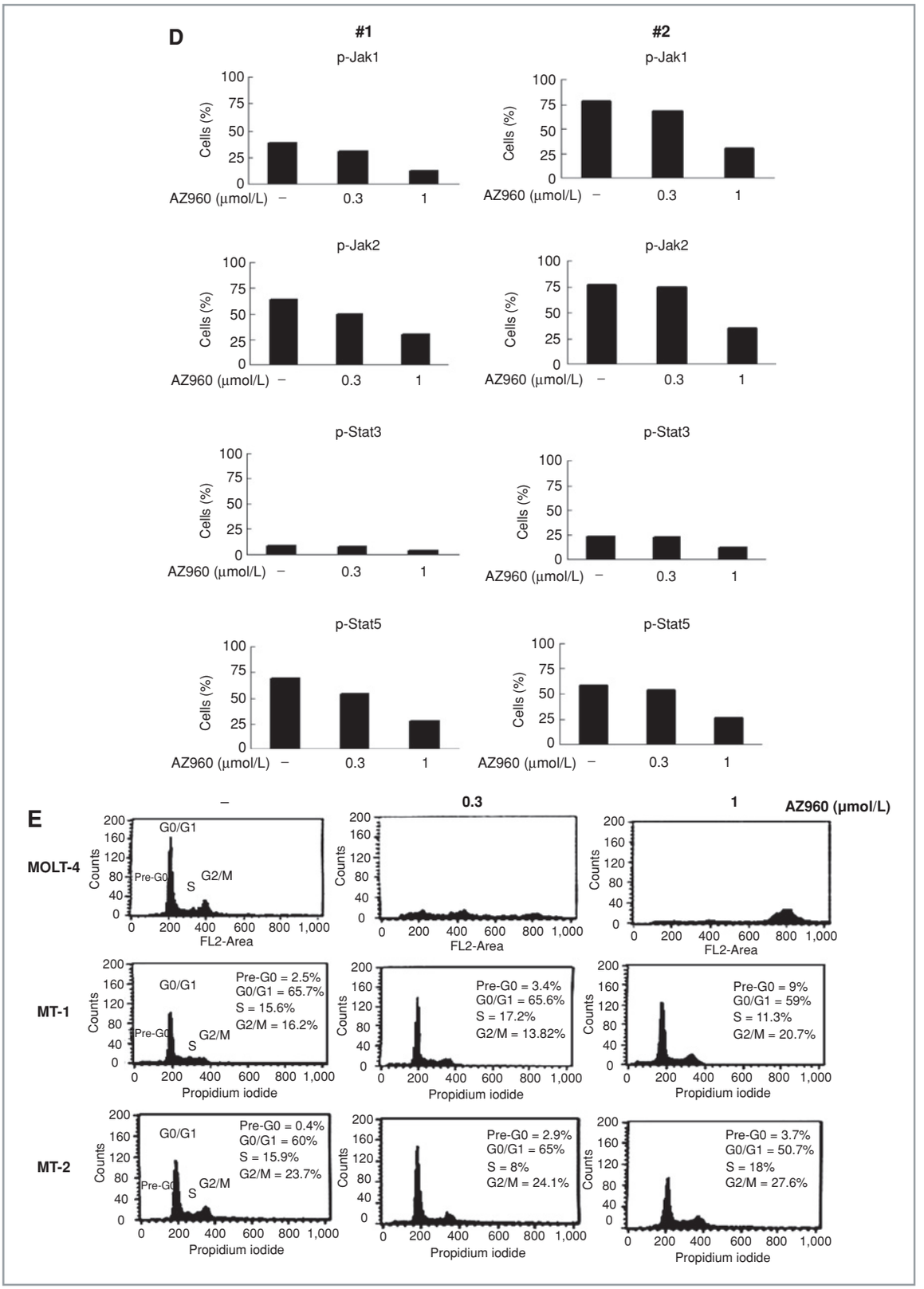

Figure 3. (*Continued*)

