http://informahealthcare.com/ipi
ISSN: 0892-3973 (print), 1532-2513 (electronic)
Immunopharmacol Immunotoxicol, 2013; 35(4): 478–486

DOI: 10.3109/08923973.2013.811597

Immunopharmacology and Immunotoxicology

informa healthcare

RESEARCH ARTICLE

Benznidazole modulates cell proliferation in acute leukemia cells

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Abstract

Context: We have previously reported that benznidazole (BZL), known for its trypanocidal action, has anti-proliferative activity against different cell lines like HeLa and Raw 264.7 among others. At the moment, it has not been reported if the anti-proliferative effect of BZL is similar for non-adherent hematopoietic cells like was reported for adherent cancer cell lines.
Objective: We aimed to investigate the efficacy of BZL on the growth of the leukemic cell lines THP-1 and OCI/AML3.
Materials and methods: We evaluated cell proliferation by [^{3}H]-thymidine incorporation and MTT reduction as well as cell death by lactate dehydrogenase (LDH) activity. We assessed apoptosis by flow cytometry for detection of annexin V-positive and propidium iodide-negative cells, along with nuclear morphology by diamidino-2-phenolindole (DAPI) staining. Western blot studies were performed to evaluate changes in cell cycle proteins in BZL-treated cells.
Results: BZL significantly reduced proliferation of both cell lines without inducing cell death. Likewise it produced no significant differences in apoptosis between treated cells and controls. In addition, flow cytometry analysis indicated that BZL caused a larger number of THP-1 cells in G0/G1 phase and a smaller number of cells in S phase than controls. This was accompanied with an increase in the expression of the CDK inhibitor p27 and of cyclin D1, with no significant differences in the protein levels of CDK1, CDK2, CDK4, cyclins E, A and B as compared to controls.
Conclusion: BZL inhibits the proliferation of leukemic non-adherent cells by controlling cell cycle at G0/G1 cell phase through up-regulation of p27.

Keywords

AML cell lines, benznidazole, cell cycle, cytotoxicity, p27

History

Received 21 March 2013
Revised 10 May 2013
Accepted 1 June 2013
Published online 12 July 2013

Introduction

Benznidazole (BZL; *N*-benzyl-2-nitroimidazole-1-acetamide), a distinctive drug currently used in the chemotherapy of Chagas' disease, acts against *Trypanosoma cruzi* through nitroreduction, leading to protein synthesis inhibition and degradation of macromolecule biosynthesis[1–4]. Besides its trypanocidal activity, BZL also has an immunomodulatory effect on macrophages by blocking the transcription of some pro-inflammatory mediators without altering interleukin 10 expression. This effect has been observed both *in vitro*, in a macrophage-derived cell line and in purified peritoneal macrophages, and *ex vivo*, in liver cells from treated animals[5–7]. This down-regulation of the synthesis of pro-inflammatory mediators in murine macrophages has been associated with an inhibition of the activation of nuclear factor kappa B (NF-κB) by the blockage of the IκB kinase complex and with an inhibition of the activation of p38 *mitogen-activated protein kinase* (MAPK) by BZL[6,8]. In fact, BZL can have selective immunomodulatory effects, as it is able not only to decrease the systemic inflammatory reaction in mice with sepsis induced by cecal ligation and puncture by lowering the levels of pro-inflammatory cytokines, leukocytes and bacteremia and the liver response, but also to increase the number of cells in the site of infection[9].

In addition to this role as a modulator of the immune response, we have previously demonstrated that BZL displays anti-proliferative activity[10]. We also demonstrated that BZL significantly reduced proliferation of RAW 264.7 but that it also leads to growth arrest in other adherent cell lines like CHO, MDCK and HeLa. Interestingly, we found that growth inhibition is a reversible process, not accompanied by significant cell death, indicating that the drug behaves mainly as a cytostatic compound.

The specific inhibition of NF-κB in RAW 264.7 and HeLa cells is sufficient to block cell proliferation. This suggests that this may be the mechanism by which BZL arrests cell growth in these cell lines[10].

Cure rates of acute monocytic leukemia (AML) patients are extremely variable, ranging between 30% and 40%; therefore, new treatment strategies are being intensively studied to improve patients' outcomes[11–13]. Since AML treatment is undergoing an adjustment during which clinical

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results have yet to reflect advances in the development of targeted therapeutics, it is still one of the hematologic neoplasms most difficult to treat[11–14]. The development of new drugs is both time- and cost-consuming. In contrast, finding new uses for existing drugs is a positive connection between the lab and the clinic[15]. Thus a better understanding of existing drugs may allow their rational redistribution in new disease settings[15,16].

Therefore to date, BZL has been shown to possess growth inhibitory effects for characterized adherent cell lines from a variety of tumors; however, the potential efficacy of BZL treatment against non-adherent AML cells remains unknown. We here evaluated the effect of BZL on cell proliferation and apoptosis in THP-1 and OCI/AML3, two characterized non-adherent AML cells, and further explored how BZL modulates THP-1 cell cycle and expression of specific cyclins and cyclin-dependent kinase inhibitor.

