Redondo-Muñoz et al., Supplementary data

Supplementary Materials and Methods

Antibodies and chemical reagents

Monoclonal antibodies (mAb) to CD5 and CD19 were from Diaclone Research (Besançon, France); anti-CD3 mAb (3T3) was a gift from Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). mAbs to poly (ADP-ribose) polymerase (PARP-1, sc-8007); Akt1 (sc-5298); PTEN (sc-7974); IκB-α (sc-1643) and rabbit polyclonal antibodies (RpAb) to Bid (sc-11423), NF-κB (sc-109), caspase-9 (sc-8355) and Hsp60 (sc-13966) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). mAbs to caspase-3 (#9668), caspase-8 (#9746), and RpAbs to X-linked inhibitor of apoptosis protein (XIAP, #2042); phospho-Akt (Ser473; #9271) and phospho-JNK (#9251) were from Cell Signalling Technology, Inc. (Beverly, MA). mAb to actin (#3853) was from Sigma-Aldrich (St. Louis, MO). mAbs to cytochrome c (#556432) and active Bax (#556467) were from BD Biosciences (Erembodegem, Belgium). RpAbs to Flip (06-864) and histone H4 (07-108) were from Upstate (Charlottesville, VA). pAbs to mouse IgG (Alexa 488) or rabbit IgG (Texas Red) were from Molecular Probes (Eugene, OR). Horseradish peroxidase (HRP)-labeled goat Abs to mouse or rabbit Abs were from Dako A/S (Glostrup, Denmark). ATO, and N-Acetyl L-Cysteine (NAC) were purchased from Sigma-Aldrich. Kinase inhibitors SB203580 (p38 MAPK), BisI (PKC), LY294002 (PI3K), Tricirbine/API-2 (Akt), UO126 (Erk1/2), SP600125 (JNK), and caspase inhibitors Z-VAD-FMK (pan-caspase), and Z-IETD-FMK (caspase-8) were from Calbiochem (Darmstadst, Germany). Mn-tetrakis-(4-benzoic acid) porphyrin (MnTBAP) was from Alexis Biochemicals (San Diego, CA). 5-(and-6)-chloromethyl-2'7'-

dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) was from Molecular Probes.

Assesment of mitochondrial damage by Immunofluorescence

Glass coverslips were coated with 10 μg/ml p-Lys for 2 h at 37ºC, washed and blocked with 1% BSA for 30 min. $5x10^5$ B-CLL cells were incubated on the coverslips at 37ºC, in the presence or absence of 3 μM ATO. After 24 h cells were fixed, permeabilized with PBS-01% SDS, and incubated with Abs to cytochrome c, active Bax or Hsp60 (4ºC, overnight), followed by a 45 min incubation at room temperature with Alexa488-labelled or Texas Red-labelled secondary Abs. Images were acquired using a Leica TCS-SP2-AOBS inverted epifluorescence confocal microscope with X63 oil immersion objective (Unidad de Microscopía Confocal, Hospital Universitario Gregorio Marañón, Madrid, Spain). The "Dye-Separation Leica software" was used for colocalization studies. Image pixels were depicted as dot-plot representations, where X and Y correspond to the fluorescence intensity value of each fluorescence channel per pixel. A colocalization region was assigned to pixels displaying high levels of fluorescence for the two analyzed colors.

Western blotting

B-CLL cells were incubated in 24-well plates ($3-5x10^6$/well) with or without 1-3 μM ATO. At appropriate times cells were lysed in 10 mM Tris, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% deoxycholate, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 5 μg/ml leupeptin, and 10 μg/ml aprotinin, pH 7.4, and analyzed by SDS-PAGE and western blotting. For Akt and JNK phosphorylation analyses,

cells were lysed in the same buffer without SDS and containing 1 mM NaPPi and 10% glycerol. To analyze multiple proteins on the same membrane, after identification of the first protein, membranes were washed with PBS/0.1% Tween^{R}20 for 10 min, followed by 3 x 30 min incubation in 1% glycine pH 2.2, 1% SDS, 0.0005% NP-40, at room temperature. Membranes were washed again 2 x 15 min with PBS/Tween, blocked with 5% nonfat milk for 1 h, and re-probed with subsequent primary and secondary Abs. Protein bands were developed using the enhanced chemiluminiscent detection method (Amersham, Buckinghamshire, UK) and quantified with the MultiGauge V3.0 program (Fujifilm Global Lifescience, Düsseldorf, Germany). Protein load was corrected using actin as internal standard.

Statistical analyses

Statistical significance of the data was determined using the two-tailed Student`s t-test. A p value of ≤0.05 was considered significant. Analyses were performed using the GraphPad InStat v 3.05 software (GraphPad Software, San Diego, CA, USA). All values are expressed as means ± standard deviation.