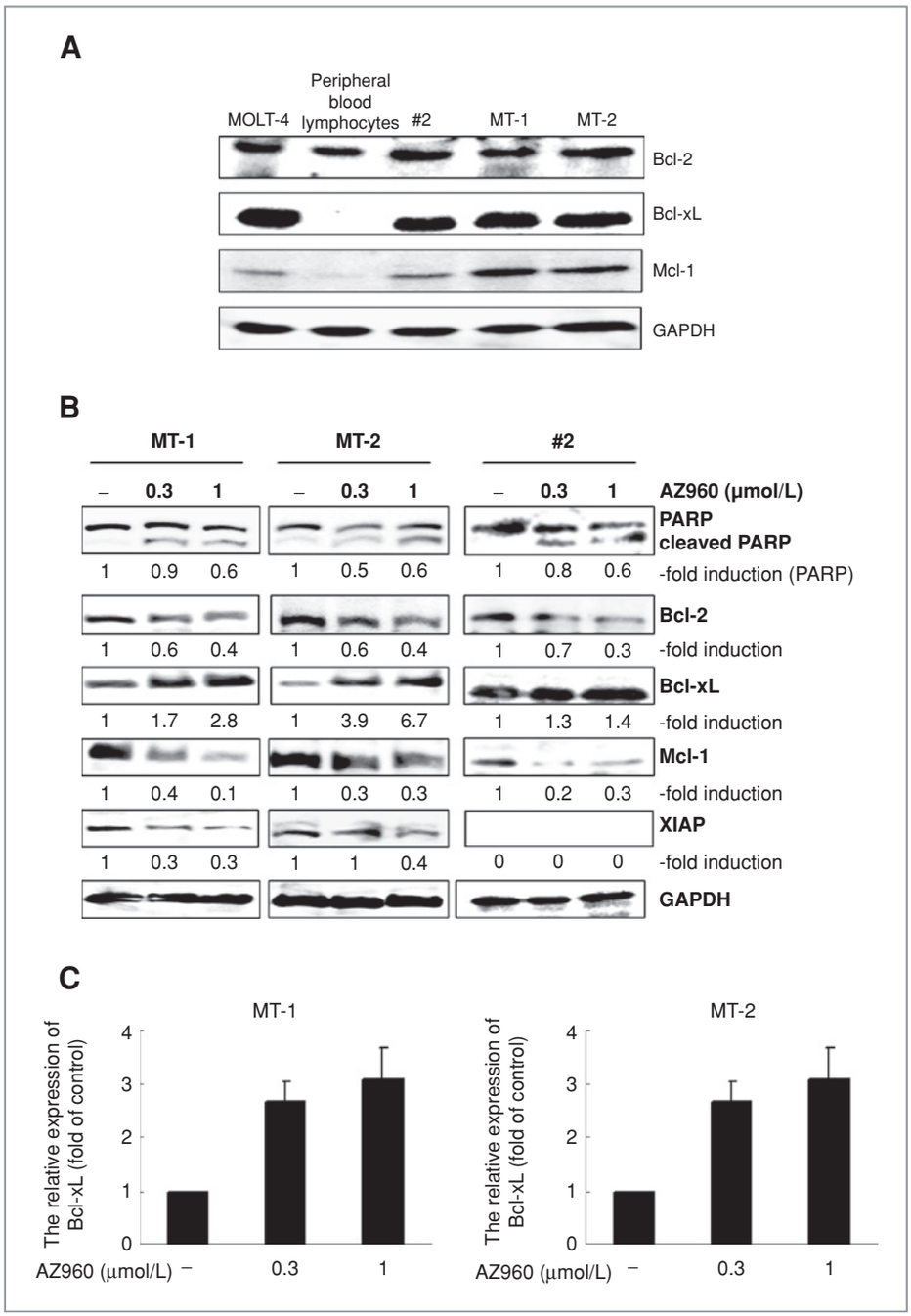

Figure 4. The levels of Bcl-2 family. A, Western blot analysis. The levels of Bcl-2 family proteins in MOLT-4, MT-1, MT-2, and freshly isolated ATL cells (case #2) and peripheral blood lymphocytes from healthy volunteer were determined by Western blot analysis. The membrane was sequentially probed with anti-Mcl-1, -Bcl-xL, -Bcl-2, and -GAPDH antibodies. Effect of AZ960 on the expression of Bcl-2 family proteins and PARP. B, Western blot analysis. MT-1, MT-2, and freshly isolated ATL (#2) cells were treated with AZ960 (0.3 or 1 μmol/L) for 48 hours. The levels of Bcl-2 family proteins and PARP were determined by Western blot analysis. The membrane was sequentially probed with the anti-PARP, -XIAP, -Mcl-1, -Bcl-xL, -Bcl-2, and -GAPDH antibodies. C, real-time RT-PCR. MT-1 and MT-2 cells were cultured with AZ960 (0.3 or 1 μmol/L) for 48 hours. Cells were harvested, and RNA was isolated and subjected to real-time RT-PCR. Results represent the mean ± SD of 3 experiments done in duplicate.

vage of PARP were dramatically induced in the Bcl-xL siRNA transfected MT-1 cells after exposure to AZ960 (1 μmol/L, 48 hours), but not in control siRNA-transfected cells (Fig. 5A).

Effect of AZ960 on levels of nuclear transcription factor CREB

Bcl-xL is one of the target genes of nuclear transcription factor CREB. We, therefore, examined the nuclear levels of CREB after exposure of MT-1 and MT-2 cells to AZ960 by Western blot analysis. As shown in Fig. 5B, AZ960 increased