Materials and methods

Reagents

BZL was kindly provided by Roche Laboratories (Argentina and Brazil); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT), propidium iodide (PI) and thymidine (T1895) were purchased from Sigma-Aldrich (Argentina). In all experiments, BZL was prepared in dimethylsulfoxide (DMSO) as a 1000× stock solution and then further diluted in cell culture medium containing 10% fetal bovine serum (FBS) in hood just before use.

Cell culture conditions

The human leukemia cell line THP-1 (established from the peripheral blood of a 1-year-old boy with acute monocytic leukemia (AML) at relapse[17] was obtained from the American Type Culture Collection (Manassas, VA) and kept at 37 °C in a 5% CO_2 atmosphere in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 2 mM HEPES. The OCI/AML3 cell line (established from peripheral blood from a 57-year-old male with AML FAB-M4[18,19] was kindly provided by Dr. Mark D. Minden (Princess Margaret Hospital, Toronto, Ontario, Canada). OCI/AML3 cells were grown in alpha-modified Eagle medium plus 20% FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 2 mM HEPES. For all experiments, cells were plated at a maximum density of 500 000 cells/mL. Cell culture reagents were from (Invitrogen Life Technologies, Buenos Aires, Argentina) and FBS was from PAA Laboratories (Capital Federal, Argentina). The cells were treated with 0.1, 0.5 or 1 mM BZL for 24 or 48 h or with 0.1% DMSO (controls).

Analytical assays

Assessment of cell viability and proliferation

Metabolic activity was determined by the MTT reduction assay. To this end, 20 000 cells in 200 μL of complete medium were incubated in quadruplicate in a 96-well plate (Lab-TekTM chamber slide, NUNC) in the presence of BZL (0.1, 0.5 and 1 mM) or vehicle (0.1% DMSO) for 24 or 48 h and then 20 μL of MTT solution (5 mg/mL in phosphate-buffered saline [PBS]) was added to each well. After 2 h at 37 °C, the MTT solution was removed and precipitated formazan was solubilized in 200 μL DMSO. Formazan production was then measured at OD_{545nm} in a micro plate spectrophotometer (Stat Fax 2100, Awareness Technologies, Inc., Palm City, FL), with DMSO as blank. Cell proliferation was evaluated by measuring the amount of [^{3}H] thymidine incorporated into DNA. Cells were grown in sextuplicate in a 96-well plate at a concentration of 20 000 cells in 200 μL of complete medium. The cells were treated with BZL (0.1, 0.5 and 1 mM) or 0.1% DMSO. Cells were pulsed with 0.5 μCi [^{3}H] thymidine 7 h before each time point evaluated, and uptake was monitored in a liquid scintillation counter (Rack beta 1214, Pharmacia)[10,20].

Cell damage and toxicity

Membrane integrity after BZL treatment was determined by measuring the release of lactate dehydrogenase (LDH) in culture supernatants. Cells were treated with BZL as described before and supernatants were collected at the indicated time points. Total lysates were obtained as internal controls by incubating the cells in sterile H_2O_d. LDH activity was evaluated by an indirect colorimetric assay using a commercial kit (Wiener Lab, Rosario, Argentina). Briefly, a 200 μL aliquot of reagent was incubated with 10 μL of supernatant or total lysate and the reaction was followed as the disappearance of NADH at 340 nm in a microplate reader (DTX 880, Becton Coulter) for 3 min. LDH activity was calculated following the manufacturer's instructions[10].

Apoptosis assay

Apoptosis was assessed by examining nuclear morphology and by flow cytometry. Cells were treated with BZL 1 mM or vehicle (0.1% DMSO) for 24 or 48 h. Apoptotic cells were identified by nuclear condensation and fragmentation, assessed by DNA-specific fluorochrome staining with 4′,6-diamidino-2-phenolindole (DAPI). The cells were washed once in cold PBS, fixed in methanol for 5 min and in ice-cold acetone for 10 min, washed again with PBS and finally stained with DAPI (Molecular Probes®, Life Technologies, Buenos Aires, Argentina) (50 μM) for 5 min in darkness and mounted with PBS:glycerol (1:1). Fluorescence localization was detected by morphological changes in the apoptotic cells and visually assessed under a laser confocal microscopy (Nikon C1SiR with inverted microscope Nikon TE200). Apoptotic cell death was then calculated as the percentage of apoptotic cells over the total cells[21]. Apoptosis was also evaluated by the detection of annexin V-positive and PI-negative cells by flow cytometry. BZL-treated and control cells were labeled with annexin V conjugated with fluorescein isothiocyanate (FITC) and PI (BD Pharmingen, San Diego, CA). Briefly, cells were washed once in PBS and once in 1× binding buffer and then 5 μL of annexin V-FITC was added to the cells. After incubating the cells at room temperature for 15 min in the dark, 300 μL 1× binding buffer and 10 μL of PI were added, and the cells analyzed by flow cytometry using a FACSAria II (Becton Dickinson, Franklin Lakes, NJ). A minimum of 10 000 cells/sample were acquired and data were analyzed with WinMDI 2.8 (Scripps Research Institute, La

Jolla, CA). Cells were gated to exclude cell debris, cell doublets and cell clumps[22].

