ARTICLE

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

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ABSTRACT

Fluorizoline is a new synthetic molecule that induces apoptosis by selectively targeting prohibitins. In the study herein, the pro-apoptotic effect of fluorizoline was assessed in 34 primary samples from patients with chronic lymphocytic leukemia. Fluorizoline induced apoptosis in chronic lymphocytic leukemia cells at concentrations in the low micromolar range. All primary samples were sensitive to fluorizoline irrespective of patients' clinical or genetic features, whereas normal T lymphocytes were less sensitive. Fluorizoline increased the protein levels of the pro-apoptotic B-cell lymphoma 2 family member NOXA in chronic lymphocytic leukemia cells. Furthermore, fluorizoline synergized with ibrutinib, 5-aminoimidazole-4-carboxamide riboside or venetoclax to induce apoptosis. These results suggest that targeting prohibitins could be a new therapeutic strategy for chronic lymphocytic leukemia.

Introduction

Chronic lymphocytic leukemia (CLL) is a malignant lymphoproliferative disorder of monoclonal B lymphocytes that accumulate in the blood, bone marrow, lymph nodes and other lymphoid tissues.[1,2] It represents the most common adult leukemia in the western world, mainly affecting elderly individuals. Although the progression-free survival (PFS) and overall survival (OS) of CLL patients have increased with the introduction of first-line therapy, there is no cure for CLL and all patients will ultimately relapse. The standard of treatment for physically fit patients is chemoimmunotherapy with fludarabine, cyclophosphamide and rituximab (FCR),[3] and for older patients bendamustine plus rituximab may be a better option.[4] Relapsed patients or those with altered *TP53* can be treated with the bruton tyrosine kinase (BTK) inhibitor ibrutinib, and also with the phosphoinositide 3-kinase (PI3K) inhibitor idelalisib or the B-cell lymphoma 2 (BCL-2) inhibitor venetoclax (ABT-199).[5] Recently, ibrutinib has been approved to treat CLL patients in first-line therapy.[5] Nevertheless, a percentage of patients are resistant to ibrutinib or do not

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tolerate the drug.[6-9] Therefore, it is necessary to identify new agents with selective toxicity for malignant B cells and to develop therapeutic strategies that can overcome cellular resistance mechanisms to current therapies, that can overcome cellular resistance mechanisms to current therapies. Hence, the nucleoside analogue 5-aminoimidazole-4-carboxamide riboside (AICAR) induces apoptosis in CLL cells independently of p53 status.[10]

Recently, our group has described novel pro-apoptotic small molecules with fluorinated thiazole scaffolds.[11] The diaryl trifluorothiazoline compound 1a, also termed fluorizoline (Figure 1A), was selected as the best apoptosis inductor in a wide range of cancer cell lines from different tissue origin, including hematopoietic cell lines, and different p53 status, proving that fluorizoline exerts its antitumor action in a p53-independent manner. Fluorizoline selectively binds to prohibitin (PHB) 1 and 2[11] and, strikingly, these proteins are necessary for apoptosis induction by this compound.[12] Fluorizoline treatment induces mitochondrial-mediated apoptosis, a pathway that is controlled by the BCL-2 family proteins. In this regard, induction of the pro-apoptotic protein NOXA is required for fluorizoline-induced apoptosis, and BIM is also involved depending on the cellular context.[12]

Prohibitins are ubiquitous, evolutionarily conserved scaffold proteins mainly localized in mitochondria and implicated in many cellular processes, including mitochondrial biogenesis, differentiation, cell survival and apoptosis. Two highly homologous proteins, PHB1 and PHB2/REA, have been described.[13,14] Increasing evidence links PHBs and tumorigenesis.[15,16]

In B lymphocytes, PHBs were identified as proteins associated with the immunoglobulin M (IgM) B cell receptor (BCR).[17] More recently, PHBs were described as pro-

