The prohibitin-binding compound fluorizoline induces apoptosis in chronic lymphocytic leukemia cells *ex vivo* but fails to prevent leukemia development in a murine model

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Supplementary data

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Materials and Methods

Primary CLL cells and healthy B cells

Peripheral blood (PB) samples from 16 patients with CLL were obtained from the Centre Hospitalier de Luxembourg (CHL) after obtaining written informed consent according to the Declaration of Helsinki. PB mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation using the LeucoSep™ Separation Medium (Greiner Bio-One) according to the manufacturer's protocol. PBMC were then incubated with 3ml 1X ACK lysing buffer (Lonza) for 3 minutes at room temperature (RT) to lyse remaining red blood cells (RBC), washed twice with phosphate buffered saline (PBS) and counted. PBMC were cultured immediately after isolation at a concentration of 4 x 10^6 cells/mL in RPMI 1640 culture medium with Ultraglutamine (Lonza) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (P/S, Lonza) at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Buffy coats were obtained from seven healthy donors (Croix-Rouge Luxembourg). B cells were isolated by positive selection with Dynabeads® CD19 pan B (Life Technologies) according to the manufacturer's protocol. After release of CD19+ B cells from beads with the DETACHaBEAD® CD19 (Life Technologies), the cells were stained with a Fluorescein isothiocyanate (FITC)- or Alexa Fluor 700-conjugated anti-human CD20 (both from BioLegend, clone 2H7) and analyzed on a CytoFlex cytometer (Beckman-Coulter) to control purity.

Cell lines

The human CLL cell lines MEC-1 (ACC-497, DMSZ) and JVM-3 (ACC-18, DMSZ) were maintained in RPMI1640 culture medium with Ultraglutamine (Lonza) supplemented with 10% FBS and 1% P/S at 37°C in a humidified atmosphere containing 5% carbon dioxide. The human stromal cell line HS-5 was maintained in DMEM culture medium (Lonza) supplemented with 10% FBS and 1% P/S at 37°C in a humidified atmosphere containing 5% carbon dioxide. Conditioned medium was harvested from sub-confluent HS-5 cells. Medium was centrifuged twice at 400 x g for 10 min and stored at -80°C until use.

Reagents and antibodies

Fluorizoline was synthesized as previously described.[1] For *in vitro* experiments, fluorizoline was dissolved in DMSO at 40mM. For *in vivo* experiments, fluorizoline was dissolved in DMSO at 27.5mg/ml. Ibrutinib (Imbruvica, Janssen) was dissolved in DMSO at 48mg/ml. Kolliphor®EL and (2-Hydroxypropyl)-β-cyclodextrin (β-HC) were from Sigma-Aldrich. Cell Counting Kit-8 (CCK8) was purchased from Dojindo Molecular Technologies.

Allophycocyanin (APC)-conjugated annexin V was purchased from ImmunoTools. 7-amino-actinomycin D (7-AAD) Viability Staining Solution was purchased from BioLegend. APC-conjugated anti-mouse CD19, phycoerythrin (PE)-conjugated anti-mouse CD5, FITC-conjugated anti-mouse CD11b, FITC-conjugated anti-mouse CD4, and FITC-conjugated anti-mouse CD8 were from BioLegend.

Specific Antibody against Bcl-2 was purchased from Santa Cruz Biotechnology, anti-NOXA was from Calbiochem®, anti-prohibitin (PHB-1) and anti-BAP-37 (PHB-2) were from BioLegend, and anti-Mcl-1 , -PUMA, -PARP, -cleaved caspase 3, -caspase 8, -phospho-ERK1/2 and -HSC70 were from Cell Signaling Technology.

Mice

Female C57BL/6 mice were purchased from Janvier labs (France). Eμ-TCL1 mice (on C57BL/6 background) were a kind gift from Pr. Carlo Croce and Pr. John Byrd (OSU, OH) and provided by Dr. Martina Seiffert (DKFZ Heidelberg, Germany). Both strains were maintained under specific pathogen-free conditions in an animal facility with the approval of the Luxembourg Ministry for Agriculture. Mice were treated in accordance with the European Union guidelines.

Adoptive transfer (AT) and treatments

For adoptive transfer (AT), 6-week-old recipient C57BL/6 mice were injected intraperitoneally (i.p.) with 15 x 10^6 splenocytes pooled from several leukemic Eμ-TCL1 donor mice in 100μl DMEM. Treatments started five days after AT. For fluorizoline treatment, fluorizoline was freshly prepared immediately before injection and diluted in 5% Kolliphor®EL/PBS as vehicle. Mice (n=8) were injected i.p. with 15mg/kg fluorizoline three times

per week. For ibrutinib treatment, ibrutinib was administered in 10% β-HC/H_2O as vehicle (n=8) *via* drinking water at 0.16mg/ml as previously described.[2] Control mice (n=10) were injected i.p. with 5% Kolliphor®EL/PBS vehicle solely and were provided with 10% β-HC/H_2O as drinking water. CLL development was monitored weekly. The blood cell count was analyzed by the MS4e Vet hematology analyzer (Melet Schloesing, France). Healthy C57BL/6 mice (n=5) were used as controls.