Cell cycle synchronization

THP-1 cells were synchronized at G1/S phase using a double thymidine block protocol. Exponentially growing cells were first treated with 3 mM thymidine for 22 h. After that, the cells were extensively washed with PBS and passed to thymidine-free medium for 12 h. Later, the cells were treated with 3 mM thymidine for additional 22 h. The cells were released from the second block by washing three times with RPMI, and incubating in standard growth medium containing BZL 1 mM or vehicle[23].

Cell cycle analysis

The effect of BZL on cell cycle distribution was determined by flow cytometric analysis of DNA content after staining with PI. Following treatment of THP-1 cells with BZL 1 mM or 0.1% DMSO for 24 and 48 h, 500 000 cells/mL were washed twice with PBS and fixed in 70% ethanol overnight at 4 °C. After fixation, cells were washed again with PBS and incubated in buffer K (1 mg/mL sodium citrate, 0.02 mg/mL RNAase, 0.3% v/v Nonidet P-40, 0.05 mg/mL PI, pH 7.4) for 15 min at room temperature. The measurements were performed on a FACSAria II flow cytometer at an excitation wavelength of 488 nm. A minimum of 10 000 cells/sample were acquired and data were analyzed with WinMDI 2.8 and Cylchred 1.0.2. Resulting DNA distributions were analyzed for the proportion of cells in G0/G1, S and G2-M phases of the cell cycle. Cells were gated to exclude cell debris, cell doublets and cell clumps[10].

Immunoblotting

Total cell extracts were prepared by homogenization with lysis buffer containing PBS, 1% Triton X-100, 0.5 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl-fluoride (Sigma), and 10 μg/mL leupeptin (Sigma). After a 30-min incubation at 0 °C and three freeze–thaw cycles, extracts were cleared by centrifugation at 12 000 rpm for 15 min at 4 °C, and supernatants were kept at −80 °C[24]. Protein concentrations were quantified according to Lowry[25]. To detect cyclins A, B, E, D1 as well as CDK1 and CDK2, 40 μg of protein were subjected to 10% sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinyl difluoride membranes (PVDF) (PerkinElmer Life Sciences, Boston, MA). Blots were blocked with 5% nonfat dry milk in PBS 0.3% Tween-20 142 (PBS-Tween). After blocking, blots were incubated overnight at 4 °C with antibodies against cyclins A (sc-596), B1 (sc-595), D1 (sc-753), E (sc-481) and CDK1 (sc-747) and CDK2 (sc-163) (Santa Cruz Biotechnology, Santa Cruz, CA), CDK4 (2906), p27 (3686) and α tubulin (3873) (Cell Signaling Technology, Inc., Danvers, MA). The membranes were then incubated with either anti-rabbit or anti-mouse IgG peroxidase conjugates (Amersham Life Sciences, Boston, MA) and the resulting bands were detected by enhanced chemiluminescence (ECL; Pierce Western blotting substrate). Autoradiographs were obtained by exposing PVDF membranes to Kodak XAR film. The bands were quantitated by densitometry using the NIH Image J program[9,26].

RNA isolation and quantitative real-time PCR (qRT-PCR)

THP-1 cells were treated with 1 mM BZL or vehicle for 24 h. Total RNA was extracted from cells using the TRIzol® method according to the manufacturer's instructions (Invitrogen). RNA was dissolved in RNAse-free water and kept at −80 °C until use. Total RNA (2.5 μg) was transcribed using 100 units of M-MLV-RT (Promega, Madison, WI) and an oligo (dT)17 primer. qRT-PCR reagents were from Thermo Scientific®-Argentina (Part of Thermo Fisher Scientific), and real-time PCR was performed using the Mx3000 (Stratagene) detection system. The transcript levels are expressed as ΔΔCt values and relative expression was normalized to GAPDH[27], since the amplification efficiency of GAPDH and cyclin-D1 were similar. Experiments were repeated at least three times and PCR reactions were always performed in triplicate. The sequences of the gene-specific primers were as follows: endogenous gene, GAPDH, forward 5′-TGTTCGTC ATGGGTGTGAAC-3′ and reverse 5′-ATGGCATGGACTGT GGTCAT-3′; D1 cyclin forward 5′-AACACGCGCAGACCT TCGTT-3′ and reverse 5′-CGGTAGTAGGACAGGAAG TT-3′. The PCR reactions were carried out in a final volume of 20 μL of the reaction mixture comprised of 10 μL of mix with eva-green (Biodynamics, Buenos Aires, Argentina), 5 μL of cDNA dilution, 1 μM of the primers and water, according to the manufacturer's instructions. The reaction was started at 95 °C for 10 min, followed by 40 rounds of amplification at 95 °C (20 s), 60 °C (30 s), and 72 °C (40 s), followed by a cooling step at 50 °C for 30 s[27].

Statistical analysis

Data are presented as mean ± SE. Student's *t* test was applied wherever necessary and statistical analysis of differences between groups was performed by one-way analysis of variance followed by Tukey's method. Differences were considered as statistically significant when $p < 0.05$.

