The influence of venetoclax, used alone or in combination with cladribine (2-CdA), on CLL cells apoptosis in vitro: Preliminary results

Aleksandra Beata Kubiak[1,B–D,F], Ewelina Izabela Ziółkowska[1,B,C,F], Anna Barbara Korycka-Wołowiec[1,A,C,E,F], Tadeusz Robak[1,E,F], Dariusz Wołowiec[2,C,E,F]

[1] Department of Hematology, Medical University of Lodz, Poland
[2] Department of Hematology, Blood Neoplasms and Bone Marrow Transplantation, Medical University of Wroclaw, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Anna Barbara Korycka-Wołowiec
E-mail: anna.korycka-wolowiec@umed.lodz.pl

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Conflict of interest
None declared

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Abstract

Background. Venetoclax (VEN), a highly selective BCL-2 inhibitor, is successfully used in the treatment of chronic lymphocytic leukemia (CLL). The purine analogue – cladribine (2-CdA) – is also administered to CLL patients, especially as a part of chemoimmunotherapy.

Objectives. To compare the effects of the VEN+2-CdA regimen with that of the 2 drugs used alone on the apoptosis of CLL lymphocytes in vitro.

Materials and methods. Mononuclear cells were collected from 103 previously untreated CLL patients. They were incubated with VEN (40 nM) or/and 2-CdA (16 μM) for 48 h. Cytotoxicity, overall apoptosis, mitochondrial transmembrane potential changes (ΔΨm), and expression of selected apoptosis-involved proteins were measured.

Results. The cytotoxicity, overall apoptosis, caspase-3 or caspase-9 expression, and ΔΨm were significantly higher after VEN+2-CdA addition compared to both drugs used alone, with a very strong synergistic effect observed. The percentage of BCL-2-positive cells decreased after VEN and VEN+2-CdA addition compared to controls. The TP53-expressing cells increased under the influence of all tested regimens. The VEN+2-CdA increased the expression of BIM, BAX and NOXA compared to either controls or VEN or 2-CdA alone. Similar increases in PUMA expression were observed after VEN, 2-CdA and VEN+2-CdA addition. The FAS-associated death-domain protein (FADD) expression was significantly higher after 2-CdA and 2-CdA+VEN addition as compared to control.

Conclusions. Our results confirm the involvement of both VEN and 2-CdA in the intrinsic apoptotic pathway. They also demonstrate that these agents have a synergistic effect on CLL cells in vitro. Further studies are needed to assess the influence of VEN+2-CdA on the expression of apoptosis-involved genes.

Key words: apoptosis, CLL, apoptosis-involved proteins, cladribine, venetoclax

Background

Despite the growing understanding of the molecular pathogenesis of hematological malignancies and the considerable progress in their treatment, many diseases, including chronic lymphocytic leukemia (CLL), still remain incurable. Chronic lymphocytic leukemia is characterized by an uncontrolled clonal accumulation of morphologically mature B cells, which in turn is believed to be caused by the inhibition of intrinsic apoptosis. In this pathway, the most important role is played by overexpression of the intracellular signaling pathway, which allows malignant cells to evade apoptosis by sequestering pro-apoptotic proteins. The key regulators of this pathway are the antiapoptotic BCL-2 family proteins.[1,2]

Recently published preclinical and clinical studies have shown that the small molecule agent venetoclax (VEN) is suitable for clinical use in CLL patients. It is a highly selective inhibitor of the BCL-2 protein that belongs to a novel class of BCL-2-antagonists able to mimic the function of pro-apoptotic BH3-only proteins (BH3s).[3–5] The intrinsic apoptotic pathway plays a crucial role in the mechanism of VEN action.[6–8] An important property of VEN is to directly induce the apoptosis, independently of *TP53*[9–13]; as such, VEN shows activity in CLL patients, including those with deletion (del) 17p and/or mutation of *TP53*, as well as in those without del17p/*TP53* mutation which failed both chemoimmunotherapy and B-cell receptor (BCR) pathway, such as ibrutinib.[9] A number of studies have confirmed the efficacy of VEN in terms of the depth of response, including eradication of minimal residual disease (MRD), which is predictive for various survival parameters such as long progression-free survival.[14]

Although VEN shows efficacy in monotherapy in CLL patients, its activity is enhanced when it is associated with drugs that have different mechanisms of action. The combination of VEN with anti-CD20 antibodies, especially rituximab and obinutuzumab, is currently widely used in the treatment of both treatment-naïve and relapsed/refractory CLL patients. The combination of VEN with ibrutinib allows for high rates of hematological responses and MRD eradication to be achieved.[10–12,15]

