The prohibitin-binding compound fluorizoline induces apoptosis in chronic lymphocytic leukemia cells through the upregulation of NOXA and synergizes with ibrutinib, 5-aminoimidazole-4-carboxamide riboside or venetoclax

Ana M. Cosialls,[1,*] Helena Pomares,[1,2,*] Daniel Iglesias-Serret,[1] José Saura-Esteller,[1] Sonia Núñez-Vázquez,[1] Diana M. González-Gironès,[1] Esmeralda de la Banda,[3] Sara Preciado,[4] Fernando Albericio,[4,5,6] Rodolfo Lavilla,[5,7] Gabriel Pons,[1] Eva M. González-Barca[2] and Joan Gil[1]

[1]Departament de Ciències Fisiològiques, Facultat de Medicina i Ciències de la Salut, Universitat de Barcelona–IDIBELL (Institut d'Investigació Biomèdica de Bellvitge), L'Hospitalet de Llobregat, Barcelona, Spain; [2]Servei d'Hematologia Clínica, Institut Català d'Oncologia (ICO)-IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain; [3]Unitat de Citohematologia, Servei d'Anatomia Patològica, Hospital Universitari de Bellvitge (HUB)-IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain; [4]Department of Organic Chemistry, University of Barcelona, Spain; [5]CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Spain; [6]School of Chemistry & Physics, University of KwaZulu-Natal, Durban, South Africa and [7]Laboratory of Organic Chemistry, Faculty of Pharmacy, University of Barcelona, Spain

AMC and HP contributed equally to this work.

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Received: December 22, 2016.

Accepted: June 8, 2017.

Pre-published: June 15, 2017.

Correspondence: jgil@ub.edu

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Supplemental data:

- **Supplemental table S1**
- **Supplemental figure S1**
- **Supplemental methods**

Patient No.	Age/Sex	Rai stage	ZAP-70*	CD38*	IGHV status#	Genomic alterations†	24h‡	48h‡
1	45/M	2	-	-	nd	normal	13	nd
2	65/M	2	+	-	UM	17p-	8,5	nd
3	60/M	0	-	-	nd	normal	5	nd
4	66/M	0	nd	-	UM	13q-	5,5	nd
5	74/M	1	+	-	M	nd	7	nd
6	67/M	1	+	+	nd	11q-	8	nd
7	70/F	0	+	+	UM	13q-	9	nd
8	66/M	0	-	-	nd	17p-; 13q-	10	nd
9	66/M	0	-	+	nd	13q-	8	nd
10	85/F	0	-	-	M	nd	4	3
11	62/M	0	-	-	nd	13q-	15	13,5
12	85/F	0	-	-	nd	13q-	10	6
13	75/M	0	-	-	nd	normal	5	2
14	74/M	0	-	-	nd	13q-	7	5
15	64/F	1	-	-	nd	17p-	7	2
16	67/F	0	-	-	nd	normal	7	6
17	69/M	1	+	-	UM	13q-	15	11
18	43/M	2	-	+	nd	normal	11	7
19	81/F	0	-	-	nd	13q-	3,5	2,5
20	61/M	0	-	-	M	normal	20	20
21	60/F	0	-	-	nd	nd	2,5	2,5
22	86/F	2	nd	+	nd	nd	6	2
23	72/M	0	-	-	nd	normal	6	3
24	58/M	0	-	-	nd	normal	9	7
25	74/M	0	nd	nd	M	13q-	10	5
26	47/M	0	-	-	nd	nd	4	2
27	59/M	1	-	-	nd	13q-	5	6
28	85/M	1	-	-	nd	11q-	8	3
29	78/M	4	+	-	nd	nd	9	7
30	69/M	0	-	-	nd	13q-	5	6
31	69/M	0	-	-	UM	13q-	12	8
32	82/F	0	-	-	nd	13q-	7	6
33	61/M	0	-	-	nd	13q-	7	8
34	83/M	2	-	-	nd	nd	7	8

Table S1. Patient characteristics. *Immunophenotyping of ZAP-70 and CD38 was performed. #V-gene (immunoglobulin heavy chain variable region, IGHV) mutations were analyzed by Sanger sequencing. †Cytogenetic alterations were determined by fluorescence *in situ* hybridization (FISH) using fluorescent-labeled DNA probes against 13q14, 17p13, 11q22-q23 and chromosome 12 loci. ‡EC_{50} values for sensitive patient samples at 24 and 48 h are shown. F: female; M: male; +: positive; -: negative; UM: unmutated; M: mutated; nd: not determined.

Figure S1.

Figure S1. Apoptosis-related gene expression profile induced by fluorizoline in primary CLL samples. CLL cells were cultured *ex vivo* and untreated (U) or treated with 10 μM fluorizoline (F) for different times ranging from 2 to 24 h (n=5 at 2, 4 and 8h; n=8 at 24h). RNA was extracted and analyzed by RT-MLPA. The results are shown as the mean±SEM of the relative expression levels of each mRNA. Two-tailed paired Student's *t* test significant *P* values are indicated: *$P \leq 0.01$ treated *versus* untreated cells.