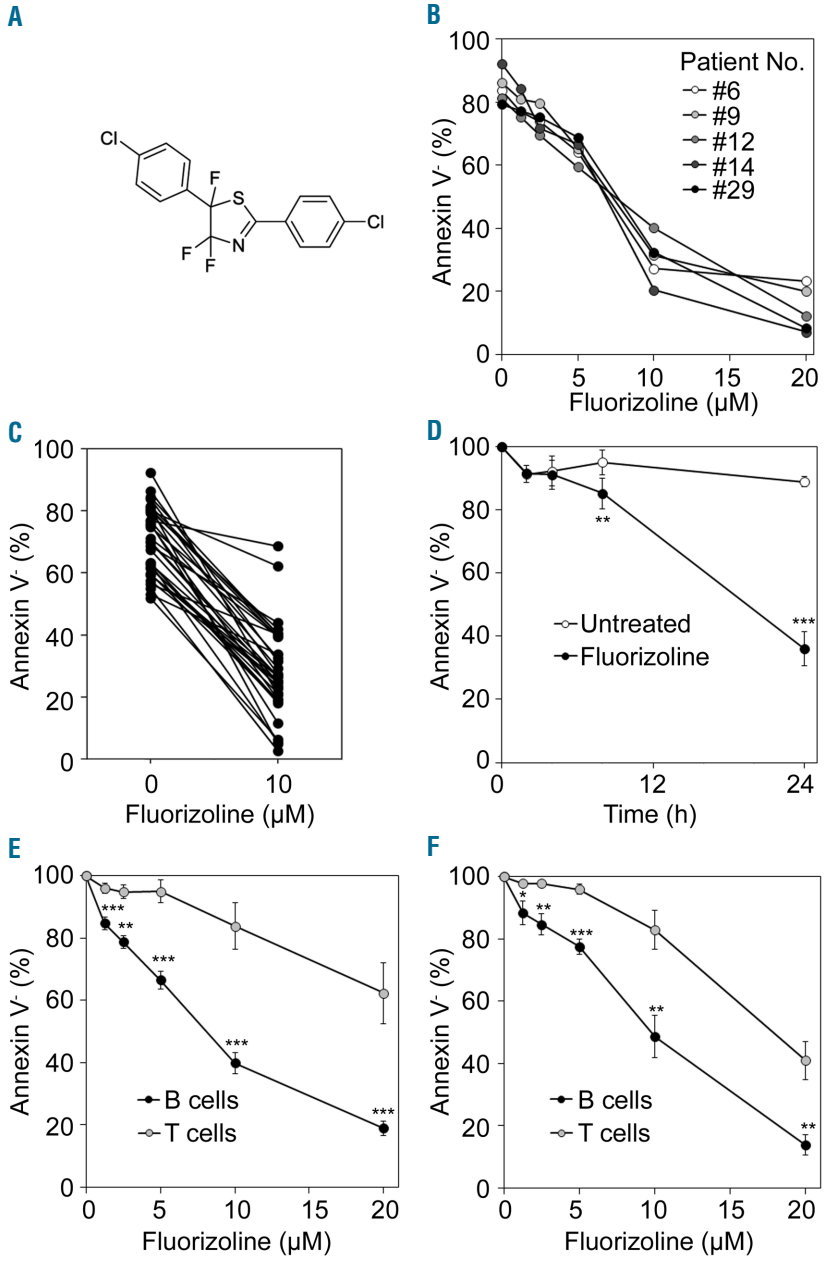

Figure 1. Cytotoxicity of fluorizoline in CLL cells *ex vivo*. (A) Chemical structure of fluorizoline. (B) Dose response of fluorizoline on primary CLL cells. PBMNC from 5 representative untreated CLL patients (#6, 9, 12, 14 and 29) out of 34 were incubated for 24 h with increasing doses of fluorizoline ranging from 1.25 to 10 μM. (C) PBMNC from 34 CLL patients were incubated for 24 h with or without 10 μM fluorizoline. (D) Time course of fluorizoline-induced apoptosis in CLL cells. Cells from 5 patients were untreated or incubated for different times ranging from 2 to 24 h with 10 μM fluorizoline. (E) Dose response of the cytotoxic effect of fluorizoline on B and T cells from CLL patients. PBMNC from CLL patients were incubated for 24 h with increasing doses of fluorizoline ranging from 1.25 to 10 μM. Viability was measured on $CD3^+$ (T cells, n=15) and $CD19^+$ (B cells, n=34) populations. (F) Dose response of the cytotoxic effect of fluorizoline on normal B and T cells. PBMNC from 12 healthy donors were incubated for 24 h with increasing doses of fluorizoline ranging from 1.25 to 10 μM. Viability was measured on $CD3^+$ (T cells) and $CD19^+$ (B cells) populations. (B, C, D, E and F) Viability (annexin V negative) was measured by analysis of phosphatidylserine exposure in total population or in $CD19^+$ and $CD3^+$ populations and is expressed (B and C) as the percentage of non-apoptotic cells or (D, E and F) as the percentage of the viability of untreated cells. (D, E and F) Data are shown as the mean±SEM. (D) Two-tailed paired Student's *t*-test was performed. (E and F) Two-tailed unpaired Student's *t*-test was performed. Significant *P* values are indicated: **P*<0.05; ***P*<0.01; ****P*<0.001 treated *versus* untreated cells or $CD19^+$ *versus* $CD3^+$ cells.