Results

BZL inhibits cell proliferation of THP-1 and OCI/AML3

Treatment of leukemic cell lines with BZL 1 mM for 48 h decreased the metabolic activity (Figure 1A) in both cell lines. This observation was further evaluated by measuring the amount of [^{3}H]-thymidine incorporated into DNA. Incubation of cells with BZL 0.1, 0.5 and 1 mM for 24 or 48 h resulted in a dose-dependent decrease in proliferation, suggesting that BZL most likely acts as an inhibitor of DNA replication (Figure 1B). The THP-1 cell line was more sensitive to BZL treatment than OCI/AML3, even at the lowest dose (0.1 mM) (Figure 1B).

BZL arrests cell growth without causing significant cell death

To test whether BZL treatment could induce cell death by necrosis, we measured LDH activity in the supernatants of cells treated with BZL for either 24 or 48 h. We found no differences in LDH activity in supernatants between BZL-treated cells and controls (Figure 1C). The observed increase in LDH activity in supernatants at 48 h is likely due to basal spontaneous lysis, but without differences between

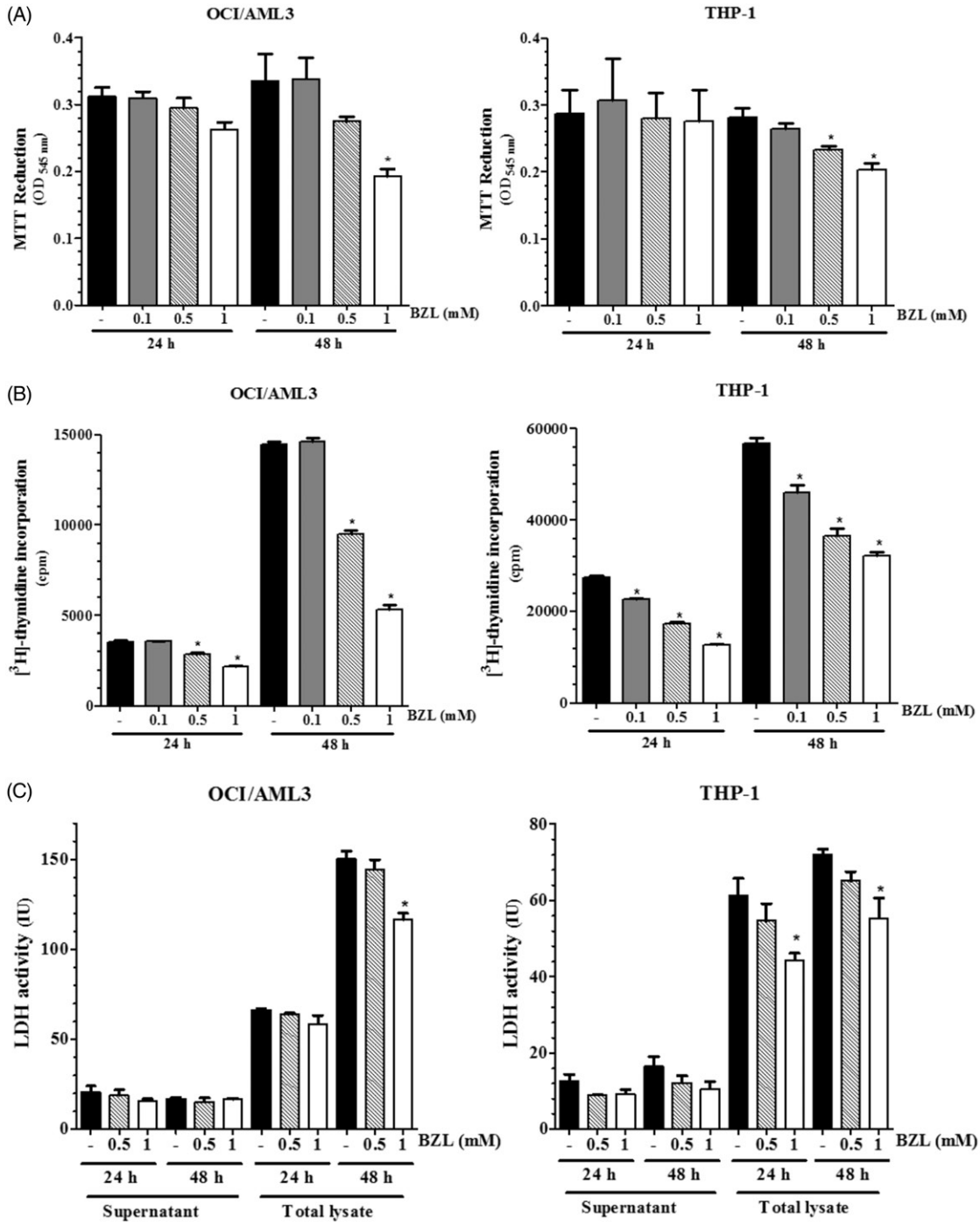

Figure 1. BZL arrests growth of leukemic cell lines but does not induce cell death. OCI/AML3 and THP-1 cells were treated with BZL for either 24 or 48 h and the following parameters were evaluated: (A) metabolic activity by MTT reduction; (B) DNA synthesis by [^{3}H]-thymidine incorporation; (C) LDH activity determined by an indirect colorimetric activity was measured in supernatants and total cell lysates. Results are shown as mean ± SD, $n = 3$–4. A representative experiment of three is shown. *BZL 0.1, 0.5 and 1 mM statistically different from control, $p < 0.05$.

treated cells and controls. As a control, we also measured LDH activity in total lysates from the same cultures and found a statistically significant decrease in LDH activity in the lysates from cells treated with 1 mM BZL for 48 h, which correlated well with the decrease observed in cell proliferation (Figure 1B).