To analyze *in vivo* cell viability, AT was performed as described above. Four weeks after AT, when CLL cells were detectable in peripheral blood (PB), mice (n=5) were injected i.p. with 15mg/kg fluorizoline three times per week for two weeks.

Sample preparation

Peripheral blood was drawn via retro-orbital puncture with EDTA as anticoagulant and processed for flow cytometry and blood cell count. The animals were sacrificed by cervical dislocation and femurs were dissected. Bone marrow cells were isolated by flushing the femurs with cold PBS (without Ca^{2+}/Mg^{2+}) followed by cell resuspension and filtration (100µm CellTrics®, Sysmex). PB cells and bone marrow cells were recovered by centrifugation (400 x g, 4°C, 10 minutes) before resuspension in ACK lysing solution (Lonza) to remove erythrocytes. Finally, cells were washed in MACS buffer (Miltenyi Biotec), counted and processed for flow cytometry.

Measure of leukemic load by flow cytometry

PB cells were directly stained with anti-CD5 and anti-CD19 for 30 minutes on ice in the dark, then red blood cells were lysed using RBC Lysis/Fixation Solution (BioLegend), according to the manufacturer's instructions. After washing twice, samples were acquired on a CytoFLEX analyzer (Beckman Coulter) and the percentage of CD5+CD19+ CLL cells was determined.

Survival analysis

Mice were monitored daily and were euthanized according to the animal welfare scoring system validated by the Luxembourg Ministry of Agriculture. The animals were sacrificed by cervical dislocation and the spleens were dissected and weighed. Overall survival (OS) was defined as the time from adoptive transfer (AT) until death and was analyzed using the Kaplan-Meier method (Graph Pad Prism 7).

Analysis of cytotoxicity by the CCK8 assay

Cell lines (5×10^4 cells/well) and human PBMC (2×10^5 cells/well) were plated in a 96-well plate in triplicate and incubated in the absence (CTRL) or in the presence of 1µM, 2.5µM, 5µM, 10µM, 20µM and 40µM fluorizoline in a final volume of 100µl. After 24, 48 or 72 hours, 10µl CCK8 was added to each well for 3 hours. The absorbance values were measured at 450nm on a multi-well plate reader. The inhibitory concentration (IC_{50}) was defined as the concentration of fluorizoline required to reduce the CCK8 metabolization ability by 50%.

Analysis of cell viability by flow cytometry

Cell viability was assessed by the detection of annexin V binding to surface-exposed phosphatidylserine and of 7-AAD binding to DNA. Cell lines (5×10^6 cells/25cm^2 flask) or primary cells (20×10^6 cells/25cm^2 flask) were incubated with DMSO (Ctrl) or with fluorizoline in a final volume of 10ml RPMI medium for 24 hours. Cells were then washed once in PBS and once in annexin V binding buffer (ABB). Cultured cells were resuspended in 100µl ABB and incubated with 5µl annexin V-APC for 15 minutes at RT in the dark. After annexin V staining, cells were washed once in ABB, resuspended in 100µl ABB, and additionally incubated with 5µl 7-AAD for 15 minutes at RT in the dark. Cells were resuspended in 400µl ABB immediately before acquisition on a CytoFLEX Flow Cytometer (Beckman Coulter). Data were analyzed using the CytExpert software (Beckman Coulter). After CLL cells were co-cultured with HS-5 cells, CLL cells were stained with a FITC-conjugated anti-human CD20 antibody (BioLegend) to gate on leukemic cells for the quantification of apoptosis.

For the analysis of cell viability after *in vivo* treatment, single cell suspensions prepared from blood and bone marrow were incubated with FITC-conjugated anti-mouse CD4, CD8, CD19, or CD11b and with Annexin V-APC/7-AAD, washed in ABB and analyzed on a CytoFLEX Flow Cytometer.

Western Blot

Freshly isolated cells as well as cultured cells treated with fluorizoline or left untreated (as described for Analysis of cell viability by flow cytometry) were used for western blotting. Cells were harvested, washed twice with PBS and whole cell protein extracts were prepared by lysing cells with RIPA buffer (Millipore) supplemented with a complete protease inhibitor cocktail (Roche) and Phosphatase Inhibitor Cocktails 2 and 3 (Sigma-Aldrich) for 30 minutes on ice before centrifugation at 12.000g for 15 minutes at 4°C. Protein concentration of the cell lysates was measured using the colorimetric protein assay (Bio-Rad). Protein lysates (15µg) were subjected to reducing conditions before electrophoresis on a polyacrylamide gel (SDS-PAGE) and electrophoretic transfer to 0.2µm PVDF membranes (Whatman). After 1 hour of blocking with 5% (w/v) non-fat milk in Tris-buffered saline with 0.1% Tween®-20 (TBS-T), the membranes were probed with specific primary antibodies against NOXA, MCL-1, PUMA, BCL-2, PARP, cleaved caspase 3, caspase 8, p-ERK1/2 and HSC70. Primary antibody binding was detected using a secondary antibody conjugated to horseradish peroxidase and the enhanced chemiluminescence (ECL) detection system (Perkin-Elmer).