teins associated with phosphorylated protein tyrosine kinase Syk[18] and the receptor CD86[19] at the inner plasma membranes of B lymphocytes, thus likely having a role in signal transduction after receptor engagement. In CLL cells, PHB is increased after phorbol ester-induced maturation,[20] and nuclear PHB is upregulated after *in vitro* treatment with fludarabine.[21] These data strongly suggest that targeting PHBs would be a candidate approach for the treatment of B-cell neoplasias, and PHB-binding compounds, such as fluorizoline, emerge as interesting new pro-apoptotic agents. Preliminary data in a small number of CLL samples showed that fluorizoline induces apoptosis in these cells.[11] The objective of the work herein was to investigate the mechanism of induction of apoptosis by fluorizoline in CLL cells and the effect of its combination with ibrutinib, AICAR or venetoclax.

Methods

Primary samples and cell isolation

Peripheral blood (PB) samples from 34 untreated patients with CLL and 12 healthy donors were included. All patients and healthy controls signed an informed consent form approved by the Institutional Review Boards according to the Declaration of Helsinki. The patients' characteristics are shown in *Online Supplementary Table S1*. Briefly, PB mononuclear cells (PBMNC) were obtained by centrifugation on a Biocoll gradient. This fraction included normal B and T cells from healthy donors or mainly B-CLL cells from patients. To ensure high B cell purity (≥80%), an initial isolation step was performed by negative selection. The complete sample handling protocol is available in the *Online Supplementary Data.*

Reagents

Fluorizoline (a diaryl trifluorothiazoline; see molecular structure in Figure 1A) was synthesized as previously described.[11] Other reagents used in this study are detailed in the *Online Supplementary Data.*

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. Cells were acquired using the FACSCanto™ II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and the data of total cells or $CD19^+$- or $CD3^+$-gated cells were analyzed using FACSDiva™ software (Becton Dickinson). A detailed protocol of cell staining can be found in the *Online Supplementary Data.*

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

Ribonucleic acid (RNA) isolated from cells was analyzed by RT-MLPA using SALSA MLPA KIT R011-C1 Apoptosis messenger (m)RNA from MRC-Holland (Amsterdam, The Netherlands) for the simultaneous detection of 40 mRNA molecules, including apoptosis-related genes.[12,22] The protocol is accurately described in the *Online Supplementary Data.*

Western blot

The antibodies used in this study and the western blot protocol are described in the *Online Supplementary Data.*

Statistical analysis

Results are shown as the mean ± standard error of the mean (SEM) of values obtained in 3 or more independent experiments as