Little is known about the usefulness of the combination of VEN with standard CLL chemotherapy containing purine nucleoside analogues (PNAs), which still holds an important place in the management of CLL, especially in first-line treatment of physically fit patients. Although fludarabine is the most commonly used purine analogue for the treatment of CLL patients, some studies have found 2-chlorodeoxyadenosine (cladribine – 2-CdA) to have similar efficacy.[16,17] It is believed to act primarily by triggering the intrinsic mitochondrial *TP53*-dependent apoptotic pathway through inducing BAX protein expression, and the *TP53*-independent pathway by directly binding to proteins located in mitochondrial membrane, leading to caspase cascade activation.[18,19] Although it is possible that the extrinsic apoptotic pathway may also play a role in 2-CdA activity, this idea remains controversial.[20,21] It is not known whether the agent may demonstrate synergic action in association with VEN towards CLL lymphocytes; however, VEN has demonstrated greater efficacy when combined with bendamustine, a drug which has also purine analogue properties.

Objectives

The aim of the present study was to evaluate the influence of VEN and 2-CdA, used either alone or in combination, on the apoptosis as well as the expression of several intracellular factors involved in apoptosis of CLL lymphocytes after short-term culture in vitro, and to determine whether such combinations are promising for the treatment of CLL patients.

Materials and methods

Patients

Peripheral blood mononuclear cells (PBMNCs) were collected from 103 CLL patients (47 females and 56 males, mean age 68 years (range: 43–88 years)) at the time of diagnosis and before any anticancer therapy, during routine diagnostic procedures in the Department of Hematology, Medical University of Lodz, Poland, and Regional Multispecialist Center of Oncology and Traumatology in Lodz, Poland, in 2019 and 2020. The diagnoses were based on the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) 2018 criteria.[22] The material obtained from 39 patients was used to evaluate the expression of selected proteins involved in the process of apoptosis (Table 1). All patients signed a written informed consent to participate in this study. The study has been approved by the Ethics Committee of the Medical University of Lodz (approval No. RNN/44/17/WE).

Table 1. General characteristics of patients

Characteristics of patients	
Total number of patients	103 (47 F, 56 M)
Age	68 years (range: 43–88 years)
Total number of patients to evaluate the expression of selected proteins	40 (15 F, 25 M)
Clinical state	
RAI 0–2	26
RAI 3–4	14
Cytogenetic aberrations	
del13q	11
del17p	2
del11q	3
tri12+	2
Normal karyotype	7
No information available	17

Drugs

Venetoclax was supplied by Abbvie (Mettawa, USA). In order to determine its cytotoxicity, VEN was used at 11 different concentrations (from 0.5 nM to 320 nM). The concentration of 40 nM was chosen for further studies. Cladribine (2-CdA; Biodribin) was purchased from Institute of Biotechnology and Antibiotics (Warsaw, Poland). In order to determine its cytotoxicity, 2-CdA was used at 21 different concentrations (from 0.1 μM to 120 μM). A concentration of 16 μM was chosen for further studies.

Isolation of peripheral blood mononuclear cells and cell cultures conditions

The PBMNCs were isolated as previously described.[23,24] Briefly, peripheral blood was layered on Histopaque-1077 cell separation medium (Sigma-Aldrich, St. Louis, USA) and centrifuged in a density gradient in 3600 rpm for 20 min. The mean B-cell ($CD19^+$) purity was found to be >95%, as measured with flow cytometry. The PBMNCs were resuspended at a concentration of 1.0×10^6 cells/mL in RPMI-1640 supplemented with 20% (v/v) heat-inactivated fetal calf serum (Gibco, Life Technologies, Waltham, USA) and antibiotics (50 mg/mL of streptomycin and 50 IU/mL of penicillin; Life Technologies, Inchinnan, UK). All cultures were incubated with the abovementioned drugs for 24 h or 48 h at 37°C, in an atmosphere of 5% CO_2 and full humidity. Cultures without drugs were used as controls.

Assessment of cytotoxicity, mitochondrial transmembrane potential changes (ΔΨm) and overall apoptosis of CLL cells

The cytotoxicity and overall level of apoptosis associated with the studied drugs were assessed at baseline and then after 24 h and 48 h of culture using flow cytometry (BD FACS Canto II; Becton Dickinson Pharmingen, San Diego, USA); cytotoxicity was tested with fluorescein isothiocyanate (FITC; Becton Dickinson Pharmingen) and conjugated propidium iodide (PI; Sigma-Aldrich), while ongoing apoptosis was examined with annexin-V (AnnV; Becton Dickinson Pharmingen), as described previously.[23,24] The term 'overall apoptosis' refers to cells which bind AnnV, since phosphatydyloserine externalization occurs in both the intrinsic and extrinsic apoptosis pathways, and is generally used to determine the percentage of apoptotic cells.