the nuclear levels of CREB. We next examined the effect of AZ960 on the levels of CREB bound on the Bcl-xL promoter by ChIP assay. Exposure of MT-1 cells to AZ960 (0.3 or 1 μmol/L) for 48 hours significantly increased levels of CREB bound on the Bcl-xL promoter in these cells (Fig. 5C).

Discussion

The present study found that inhibition of Jak/Stats by AZ960 effectively induced growth arrest and apop-

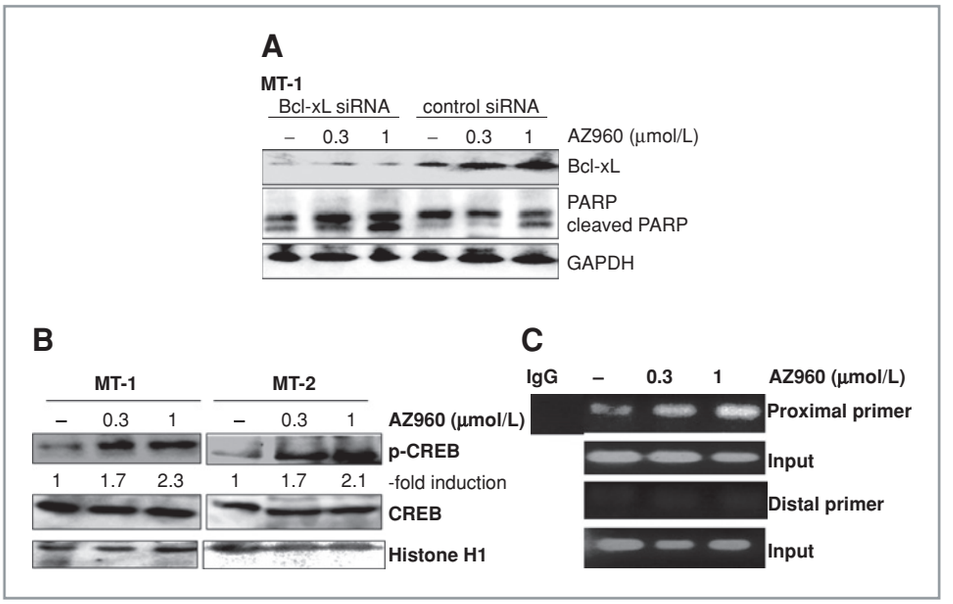

Figure 5. Knockdown of Bcl-xL by siRNA sensitizes MT-1 cells to AZ960-induced apoptosis. A, Western blot analysis. MT-1 cells were transiently transfected with either control or Bcl-xL siRNA. After 24 hours, cells were exposed to AZ960 (0.3 or 1 μmol/L) for 48 hours and proteins were extracted and subjected to Western blot analyses. The membrane was probed sequentially with the indicated antibodies. B, effect of AZ960 on the expression of p-CREB protein. MT-1 and MT-2 cells were treated with AZ960 (0.3 or 1 μmol/L) for 48 hours. The levels of p-CREB and total CREB proteins were determined by Western blot analyses. C, ChIP assay. MT-1 cells were treated with AZ960 (0.3 or 1 μmol/L) for 48 hours. Soluble chromatin was immunoprecipitated with antibodies against CREB or control IgG. Extracted DNA was amplified using pairs of either proximal or distal primers. Images are representative of 2 separate experiments. The experiments were repeated twice and identical results were obtained. Band intensities were measured by ImageJ software (Wayne Rasband, NIH).