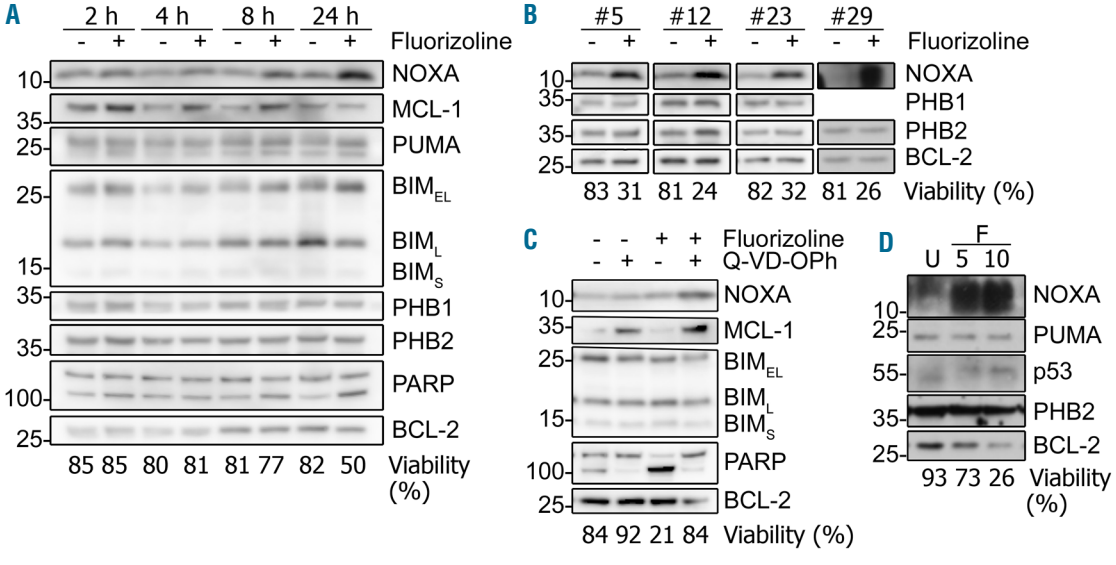

Figure 2. Induction of NOXA protein by fluorizoline in primary CLL cells. (A) PBMNC from patient #11 were incubated for different times ranging from 2 to 24 h with 10 μM fluorizoline. After the times stated on the figure, cells were collected. (B) PBMNC from patients #5, 12, 23 and 29 were incubated with 10 μM fluorizoline for 24 h. (C) PBMNC from patient #5 were pre-incubated with 20 μM caspase inhibitor Q-VD-OPh for 30 min and then treated with 10 μM fluorizoline for 24 h. (D) PBMNC from patient #8 with 17p deletion were untreated (U) or treated with 5 and 10 μM fluorizoline (F) for 48 h. (A, B, C and D) Cells were lysed and analyzed by western blot. BCL-2 was used for loading normalization. Viability was measured by analysis of phosphatidylserine exposure and is expressed as the percentage of non-apoptotic (annexin V negative) cells. These are representative patients of at least 3 analyzed (n=5 for A; n=8 for B; n=4 for C; n=1 for D).

indicated in each figure legend. Data were analyzed using SPSS® Statistics v22.0 software package (IBM®, Armonk, NY, USA). Two-tailed paired or unpaired Student's *t*-test with normal-based 95% confidence interval was used to compare the differences between samples, as required. Differences were considered statistically significant at *P* values below 0.05. In two-drug combination studies, the combination index (CI) was calculated according to the Chou-Talalay method[23] by using CalcuSyn software version 2.11 (Biosoft, Cambridge, UK). A CI of below 1 indicates a synergistic effect.

Results

Fluorizoline induces apoptosis in primary CLL cells *ex vivo*

The cytotoxicity of fluorizoline (Figure 1A) was evaluated in samples obtained from patients with CLL prior to any treatment (see *Online Supplementary Table S1* for details of patient samples). PBMNC from 34 different patients were exposed *ex vivo* to a range of fluorizoline concentrations (from 1.25 to 20 μM). Incubation with fluorizoline strongly reduced cell viability in a dose-dependent manner (Figure 1B). All CLL samples were sensitive to fluorizoline, and cell viability decreased from 70.0±1.9% to 28.1±2.6% (n=34) after incubation with 10 μM fluorizoline for 24 hours (Figure 1C), with half-maximal effective concentration (EC_{50}) values ranging from 2.5 to 20 μM (mean 8.1±0.6 μM; *Online Supplementary Table S1*). Longer exposition to fluorizoline for 48 hours slightly reduced the mean EC_{50} value to 5.5±0.6 μM (n=25; *Online Supplementary Table S1*). Treatment with 10 μM fluorizoline induced a time-dependent decrease of cell viability that was detected after the first 8 hours of incubation (Figure 1D).

Some patients included in this study harbored alterations of the *TP53* and *ATM* genes detected by fluorescent *in situ* hybridization (FISH; *Online Supplementary Table S1*), which are associated with poor response to chemotherapy and chemoimmunotherapy and a worse prognosis.[1,2] Interestingly, samples from these patients had similar sensitivity to *ex vivo* treatment with fluorizoline (mean EC_{50} value of 8.3±0,5 μM at 24 hours for samples from patients with 17p or 11q deletion, n=5) compared to samples from patients without these alterations (mean EC_{50} value of 8,1±0,8 μM at 24 hours, n=29). Similarly, *ex vivo* cytotoxicity of fluorizoline was similar in cells from CLL patients with unmutated and mutated immunoglobulin heavy chain variable region (IGHV) genes (mean EC_{50} values at 24 hours of 10,0±1,6 μM, n=5; and 10,3±3,5 μM, n=4; respectively) (*Online Supplementary Table S1*).