Apoptosis was also assessed after 48 h of culture by changes in mitochondrial transmembrane potential (ΔΨm) for the initial step of intrinsic apoptosis. The ΔΨm were evaluated using MitoTracker Red CMX Ros kit (Invitrogen, Waltham, USA), as previously described.[23,24] Briefly, PBMNCs were centrifuged and the cell pellet was gently resuspended in the staining solution containing the MitoTracker probe, prepared according to the manufacturer's protocol, and incubated for 30 min at 37°C. The cells were re-pelleted by centrifugation and resuspended in fresh warmed-up medium. The assays were performed in duplicate and analyzed with flow cytometry (BD FACS Canto II) at 490"20 nm using FL1 standard fluorescent filter, and at 530"20 nm using FL3 standard fluorescent filter.

Assessment of the active forms of caspase-3, caspase-9 and caspase-8

The expression of the active forms of caspase-3, caspase-8 and caspase-9 was evaluated in the PBMNCs before culture and after 48 h of incubation with the tested drugs, used alone or in combination. The analysis of the expression of active forms of caspase-3, caspase-8 and caspase-9 was performed on a flow cytometer. The percentage of cells expressing the active form of caspase-3 was assessed using PE Active Caspase-3 Apoptosis Kit (Becton Dickinson Pharmingen), according to the manufacturer's protocol. Similar analyses of the active forms of caspase-8 and caspase-9 were also performed according to the manufacturer's protocol using FAM-FLICA in vitro Caspase 8 Kit or FAM-FLICA in vitro Caspase 9 Kit (ImmunoChemistry Technologies, Bloomington, USA), respectively. All assays were performed in duplicate.

Detection of apoptosis-regulatory proteins

The following proteins expression was evaluated in fixed, permeabilized cells after 48 h of incubation using direct staining of specific labeled antibodies:

1. P53 (FITC Mouse Anti-Human P53 Set; Becton Dickinson Pharmingen);
2. BCL-2 (FITC Mouse Anti-Human BCL-2; Becton Dickinson Pharmingen);
3. BIM (Rabbit mAb to BIM [Y36] Alexa Fluor 488; Abcam, Cambridge, UK);
4. BAX (Rabbit Anti-BAX antibody [T22-A]; Abcam);
5. PUMA (Rabbit Anti-PUMA Antibody; Abcam);
6. NOXA (Mouse Anti-NOXA [114C307]; Abcam);
7. FADD (Rabbit Anti-FADD Antibody; Abcam).

All investigated proteins apart from P53 (FITC-conjugated) were used with an appropriate secondary antibody (Goat polyclonal Antibody to Rabbit IgG (Abcam); FITC appropriate isotype controls). The fluorescence was measured using BD FACS Canto II flow cytometer at 490"20 nm using FL1 standard fluorescent filter, and at 530"20 nm using FL3 standard fluorescent filter.

Statistical analyses

The normality of data distribution was checked and the distribution of the variables was not normal. All the measurements (control cultures, cultures with 40 nM VEN, 16 μM 2-CdA and their combination) were performed on the blood sample drawn from the same patient. The data regarding cytotoxicity, apoptosis, caspase

expression, mitochondrial transmembrane potential changes, and differences in protein expression profiles were analyzed using the Wilcoxon signed-rank test with Bonferroni's correction for multiple comparisons, where $p < 0.008$ was considered statistically significant. STATISTICA v. 13.1 (StatSoft Inc., Tulsa, USA) software was used for analyses (the p-value was calculated according to the following formula: $p < \alpha/k$; $\alpha = 0.05$ – significance level; $k = 6$ – number of comparisons).

GraphPad Prism v. 5.0 (GraphPad Software, San Diego, USA) and CompuSyn v. 1.0 (https://www.combosyn.com/) software was respectively used to present the cytotoxic effect of drugs as isobolograms, and to calculate IC_{50} (values defined as the concentration of drug that achieved 50% cytotoxicity).