To check whether BZL treatment could induce cell death by apoptosis, we evaluated the number of cells with apoptotic nuclei by DAPI stain in cells treated with BZL for 24 h. We found no differences between treated cells and controls (Figure 2A). BZL treatment did not increase the percentage of annexin V-positive cells after 24 or 48 h of incubation (Figure 2B).

These results suggest that BZL inhibits proliferation of AML cells without inducing cell death by apoptosis or necrosis in either of the cell lines studied.

BZL delays the entry of THP-1 cells into mitosis

Evaluation of the effect of BZL on the cell cycle was performed in THP-1 cell line, which exhibited the higher anti-proliferative response to the drug. The cell cycle

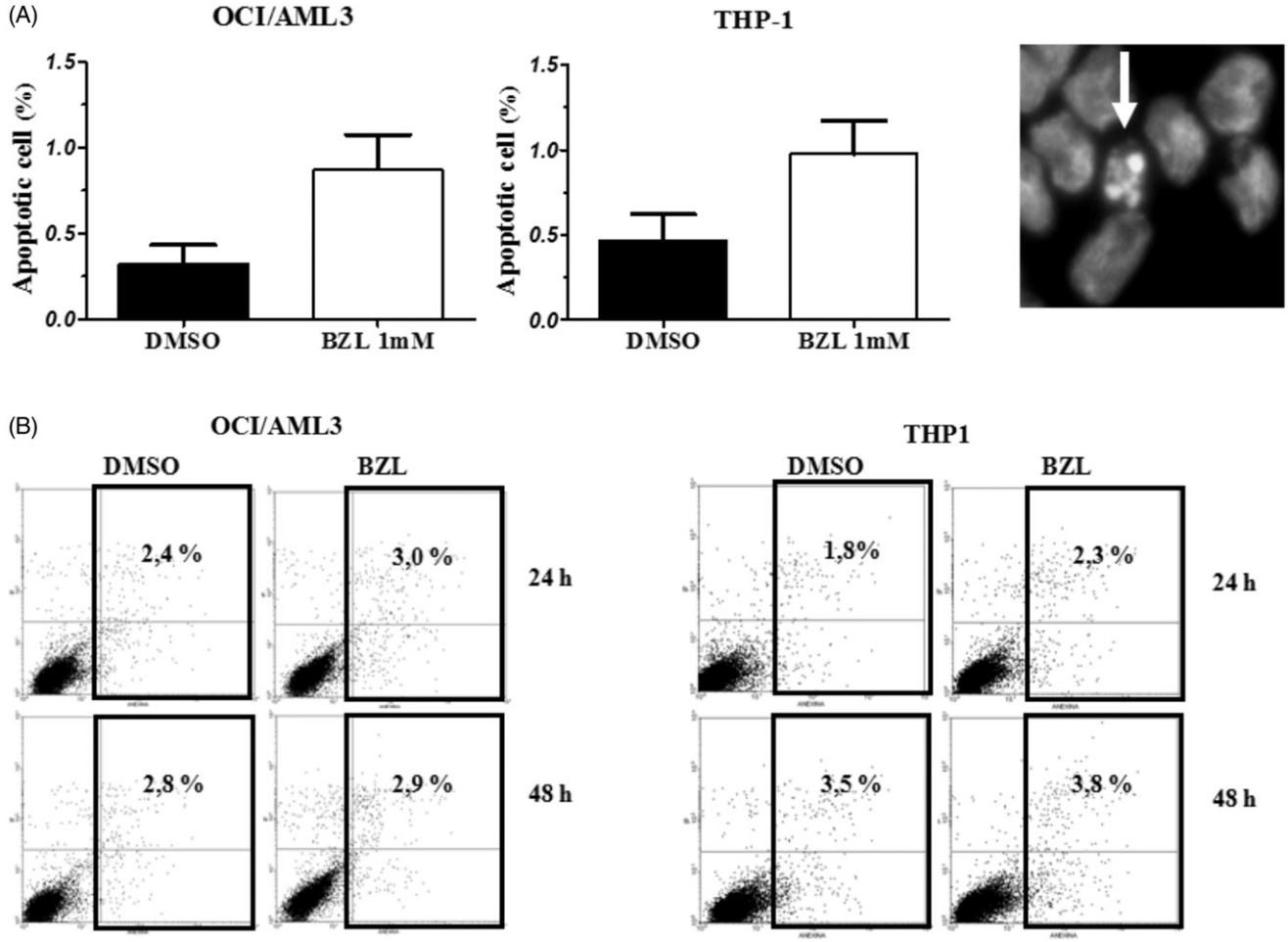

Figure 2. Apoptosis by DAPI stain. (A) Apoptotic nuclei were quantified and normalized to total nuclear morphology. A representative fluorescence micrography of a typical THP-1 apoptotic cell (arrow) is shown (×1000); (B) Apoptosis was also evaluated by fluorescence-activated cell sorter for detection of annexin V-positive cells.