To examine the effects on the non-leukemic T lymphocytes of CLL patients, apoptosis induction was assayed in the $CD3^+$ population of 15 CLL samples. As depicted in Figure 1E, the reduction in cell viability in the presence of fluorizoline was higher within the leukemic $CD19^+$ population (35.3±34.9% of viable cells at 24 hours treatment with 10 μM fluorizoline) compared to the normal $CD3^+$ population (83.8±7.5% of viable cells at 24 hours treatment with 10 μM fluorizoline, with EC_{50} values higher than 20 μM in 9 samples out of 15), demonstrating that fluorizoline preferentially induces apoptosis in malignant B lymphocytes.

Additionally, to evaluate the cytotoxicity of fluorizoline in non-malignant B cells, the effect of fluorizoline on normal PBMNC from healthy donors was assessed. Incubation with increasing doses of fluorizoline reduced the percentage of viable normal B and T cells (48.6±6.8% and 82.8±6.3% of viable cells at 24 hours of treatment with 10 μM fluorizoline in normal $CD19^+$ and $CD3^+$ populations, respectively) (Figure 1F) with a mean EC_{50} value of 10.9±0.8 μM and 19.1±2.2 μM at 24 hours for normal B and T cells, respectively. Thus, CLL cells are slightly more sensitive to fluorizoline than normal B lymphocytes.

NOXA is upregulated by fluorizoline in primary CLL cells

To analyze the mechanism of apoptosis induction upon fluorizoline treatment in B lymphocytes of CLL samples we sought to examine the changes in the protein levels of NOXA and BIM, 2 members of the BCL-2 family that have been involved previously in fluorizoline-induced apoptosis.[12] We observed a time-dependent upregulation of NOXA that was detected after 8 hours of incubation, occurring simultaneously with the decrease of cell viability (Figure 2A), and was also clearly found upregulated after 24 hours in all samples analyzed (Figure 2B). The protein levels of MCL-1, the anti-apoptotic counterpart of NOXA, were slightly upregulated during the first hours of incubation with fluorizoline and were not altered after 24 hours. PUMA and BIM, as well as PHBs protein levels, were not modified upon fluorizoline treatment (Figure 2A). In addition, fluorizoline clearly induced poly(ADP-ribose) polymerase (PARP) cleavage (Figure 2A), thus confirming an apoptotic mechanism. The induction of NOXA preceded caspase activation, as pre-incubation with the pan-caspase inhibitor Q-VD-OPh did not block its upregulation (Figure 2C). As expected, BIM protein expression was not modified upon caspase inhibition, whereas MCL-1 protein levels were increased at 24 hours after caspase activity arrest, both in the absence and the presence of fluorizoline, indicating a late caspase-dependent degradation of this protein (Figure 2C). Finally, the increase of NOXA protein expression was also detected in CLL samples from patients with chromosomal alterations that cause loss of p53 expression (Figure 2D), thus corroborating the fact that fluorizoline-induced NOXA upregulation occurs in a p53-independent manner. Altogether, these results indicate that fluorizoline causes an increase of NOXA protein levels prior to caspase activation and these modulations could explain the apoptotic outcome observed in primary CLL cells.

The induction of NOXA protein by fluorizoline could be due to the modulation of the corresponding mRNA levels. To that purpose, we analyzed the changes in the overall apoptosis mRNA expression profile by RT-MLPA. *NOXA* levels were not modified after fluorizoline treatment, neither at the initial stages nor at 24 hours of incubation of CLL cells (*Online Supplementary Figure S1*). Among all apoptosis-related genes analyzed, only the pro-apoptotic BCL-2 family member *MOAP1* and the anti-apoptotic *HIAP1* and *HIAP2* were weakly upregulated and downregulated upon fluorizoline treatment, respectively. This result indicates that fluorizoline-induced NOXA protein upregulation does not result from mRNA modulation in CLL cells.