CompuSyn v. 1.0 was also used to calculate combination index (CI), where CI < 0.1 indicates very strong synergy, CI = 0.1–0.3 strong synergy, CI = 0.3–0.7 synergy, CI = 0.7–0.85 moderate synergy, CI = 0.85–0.9 slight synergy, CI = 0.9–1.1 nearly additive effect, and CI > 1.1 antagonistic effect.[25]

Drug-induced cytotoxicity (DICy) was expressed by the difference between the mean percentage of dead cells obtained after incubation with drug(s) studied and the mean percentage of cells in the control culture, calculated for each sample. Drug-induced apoptosis (DIA) was assessed as the difference between the percentage of cells which underwent apoptosis ($AnnV^+$ cells) after incubation with studied drug(s) and the percentage of cells undergoing spontaneous apoptosis in the control cultures, calculated for each sample. Drug-induced $\Delta\psi m$ ($DI\Delta\psi m$) was expressed as the difference between the mean percentage of cells with $\Delta\psi m$ after incubation with the drug(s) studied and the percentage of cells with $\Delta\psi m$ in the control culture, calculated for each sample. Drug-induced caspase expression (DICE) was assessed by the difference between the mean value of the percentage of cells expressing the active forms of caspases after incubation with drug(s) studied and the percentage of cells with expression of the active form of caspases in the control culture, calculated for each sample. Drug-induced protein expression (DIPE) of protein-expressing CLL cells was expressed by the mean value of the differences between the percentage of cells expressing protein after 48 h incubation with drug(s) and the percentage of cells expressing protein in the control culture, calculated for each sample.

Results

Cytotoxic effect of VEN and 2-CdA used alone or in combination on CLL cells in culture in vitro, and thetype of interaction

Eleven concentrations of VEN (from 0.5 nM to 320 nM) and 21 concentrations of 2-CdA (from 0.1 μM to 120 μM) were used to determine the cytotoxicity. At all concentrations, VEN and 2-CdA, used alone, demonstrated significantly stronger cytotoxic effects after 48 h compared to the 24-h period (Fig. 1A,B). After 48 h of incubation, VEN demonstrated higher cytotoxic effects than 2-CdA (Fig. 1C), with the highest cytotoxic effect (48.8%) observed at the concentration of 40 nM (Table 2). In contrast, the highest cytotoxic effect of 2-CdA was 44%, achieved at a concentration of 16 μM (Table 2).

Venetoclax demonstrated significantly higher DICy than 2-CdA (31.7% and 26.9%, respectively, $p < 0.001$) (Table 3). The IC_{50} observed for VEN was 143 nM. The cytotoxicity of 2-CdA was below 50% at all concentrations used, and IC_{50} estimated by extrapolation was 2290 μM (Fig. 1C).

The highest cytotoxic effect for VEN (40 nM) used in combination with 2-CdA (16 μM) was 61.2% and DICy was 44.1%. These values were significantly higher than these found for each drug used separately (Table 3). The combination demonstrated strong synergistic effects at the above concentrations (CI = 0.035; Table 2). In further