Supplementary Figures

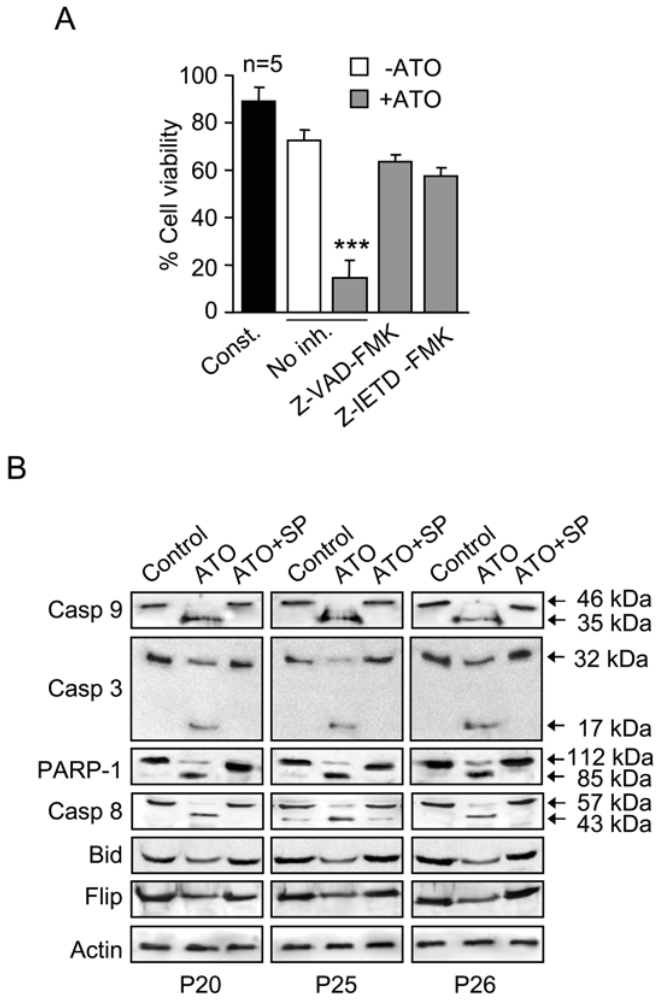

Figure S1

Figure S1. ATO induces caspase activation in B-CLL cells. (A) B-CLL cells (5 different patients) treated or not with caspase inhibitors (50 μM, 30 min) were cultured with or without 3 μM ATO for 48 h and viability measured by flow cytometry. Const. (constitutive) indicates fresh cell viability. (B) B-CLL cells (three patients) were incubated with or without 3 μM ATO and in the presence or absence of the JNK inhibitor SP600125 (SP). After 24 h cells were lysed and caspase activation was analyzed by western blotting. *** P≤0.001.

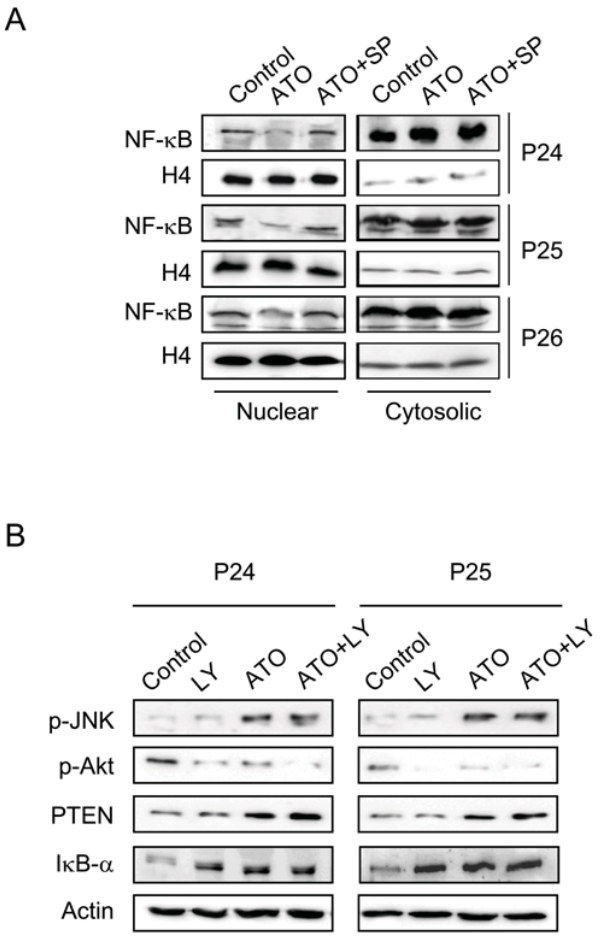

Figure S2

Figure S2. ATO-induced JNK activation leads to NF-κB inactivation and PTEN upregulation. (A) B-CLL cells (three different patients), treated or not with 10 μM SP600125 (SP) for 30 min, were incubated with or without 3 μM ATO. After 2 h, cells were lysed, and the nuclear and cytoplasmic fractions were analyzed by western blotting. Histone H4 was used as an internal control. (B) B-CLL cells were untreated (Control) or treated with 5 μM LY294002 (LY) for 30 min and further incubated with or without 2 μM ATO. In parallel, cells were incubated with ATO alone. After 24 h, cells were lysed and protein expression analyzed by western blotting.