Supplemental methods

Primary samples and cell isolation

Peripheral blood (PB) samples from 34 untreated patients with CLL (**Table S1**) and from 12 healthy donors were studied. CLL was diagnosed according to standard clinical and laboratory criteria. CLL blood samples were obtained from Hospital Universitari de Bellvitge, Barcelona, Spain. Written informed consent was obtained from all patients in accordance with Human Research Ethics Committees of the Hospital Universitari de Bellvitge, L'Hospitalet de Llobregat, Barcelona, Spain. Healthy blood samples were obtained from donors from the Banc de Sang i Teixits (BST), L'Hospitalet de Llobregat, Barcelona, Spain. Written informed consent was obtained from all donors in accordance with Human Research Ethics Committees of the BST. All procedures with human individuals were in accordance with the Helsinki Declaration of 1975. PB mononuclear cells (PBMNC) were isolated by centrifugation on a Biocoll (Biochrom AG, Berlin, Germany) gradient and cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide (Sigma-Aldrich Inc, St Louis, MO, USA). If required, a negative selection step (RosetteSep™ Human B Cell Enrichment Cocktail, StemCell Technologies SARL, Grenoble, France) was performed before density gradient centrifugation to ensure high B-CLL cell purity (≥80%) for subsequent RNA or protein extraction, following the manufacturer's instructions. PBMNC were cultured immediately after thawing or isolation at a concentration of $1x10^6$ cells/mL in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Biological Industries) at 37 ºC in a humidified atmosphere containing 5% carbon dioxide.

Reagents

Fluorizoline was dissolved in DMSO at 20 or 100 mM. Ibrutinib and venetoclax (ABT-199) were obtained from Selleck Chemicals LLC (Houston, TX, USA). AICAR (5-aminoimidazole-4-carboxamide [AICA] riboside, acadesine) was synthesized by Kyowa-Hakko Europe GmbH (Düsseldorf, Germany) and kindly provided by ADVANCELL-Advanced In Vitro Cell Technologies S.A. (Barcelona, Spain). Peridinin Chlorophyll (PerCP)-labeled antibody against human CD3 was

purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Allophycocyanin (APC)-H7- or phycoerythrin (PE)-labelled antibody against human CD19 were from BD Biosciences (Franklin Lakes, NJ, USA). APC-conjugated annexin V was purchased from eBiosciences (San Diego, CA, USA).

Analysis of cell purity and cell viability by flow cytometry

Cell viability was assessed by phosphatidylserine exposure and measured as the percentage of annexin V negative cell population. After incubation of $1x10^6$ cells with the indicated factors and times, cells were washed in annexin-binding buffer (ABB) and incubated in 100 µL ABB with 0.5 µL annexin V-APC for 10 min in the dark and then diluted with ABB to a final volume of 150 µL. To distinguish the effect of fluorizoline on B and T cells from both CLL patients and healthy donors, triple staining with anti-CD19, anti-CD3 and annexin V was performed. Primarily, in CLL samples with lower than 80% of B lymphocytes, triple staining was mandatory to avoid considering the effect of the drug in non-leukemic T cells that would cover up the result. In brief, cells were washed in ABB and incubated in 50 µL ABB containing 0.5 µL anti-CD19 and 0.5 µL anti-CD3 for 10 min in the dark and thereafter stained with annexin V, as previously mentioned. Cells were acquired using the FACSCanto^{TM} II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and data of total cells or $CD19^+$- or $CD3^+$-gated cells were analyzed using the FACSDiva^{TM} software (Becton Dickinson).

Reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA)

RNA was isolated from cultured $3\text{-}5x10^6$ cells by the RNeasy Micro kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. RNA samples (200 ng total RNA) were first reverse transcribed using a gene-specific primer mix. The resulting cDNA was annealed overnight at 60 ºC to the RT-MLPA probe mix. Annealed oligonucleotides were ligated by adding Ligase-65 (MRC-Holland) and incubated at 54 ºC for 15 min. Ligation products were amplified by PCR (35 cycles, 30 s at 95 ºC; 30 s at 60 ºC, and 1 min at 72 ºC) with one unlabeled and one FAM-labelled primer. The final PCR fragments

amplified were separated by capillary electrophoresis on a 96-capillary ABI-Prism® 3730XL Genetic Analyzer (Applied Biosystems/Hitachi, Carlsbad, CA, USA). Peak area and height were measured using GeneMapper™ v3.0 analysis software (Applied Biosystems). Ratios of individual peaks relative to the sum of all peaks were calculated, resulting in the relative abundance of mRNAs of the genes of interest.

Western blot

Whole cell protein extracts were obtained by lysing 2-5x10^6 cells with Laemmli sample buffer. Protein concentration was measured with the Micro BCA™ Protein Assay Reagent kit (Thermo Scientific Pierce, Rockford, IL, USA). Protein extracts (25 µg) were subjected to reducing conditions before being subjected to electrophoresis on a polyacrylamide gel and then transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). One hour after blocking with 5% (w/v) non-fat milk in Tris-buffered saline with 0.1% Tween®-20, the membranes were incubated with the specific primary antibodies against BCL-2 (clone 124, #M0887, Dako, Denmark), BIM (clone C34C5, #2933, Cell Signaling, Danvers, MA, USA), NOXA (clone 114C307, #ab13654, Abcam, Cambridge, UK), MCL-1 (clone S-19, #sc-819, Santa Cruz Biotechnology, Dallas, TX, USA), PHB1 (clone H-80, #sc-28259, Santa Cruz Biotechnology), PHB2 (anti-REA, #07-234, Millipore), PARP (#9542, Cell Signaling), PUMA (#4976, Cell Signaling) and p53 (Ab-5, clone DO-7 #MS-186-P1, NeoMarkers; Fremont, CA, USA). Antibody binding was detected using a secondary antibody conjugated to horseradish peroxidase and the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Amersham Place, Buckinghamshire, UK).