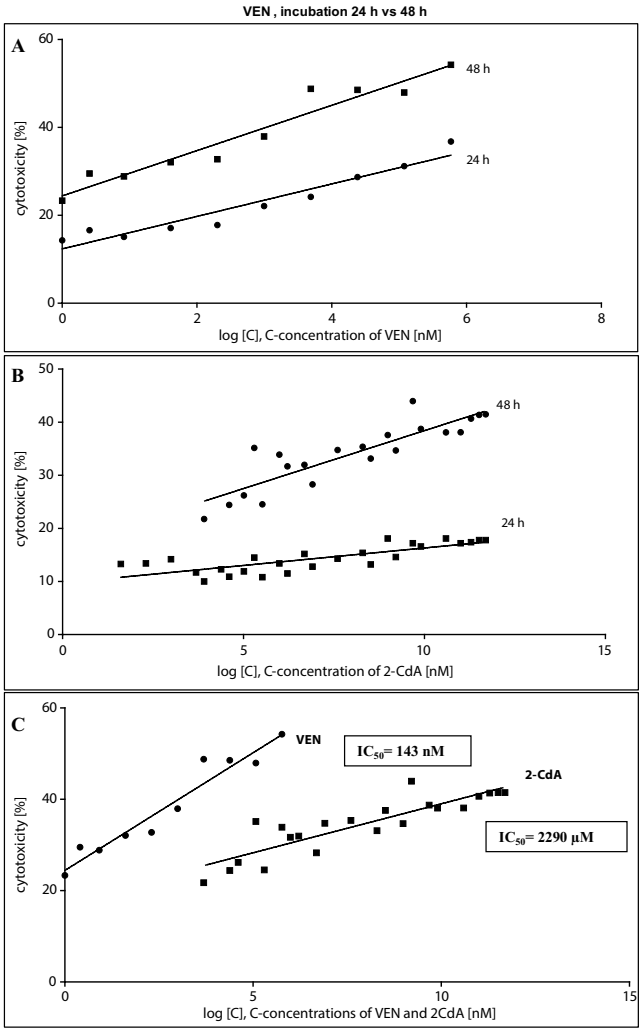

Fig. 1. Isobolograms presenting the relationship between drug concentration and its cytotoxic effect on chronic lymphocytic leukemia (CLL) cells after 24 h and 48 h of incubation. A. Venetoclax (VEN); B. Cladribine (2-CdA); C. VEN and 2-CdA, after 48-h incubation only

Table 2. Cytotoxicity of VEN and 2-CdA used alone, or in combination, and the combination index for both drugs used in 3 selected concentrations after 48 h of incubation

Drug	VEN	VEN 40 nM	VEN 80 nM	VEN 160 nM
2-CdA	cytotoxicity cytotoxicity	48.8% n = 78	48.5% n = 36	47.9% n = 41
2-CdA 4 μM	35.4% n = 47	44.8% n = 33	48.0% n = 16	52.7% n = 11
		CI = 0.75088	CI = 0.81526	CI = 0.69612
2-CdA 8 μM	37.6% n = 44	47.0% n = 33	51.1% n = 11	53.2% n = 11
		CI = 0.50452	CI = 0.46875	CI = 0.62496
2-CdA 16 μM	**44.0%** n = 58	**61.2%** n = 52	50.2% n = 11	52.9% n = 23
		CI = 0.03539	CI = 0.55668	CI = 0.66280

VEN – venetoclax; 2-CdA – cladribine; n – number of samples; CI – combination index: CI < 0.1 very strong synergy, CI 0.1–0.3 strong synergy, CI 0.3–0.7 synergy, CI 0.7–0.85 moderate synergy, CI 0.85–0.9 slight synergy, CI 0.9–1.1 nearly additive, CI > 1.1 antagonistic. Values in bold are the most important.

Table 3. The influence of VEN and 2-CdA used alone or in combination on cytotoxicity or apoptosis of CLL cells in vitro after 48 h of incubation

Drug	Control		VEN 40 nM		2-CdA 16 M		VEN 40 nM + 2-CdA 16 μM		Statistical analysis	
	PI(+)	AnnV(+)	PI(+)	AnnV(+)	PI(+)	AnnV(+)	PI(+)	AnnV(+)	PI(+)	AnnV(+)
	1	2	3	4	5	6	7	8		
n	93	36	78	36	58	33	52	33	**1 vs 3 < 0.001** **1 vs 5 < 0.001** **1 vs 7 < 0.001** 3 vs 5 = 0.033 **3 vs 7 < 0.001** **5 vs 7 < 0.001**	**2 vs 4 < 0.001** **2 vs 6 < 0.001** **2 vs 8 < 0.001** 4 vs 6 = 0.071 **4 < 8 = 0.001** **4 vs 8 < 0.001**
Median	15.3	4.0	49.0	7.1	42.4	8.7	62.0	11.7		
IQR	9.1–22.0	4.9–12.7	36.3–60.1	5.9–14.8	30.9–54.1	6.9–18.8	47.1–75.1	1.3–7.1		
DICy [%]	N/A	N/A	**31.7**	N/A	**26.9**	N/A	**44.1**	N/A	**3 vs 5 < 0.001** **3 vs 7 < 0.001** **5 vs 7 < 0.001**	N/A
DIA [%]	N/A	N/A	N/A	**4.8**	N/A	**5.3**	N/A	**8.9**	N/A	**4 vs 6 < 0.001** **4 vs 8 < 0.001** **6 vs 8 < 0.001**

n – number of samples; PI – propidium iodide; AnnV – annexin-V; IQR – interquartile range (Q1–Q3); VEN – venetoclax; 2-CdA – cladribine; DICy – drug-induced cytotoxicity; DIA – drug-induced apoptosis; N/A – not applicable. Wilcoxon signed-rank test with Bonferroni's correction for multiple comparisons; p < 0.008. Values in bold are statistically significant.

experiments, we used VEN at a concentration of 40 nM and 2-CdA at a concentration of 16 μM.