distribution was monitored by flow cytometry. Figure 3(A) shows representative histograms of cells incubated in the presence or absence of BZL with the respective percentage over the cell cycle obtained with Cylchred 1.0.2. Treatment with 1 mM BZL for 24 h caused a transient G0/G1 accumulation of 65.3% compared to 48% of the untreated cells (percentage expressed as median) (Figure 3B). THP-1 cells were synchronized at G1 phase[23]. After release from the double thymidine block, they showed comparable results (unpublished results). Consequently, BZL is likely to interfere with cellular processes that occur in early S phase including DNA replication. This finding, along with the absence of apoptotic nuclei in BZL treated cultures suggest that the presence of BZL delays the onset of mitosis without inducing cell death.

BZL induces an increase in p27 and cyclin-D1 levels

In view of the data demonstrating modulation of the cell cycle by BZL, and to further analyze whether this effect could result from changes in the expression of proteins involved in cell cycle progression, THP-1 cells were incubated with BZL 1 mM or DMSO for 24 h. Immunoblotting demonstrated that the levels of p27, a strong cyclin-dependent kinase inhibitor of cyclin E-CDK2, increased after the addition of BZL (Figure 4A). We also observed cyclin D1 accumulation at protein level after BZL treatment (Figure 4B) with no differences at transcriptional level. qRT-PCR showed a $\Delta\Delta$Ct of -0.27 (± 0.32).

This result strengthens the notion that the arrest in G1 phase of THP-1 cells by BZL was possible because p27 inhibits the phase transition from S.

Discussion

We have previously described a novel anti-proliferative activity of BZL on several cell lines of different host and tissue origin (RAW 264.7, CHO, MDCK and HeLa cells), an effect that might be related to NF-κB blockage[10]. NF-κB has been demonstrated to be abnormally activated in some patients with AML[28], and is thought to be involved in the pharmacological resistance to many chemotherapeutic agents. This suggests that NF-κB inhibitors can be exploited as a strategy to increase the rate of chemotherapy sensitivity[29].

Limited progress has been achieved in improving the long-term disease-free survival, except for certain subtypes of AML (e.g. acute promyelocytic leukemia). Cytarabine (Ara-C), etoposide (VP-16) and daunorubicin (DNR) remain frontline agents[30]. Our previous results demonstrated an anti-proliferative activity of BZL on well characterized adherent cell lines[10]. In the present study, we investigated the role of BZL in the proliferation of two non-adherent leukemic cell