Fluorizoline synergizes with ibrutinib, AICAR or venetoclax to induce apoptosis in CLL cells

Finally, we sought to analyze the effect of the combination of fluorizoline with other drugs. For that purpose we chose the BTK irreversible inhibitor ibrutinib, the nucleo-

side analogue AICAR, which has demonstrated selective anti-tumor activity in CLL *ex vivo*[10,24] and was tested in a phase I/II clinical trial for relapsed/refractory CLL,[25] and the BCL-2 inhibitor venetoclax (ABT-199). Ibrutinib therapy *in vivo* causes an intracellular MCL-1 protein decrease,[26] and partially downregulates MCL-1 protein levels *in vitro* in some CLL patient samples.[27] Interestingly, the combination of fluorizoline with ibrutinib was more effective than single drug treatment in all patients analyzed. Thus, the addition of ibrutinib enhanced fluorizoline cytotoxic effect (CI values ranging from 0.192 to 0.797, indicating a synergistic effect; the lowest CI values correspond to the combination of fluorizoline with 10 μM ibrutinib) (Figure 3A). Similarly, the combination of fluorizoline with AICAR increased cell death compared to each drug alone (CI values ranging from 0.643 to 0.991; the lowest CI values correspond to the combination of AICAR with 10 μM fluorizoline) (Figure 3B). Finally, the combination of fluorizoline and venetoclax showed a synergic effect in all conditions analyzed (CI values ranging from 0.492 to 0.824; the lowest CI values correspond to the combination of venetoclax with 10 μM fluorizoline) (Figure 3C). Thus, these results show a synergistic interaction between fluorizoline and ibrutinib, AICAR or venetoclax in CLL cells.

Discussion

In the study herein we describe the mechanism of apoptosis induction by the prohibitin-binding compound fluorizoline in CLL cells. It was previously described that fluorizoline directly binds to PHB1 and PHB2[11] and reduces cell viability through the upregulation of NOXA and BIM.[12] Although PHBs are necessary for apoptosis induction by fluorizoline,[12] we cannot discard that it could interact with other proteins. Expression analysis revealed a consistent upregulation of the BH3-only protein NOXA in CLL cells upon fluorizoline treatment. Fluorizoline induced increases in NOXA protein levels prior to caspase activation, which could explain the apoptotic outcome. Formerly, the effects of fluorizoline in primary cancer cells have been analyzed in cells from patients with chronic myeloid leukemia in blast crisis, mantle cell lymphoma, B cell chronic lymphoproliferative syndrome, adult T-cell leukemia/lymphoma,[12] and in acute myeloid leukemia cells.[28] Interestingly, treatment with fluorizoline resulted in a decrease in viability and an increase in NOXA protein levels, whereas BIM protein levels were not modified. Hence, fluorizoline seems to mainly increase NOXA protein levels in leukemia cells.

NOXA has been described as a particularly relevant pro-apoptotic BCL-2 family member in CLL cells. NOXA protein is induced by histone deacetylase (HDAC) inhibitors,[29,30] proteasome inhibitors,[31] bendamustine,[32] Akt inhibitors,[33] AICAR,[10] cyclin-dependent kinase inhibitors[34] and microtubule disrupting agents.[35] Furthermore, CLL development is accelerated in mice with a deficiency of NOXA.[36] NOXA is a pro-apoptotic BH3-only member that has been classified as a "sensitizer" because it was considered as an inhibitor of the anti-apoptotic MCL-1 and A1 proteins.[37] However, recent data indicate that NOXA is also an "activator" of the BAX and BAK multidomain pro-apoptotic BCL-2 family members.[38]

Related to our study with a PHB-binding compound in CLL, rocaglamide silvestrol induces apoptosis of CLL