The influence of VEN and 2-CdA, used alone or in combination, on ΔΨm, overall apoptosis, and the expression of active forms of caspase-3, caspase-8 and caspase-9 in CLL cells in vitro

Mean Δψm was 29.3% for VEN, 34.0% for 2-CdA and 42.9% for VEN+2-CdA. Significantly higher DIΔψm was observed for drugs used in combination than separately (p < 0.0001) (Fig. 2A).

Venetoclax (40 nM) induced apoptosis with DIA = 4.8% and 2-CdA with DIA = 5.3% (Table 3). This difference was not statistically significant. Apoptosis induced by VEN+2-CdA was 11.7%; this was significantly higher than the apoptosis induced by either drug used separately (p < 0.001) (Table 2).

No significant differences were found between the effects of VEN and 2-CdA used alone and the percentage of cells expressing caspase-3, caspase-9 and caspase-8. In contrast, VEN+2-CdA elicited significantly higher percentages of cells expressing caspase-3 and caspase-9 (p < 0.001) (Fig. 2B); however, for caspase-8, a significant difference was observed only between VEN+2-CdA and 2-CdA alone (p < 0.001).

The influence of VEN and 2-CdA used alone and in combination on selected protein expression in CLL cells in vitro

For all studied proteins, except BCL-2, the percentage of positive cells increased under the influence of VEN compared to controls (Fig. 3A–G). Additionally, VEN application resulted in a significantly greater increase