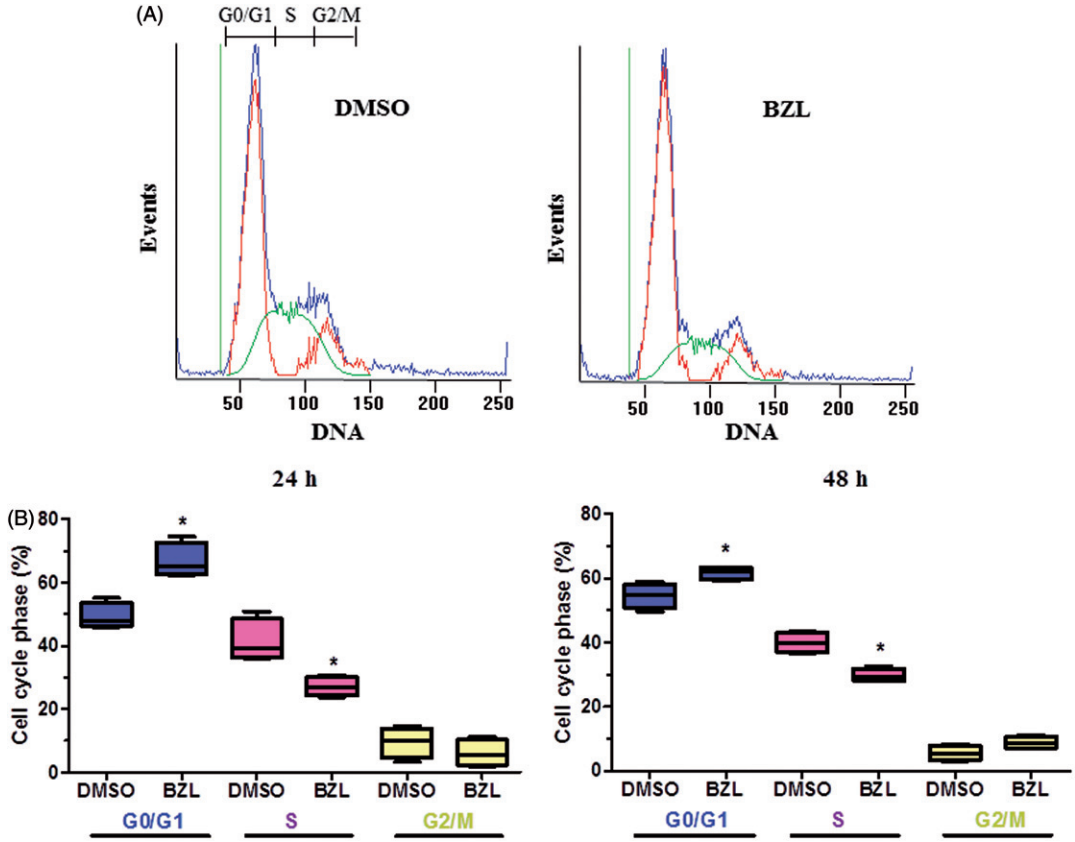

Figure 3. BZL delays the entry of THP-1 cells into mitosis. DNA content was measured by flow cytometry after PI staining. (A) A representative histogram is shown for BZL-treated and control cultures. These graphics are made by the equipment software (Cylchred 1.0.2.) in order to calculate the events of each phase; the blue line corresponds to total DNA content, the first red curve represents the G0/G1 phase, the green curve corresponds to the S phase and the second red curve is representative of the G2/M phase. (B) Cell cycle distribution was studied in cells treated with BZL for 24 or 48 h. Results are shown as the percentage of cells in the different phases.

lines THP-1 (AML M5 subtype) and OCI/AML3 (AML M4 subtype). Because of the non-adherent, undifferentiated, nature of these cell lines, results from the current study could be more reliably associated to cell proliferation under AML conditions. BZL was applied at millimolar concentrations because it has been shown that at such levels inhibit cell growth[10]. We found growth inhibition, assessed by a reduction in metabolic activity, and no significant cell death after 48 h of BZL treatment, as assessed by the release of the cytosolic enzyme LDH in culture supernatants. Estimation of apoptosis by flow cytometric detection of annexin V-positive and PI-negative cells as well as by the morphology of the nuclei stained with DAPI showed no significant differences between treated and untreated cells.

Both cell lines were shown to decrease [^{3}H]-thymidine incorporation, suggesting that BZL is a potential inhibitor of DNA replication. THP-1 cells were more sensitive to BZL treatment than OCI/AML3 even at the lowest dose used (0.1 mM). The OCI/AML3 cell line carries a gene mutation in nucleophosmin (NPM). NPM is a nucleocytoplasmic shuttling protein with prominent nuclear localization, implicated in ribosomal protein assembly and transport as well as in prevention of nucleolar protein aggregation[31,32]. Mutations in NPM exon 12 and the resulting relocalization of NPM from nucleus to the cytoplasm are the most specific and frequent cellular events in AML patients with normal karyotype[33–35]. Cilloni et al. demonstrated that NF-κB and NPM proteins interact physically within the cytoplasm. These proteins do not interact in normal cells because NPM has mainly nucleolar localization. However, this interaction becomes biologically significant when mutations relocalize the protein into the cytoplasm[36]. This interaction could explain the difference in NF-κB activity in AML patients with NPMc$^+$ mutation; the reduced nuclear level of NF-κB in NPMc$^+$ cells may be due to the sequestration into the cytoplasm by the abnormally localized NPM mutant protein of the active form of NF-κB[36]. We hypothesized that this situation could be connected with the reduced susceptibility of OCI-AML3 cells to cell cycle arrest observed following the addition of BZL (unpublished results).

The cell cycle distribution of THP-1 cells was monitored by flow cytometry. Asynchronously growing cells treated with BZL were delayed in the cell cycle distribution; we found a larger number of cells in G0/G1 phase and a smaller number of cells in S phase than in controls. Similar results were observed when the cells were previously synchronized at G0/G1 using a double thymidine block protocol.

Immunoblotting demonstrated high levels of cyclin D1 and p27 in THP-1 cells treated with BZL and no significant differences in the protein levels of CDK1, CDK2, CDK4, cyclin E, cyclin A and cyclin B as compared to controls. Consequently, the cell arrest in G0/G1 phase was accompanied by an increase in the expression of p27, an important negative regulator of the CDK2-cyclin E complex necessary for the transition of G1/S[37,38]. p27 also appears to promote the assembly of p27-cyclin D1-CDK4 and CDK6 complexes