tosis of HTLV-1–infected T cells in association with downregulation of antiapoptotic proteins Bcl-2, Mcl-1, and XIAP (Fig. 4B). Similarly, previously studies also found that inhibition of Jak/Stats by AG490 effectively induced growth arrest of HTLV-1–infected HUT102 cells in conjunction with downregulation of Bcl-2 (13). These observations suggest that Jak/Stats upregulates expression of Bcl-2 family proteins and stimulates proliferation of ATL cells.

Interestingly, AZ960 also inhibited the proliferation of acute T-lymphoblastic leukemia MOLT-4 cells that are not infected with HTLV-1. The phenotype of MOLT-4 after exposure to AZ960 was consistent with that of cells after exposure to an Aurora A inhibitor (29). AZ960 was shown to inhibit the kinase activity of Aurora A (25). Thus, AZ960 probably inhibited proliferation of MOLT-4 cells via Aurora A inhibition.

Bcl-xL is a critical antiapoptotic molecule in HTLV-1–infected T cells. Levels of Bcl-xL are regulated by nuclear factor-κB (NF-kB; ref. 30). NF-kB is constitutively activated in HTLV-1–infected T cells and inhibition of this nuclear transcription factor by BAY11–7082 effectively induced apoptosis of ATL cells in association with downregulation of Bcl-xL (31). We also showed that triterpenoid oridonin induced apoptosis of ATL cells in conjunction with downregulation of NF-kB and Bcl-xL (32). Surprisingly, this study found that inhibition of Jak2/Stats signaling by AZ960 caused upregulation of Bcl-xL in HTLV-1–infected cells. We first hypothesized that AZ960 could activate NF-kB and increase expression of Bcl-xL in MT-1 and MT-2 cells. Contrary to our expectation, AZ960 did not affect nuclear levels of NF-kB (data not shown). Another transcription factor that regulates expression of Bcl-xL is CREB. After exposure of HTLV-1–infected T cells to AZ960, nuclear levels of p-CREB were upregulated as measured by Western blot analysis (Fig. 5B), which suggested that AZ960-induced upregulation of Bcl-xL was probably via induction of CREB. Further experiments found that levels of p-CREB bound around the promoter region of Bcl-xL were increased in MT-1 cells after exposure to AZ960 (Fig. 5C), confirming that AZ960 stimulated expression of Bcl-xL via activation of CREB. CREB could be activated to compensate the impaired function of Jak/Stats and blunt the proapoptotic effect of AZ960 in HTLV-1–infected T cells. Recent studies identified a new biological function of Bcl-xL, which was distinct from its antiapoptotic activity. Exposure of malignant lymphoma cells to cytotoxic drugs induced expression of Bcl-xL, which interacted with cyclin-dependent kinase 1 and abrogated its ability to trigger mitotic entry, resulting in accumulation of cells in the G_2-M phase of the cell cycle (33). Exposure of MT-2 cells to AZ960 accumulated these cells in the G_2-M

phase of the cell cycle (Fig. 3E) in parallel with upregulation of Bcl-xL. Upregulation of Bcl-xL might cause G_2-M cell-cycle arrest in these cells.

Jak2 is a major focus in treatment of myeloproliferative diseases. V617F mutation in the *JAK2* gene was found in approximately 90% of polycythemia vera (34) and 50% of primary myelofibrosis (34). The specific Jak2 inhibitor including INCB018424 (35) and TG101348 (36) are under clinical evaluation in individuals with these diseases with favorable responses.

Taken together, concomitant blockade of Jak2/Stats and Bcl-xL represents a promising treatment strategy for individuals with ATL. Further studies are warranted to evaluate the effect and safety of the Jak2 inhibitor in this lethal disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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