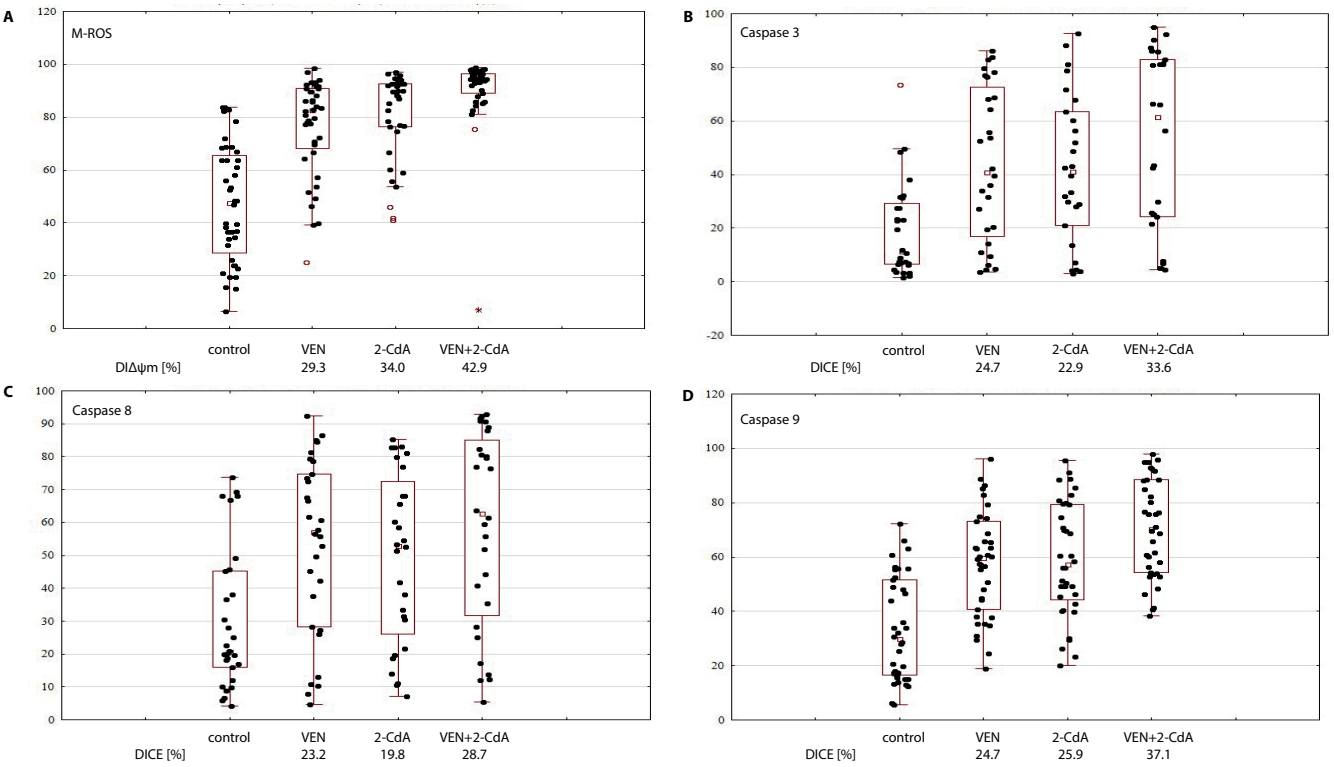

Fig. 2. The influence of 40 nM venetoclax (VEN) and 16 µM cladribine (2-CdA) used alone or in combination on the chronic lymphocytic leukemia (CLL) cells after 48 h of incubation in vitro. A. Percentage of cells expressing mitochondrial potential changes (Δψm); B. Percentage of cells expressing caspase-3 activity; C. Percentage of cells expressing caspase-8 activity; D. Percentage of cells expressing caspase-9 activity

VEN – venetoclax; 2-CdA – cladribine; DICE – drug-induced caspase expression; DIΔψm – drug-induced mitochondrial potential changes.

in the percentage of cells expressing BIM, BAX and NOXA proteins than 2-CdA (Table 4). However, VEN and VEN+2-CdA significantly decreased BCL-2 expression (Table 4): the median percentage of cells expressing BCL-2 was 77.3% for culture with VEN, 74.3% for 2-CdA and 74.4% for VEN+2-CdA (Fig. 3A). The DIPE values for BCL-2 were –10.7%, –11.2% and –13.9% for VEN, 2-CdA and VEN+2-CdA, respectively, and were significantly different only between 2-CdA and VEN+2-CdA (Fig. 3A).

The expression of P53 and PUMA increased under the influence of VEN, 2-CdA and VEN+2-CdA; however, no significant differences were observed between VEN and 2-CdA used alone (Table 4), and only a borderline difference was found between 2-CdA and VEN+2-CdA. The FAS-associated death-domain protein (FADD) expression increased after VEN, 2-CdA and VEN+2-CdA compared to controls, but the increase observed after VEN was not significant. The VEN+2-CdA treatment resulted in a significantly higher expression of FADD than of VEN alone (Table 4, Fig. 3G).

Discussion

Venetoclax is a new promising small molecule targeted against the antiapoptotic BCL-2 protein, the overexpression of which is one of the molecular hallmarks of CLL. Venetoclax, as a BH3-only mimetic, binds directly to BCL-2 and displaces the BH3 activator BIM from BCL-2, leading to BAX/BAK homooligomerization. The BAX/BAK complex permeabilizes the mitochondrial outer membrane (MOM), promotes the release of cytochrome c from the mitochondria to the cytosol, and induces caspase-mediated apoptosis.[26]

An important clinical advantage of VEN is its independence from functional P53. Although the treatment gives a high percentage of responses when used in monotherapy, it is not curative because of the complexity of the pathogenesis of CLL.[15,27] It is hence reasonable to assume that its efficacy will be enhanced by combination with other drugs which demonstrated the activity in CLL via different mechanisms of action.

The efficacy and safety of VEN have been already extensively studied in combination with anti-CD20 antibodies and BCR inhibitors.[11,15,28,29] Venetoclax has also demonstrated clinical efficacy in combination with bendamustine, a drug combining both alkylating and purine analogue properties.[30–32] The present study examines the influence of VEN and 2-CdA, used alone or in combination, on the viability, apoptosis and expression of selected apoptosis-related factors in vitro, i.e., in lymphocytes obtained from the peripheral blood of CLL patients.

The 2-CdA is a purine analogue, which may be used as an alternative for CLL treatment to fludarabine.

Table 4. The influence of VEN and 2-CdA used alone or in combination on selected proteins expression in CLL cells after 48 h of incubation