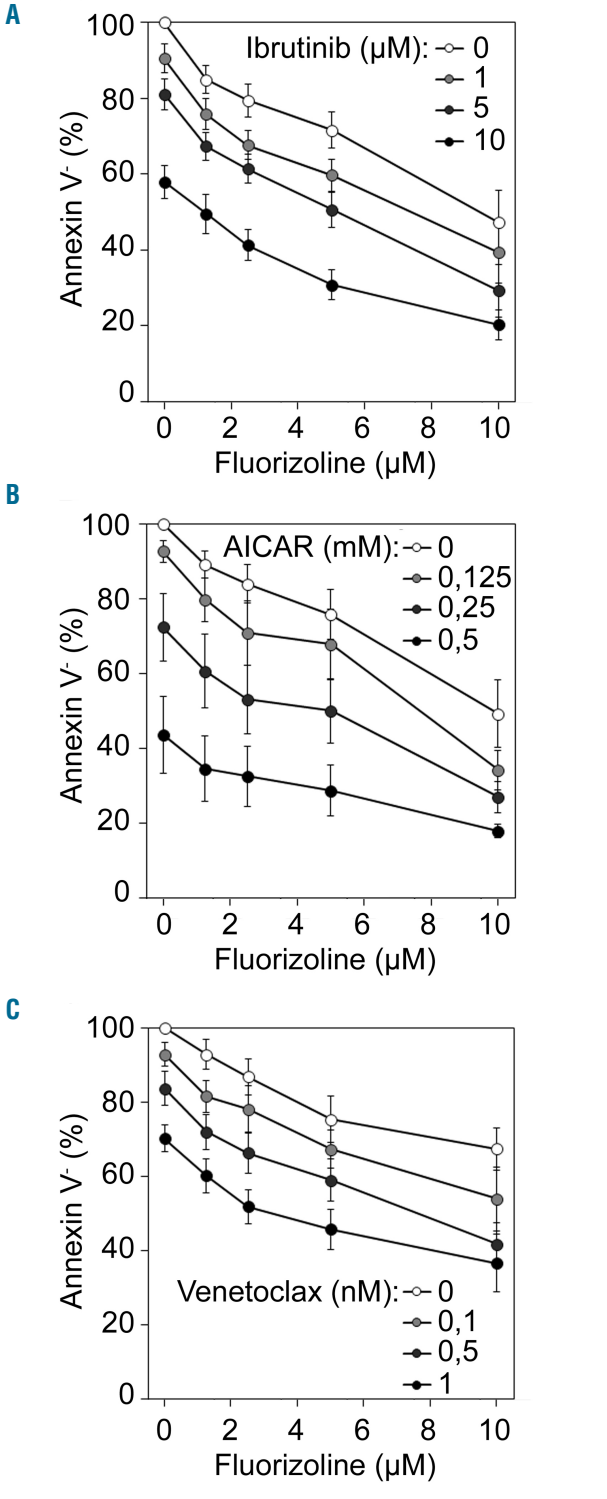

Figure 3. Combination of fluorizoline and other drugs in CLL cells *ex vivo*. (A) PBMNC from 5-9 untreated CLL patients (#11, 12, 15, 16, 17, 18, 23, 28 and 30) were incubated for 24 h with increasing doses of fluorizoline ranging from 1.25 to 10 μM and combined with increasing doses of ibrutinib ranging from 1 to 10 μM. (B) PBMNC from 4-5 untreated CLL patients (#5, 11, 17, 18 and 23) were incubated for 24 h with increasing doses of fluorizoline ranging from 1.25 to 10 μM and combined with increasing doses of 5-aminoimidazole-4-carboxamide riboside (AICAR) ranging from 0.125 to 0.5 mM. (C) PBMNC from 5-6 untreated CLL patients (#10, 13, 14, 16, 23 and 25) were incubated for 24 h with increasing doses of fluorizoline ranging from 1.25 to 10 μM and combined with increasing doses of venetoclax (ABT-199) ranging from 0.1 to 1 nM. Viability was measured by analysis of phosphatidylserine exposure and is expressed as the percentage of the viability (annexin V negative) of untreated cells. Data are shown as the mean±SEM.

cells.[39] Interestingly, some rocaglamides bind to PHB and it has been described that this binding mediates its anti-proliferative effects through inhibition of the Raf-MEK-ERK pathway.[40] However, whether or not silvestrol binds to PHB is presently unknown.

Our study has shown that T cells from CLL patients are less sensitive to fluorizoline-induced apoptosis. Thus, the differential effect of fluorizoline in B and T lymphocytes is of great interest and may be useful in the therapy of CLL, since immunosuppression caused by classic chemotherapy could be avoided or reduced. *In vivo* experiments could be necessary in the future and it is possible that the effective concentration of fluorizoline would be higher than in the *in vitro* conditions. Thus, further testing the effect of fluorizoline in CLL cells co-cultured with bone marrow stromal cells would be interesting in order to better reproduce the CLL microenvironment conditions.[41]

Finally, our results show that the combination of fluorizoline with ibrutinib, AICAR or venetoclax has synergistic effects in the induction of apoptosis in CLL cells. Likely the induction of NOXA by fluorizoline is involved in these synergistic effects and could overcome resistance to BCL-2 inhibitors that do not inhibit MCL-1.

Altogether, our results suggest that fluorizoline could be an alternative therapy for resistant/refractory patients to classic chemotherapy or novel drugs such as kinase inhibitors or BCL-2 antagonists approved for the treatment of CLL.

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