Immunohistochemistry

Mice were euthanized by cervical dislocation and dissected to collect spleens. A portion was cut out, fixed in 10% neutral buffered formalin (HistoTainer™ prefilled specimen containers, Simport) and directly shipped to HistoWiz Inc. (histowiz.com). Samples were processed, embedded in paraffin, sectioned at 4µm, and stained with an anti-mouse cleaved-caspase 3 or with TUNEL assay to detect apoptotic cells. Sections were then counterstained with hematoxylin, dehydrated and film coverslipped using a TissueTek-Prisma and Coverslipper (Sakura). Whole slide scanning (40x) was performed on an Aperio AT2 (Leica Biosystems).

Positive cells were counted in ten fields per sample in three animals. A representative picture is presented for each animal.

Statistical analysis

Statistical significance was determined using the two-sided unpaired t test for *in vitro* analysis or the two-sided Mann Whitney U test for *in vivo* analysis. *p*-values lower than 0.05 were considered statistically significant. Analyses were performed with GraphPad Prism 7. Significance displayed in each figure is explained in figure legends.

References

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Supplementary Figure legends

Supplementary Figure S1.

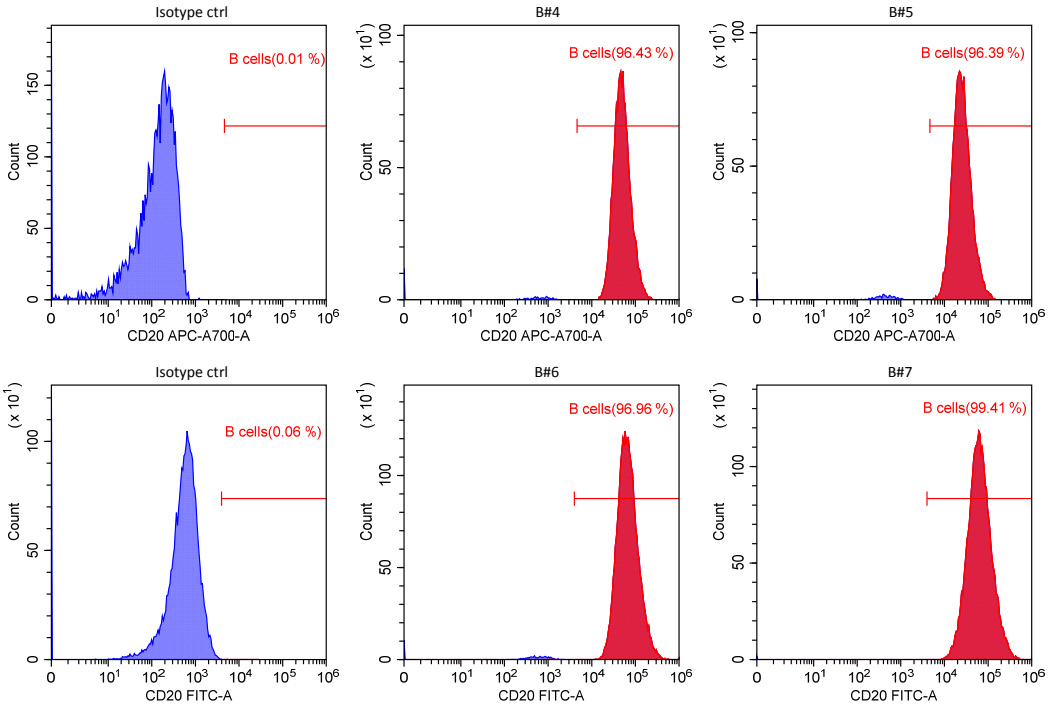
Purification of human B cells.

B cells were isolated by positive selection from buffy coats obtained from healthy donors. After release of CD19+ B cells from beads, the cells were stained with a FITC- or AF700-conjugated anti-human CD20 antibody and analyzed on a CytoFlex cytometer (Beckman-Coulter) to control purity.

Supplementary Figure S2.

Number of normal B cells, T cells, monocytes, and granulocytes in the blood of mice treated with fluorizoline. Following adoptive transfer of Eμ-TCL1 splenocytes, mice were treated for five weeks with 15mg/kg fluorizoline or with vehicle (DMSO). Blood was analyzed weekly for cell count with a MS4e Vet hematology analyzer and the percentage of each cell subset was determined by flow cytometry.

Supplementary Figure S1



Supplementary Figure S2