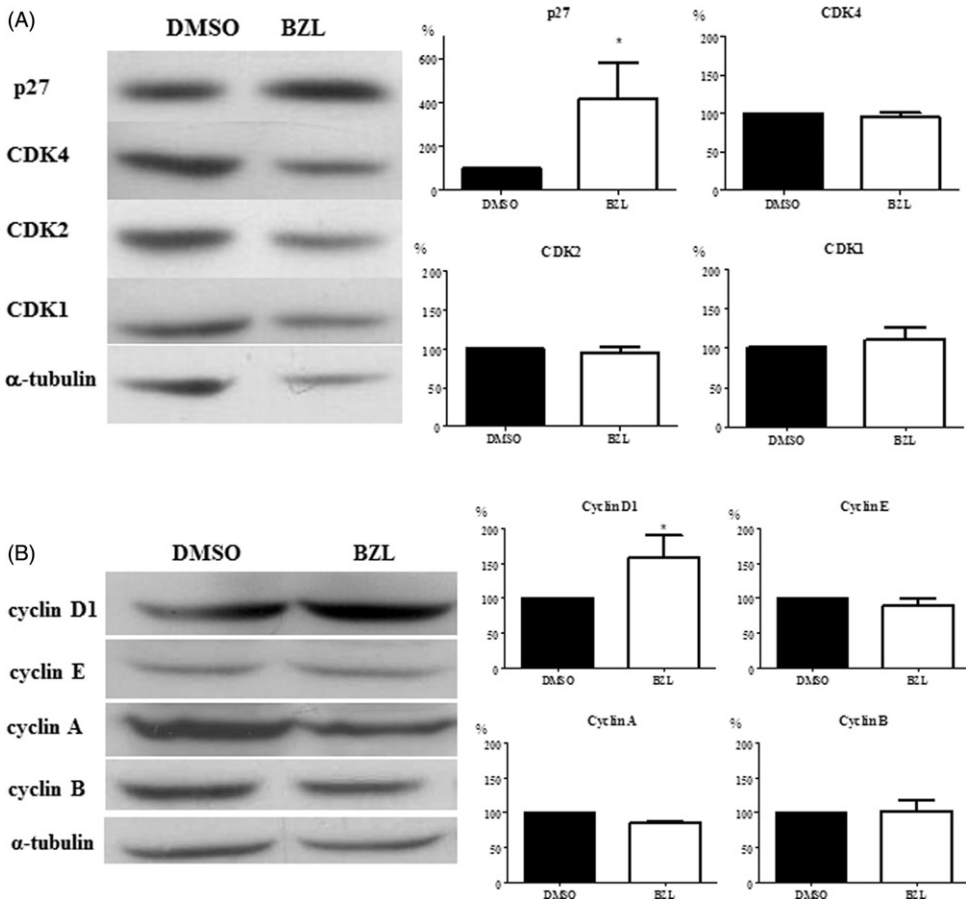

Figure 4. Immunoblotting analysis in total THP-1 total cell extracts. Cells were incubated with 1 mM BZL or in absence (DMSO) for 24 h. Cell lysates were prepared as described in Materials and methods, and were resolved on 12% SDS-PAGE gels for immunoblotting analysis of (A) p27, CDK4, CDK2, CDK1 and (B) cyclin-D1, cyclin-E, cyclin-A, cyclin-B. Levels of α-tubulin were measured to confirm equal protein loading. The accompanying bars represent the densitometry of each specific protein relative to α-tubulin represented as percentage considering control as 100%. This figure is representative of three separate experiments. Data were analyzed by Student's *t* test. Results are shown as mean ± SD, $n = 4$. *BZL 1 mM statistically different from control, $p < 0.05$.

in vitro[39] and/or the stabilization of these complexes in cells[39–41]. Probably, the unexpected increase in cyclin D1 was due to a prolonged half-life of the protein since there were no differences at transcriptional level as assessed by RT-PCR.

Although the molecular mechanism is not definitively clarified, a negative modulation of the NF-κB activation process probably occurred. Transcription factors of the NF-κB family are regulators of cell proliferation and survival and control expression of several genes relevant to the tumorigenic process[42]. Indeed, NF-κB can be activated by a number of pathways like lipopolysaccharide (LPS) among others. On this regard, we have found that THP-1 cells, pretreated with BZL (1 mM) and stimulated with LPS exhibited an important delay in the translocation of p65 subunit of NF-κB to the nucleous when compared to cells not treated with BZL (unpublished results). Even when we did not demonstrate whether BZL inhibits NF-κB activation upon stimulation[8,9], participation of this transcription factor cannot be rule out as a mediator of BZL effects.

Developing new drugs is cost-consuming and naturally deters the development of drugs in less common diseases[15,43,44]. One approach to overcome this hindrance is to develop a better understanding of old drugs[15]. For example, diverse groups have shown the anti-leukemic actions of the lipid lowering drug bezafibrate (BEZ) and the contraceptive steroid medroxyprogesterone acetate (MPA) against AML cells[16,45,46]. Treatment of AML cells with combined BEZ and MPA resulted in growth arrest, and induction of differentiation and apoptosis[46]. Thus it is reasonable to administer a combination of BZL with a chemotherapeutic agent to obtain a cooperative effect, which could be effective even using BZL at a lower dose than 1 mM. This rationale for a combined therapy is further sustained because it has been shown that most chemotherapeutic agents activate NF-κB. Doxorubicin, DNR, VP-16, Ara-C and cisplatin, drugs frequently used in AML treatment[47], are among these agents.

Whether BZL would be able to reduce proliferation *in vivo*, it needs to be explored. However, results from our laboratory using an experimental rat model inoculated with a fibrosarcoma by subcutaneous route showed that BZL induced a regression in tumor size and was devoid of hematological and hepatic toxicity, consistent with BZL treatment indeed (manuscript in preparation).

Conclusion

In this approach, we evaluated the potential use of an established drug in a new disease setting. The present work demonstrates for the first time the anti-proliferative

activity of BZL in non-adherent leukemic cell lines and open expectations about its potential therapeutic use in combination with classical chemotherapy drugs. However, further investigation is required to elucidate the exact mechanism by which this drug leads to cell cycle arrest in AML cells.

Declaration of interest

This work was supported by Research Grant PICT 01865 from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), and by Se C-T-I-UNR (MED 259). Karina Lucrecia Calvo was supported by a doctoral fellowship from CONICET.

The authors report no declarations of interest.

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