Protein		Control	VEN 40 nM	2-CdA 16 µM	VEN 40 nM +2-CdA 16 µM	Statistical analysis (p-value)
		1	2	3	4	
BCL-2	n	36	36	36	36	**1 vs 2 < 0.001** 2 vs 3 = 0.1573 **1 vs 3 < 0.001 2 vs 4 < 0.0001** **1 vs 4 < 0.001** 3 vs 4 = 0.1270
	median	87.0	77.3	74.3	74.4	
	IQR	80.5–90.1	69.0–82.8	65.3–81.8	59.9–80.1	
	DIPE	N/A	10.7↓	11.2↓	13.9↓	
P53	n	36	36	35	35	**1 vs 2 < 0.001** 2 vs 3 = 0.1819 **1 vs 3 = 0.001** 2 vs 4 = 0.3547 **1 vs 4 < 0.001** 3 vs 4 = 0.0116
	median	3.0	3.85	4.4	5.6	
	IQR	1.6–4.6	2.6–10.8	2.6–5.8	3.2–9.8	
	DIPE	N/A	1.8↑	2.6↑	3.4↑	
BIM	n	36	36	36	36	**1 vs 2 < 0.001 2 vs 3 < 0.001** **1 vs 3 < 0.001 2 vs 4 < 0.001** **1 vs 4 < 0.001 3 vs 4 < 0.001**
	median	1.7	20.45	11.0	23.25	
	IQR	1.1–2.4	18.2–23.4	10.3–13.8	21.2–25.5	
	DIPE	N/A	18.5↑	10.2↑	21.3↑	
BAX	n	35	35	35	35	**1 vs 2 < 0.001 2 vs 3 < 0.001** **1 vs 3 < 0.001 2 vs 4 < 0.001** **1 vs 4 < 0.001 3 vs 4 < 0.001**
	median	15.0	22.5	29.9	24.7	
	IQR	10.1–17.3	17.9–26.6	15.5–23.8	21.1–28.1	
	DIPE	N/A	8.3↑	5.6↑	10.3↑	
PUMA	n	34	34	34	34	**1 vs 2 < 0.001** 2 vs 3 = 0.6505 **1 vs 3 < 0.001** 2 vs 4 = 0.9182 **1 vs 4 < 0.001** 3 vs 4 = 0.6505
	median	19.8	28.3	27.95	26.95	
	IQR	17.3–23.2	22.5–32.8	19.8–32.4	23.4–30.4	
	DIPE	N/A	6.5↑	6.3↑	6.5↑	
NOXA	n	36	36	36	36	**1 vs 2 < 0.001 2 vs 3 < 0.001** **1 vs 3 < 0.001 2 vs 4 < 0.001** **1 vs 4 < 0.001 3 vs 4 < 0.001**
	median	11.25	21.5	18.5	23.85	
	IQR	9.1–14.2	17.5–26.6	14.3–21.5	19.4–28.3	
	DIPE	N/A	10.3↑	6.5↑	18.1↑	
FADD	n	33	33	34	34	1 vs 2 = 0.047 2 vs 3 = 0.148 **1 vs 3 = 0.007 2 vs 4 = 0.003** **1 vs 4 < 0.001** 3 vs 4 = 0.015
	median	2.2	2.5	3.4	4.5	
	IQR	1.6–3.0	2.0–3.8	1.9–4.9	2.0–8.7	
	DIPE	N/A	0.9↑	1.2↑	2.9↑	

VEN – venetoclax; 2-CdA – cladribine; CLL – chronic lymphocytic leukemia; n – number of samples; DIPE – drug-induced protein expression; ↑ – increase of expression; ↓ – decrease of expression; IQR – interquartile range (Q1–Q3). Wilcoxon signed-rank test with Bonferroni's correction for multiple comparisons; $p < 0.008$. Values in bold are statistically significant.

The 2-CdA remains a backbone of chemotherapy regimens for physically fit patients. Its mechanism of action is based on the ability to incorporate into a newly synthesized DNA chain and inhibit DNA repair.[18,33,34] In the intrinsic *TP53*-dependent apoptotic pathway, P53 induces BAX protein expression, leading to the release of cytochrome c from mitochondria to the cytoplasm, apoptosome formation and caspase cascade activation. Moreover, 2-CdA can also act independently of *TP53* by directly binding to proteins located in the mitochondrial membrane, resulting in cytochrome c release and apoptosome formation, or formation of apoptosis-inducing factor (AIF); this in turn can directly lead to chromatin condensation and DNA fragmentation, but without caspase cascade activation.[18,33,34] Despite this, the role of the extrinsic pathway in 2-CdA-induced apoptosis remains controversial.[20,21] The mechanism of action of 2-CdA is therefore different than the mechanism of VEN; however, the common feature is that both drugs trigger the intrinsic pathway of apoptosis.

The present study tested the influence of VEN and 2-CDA on the viability of CLL lymphocytes (drug cytotoxicity), as determined by the percentage of cells incorporating PI and overall apoptosis, measured by AnnV binding capacity. Both phenomena were more visible in cells incubated with the drugs than in control cultures, and higher results were obtained in cells subjected to VEN+2-CdA compared to cells incubated with 1 drug only.

Additionally, both drugs used at the tested concentrations showed a synergistic effect with very low CI value (CI = 0.035), which proves a strong synergy. Similar conclusions can be drawn from the analysis of $\Delta\Psi m$ made at the very early stage of intrinsic pathway of apoptosis.

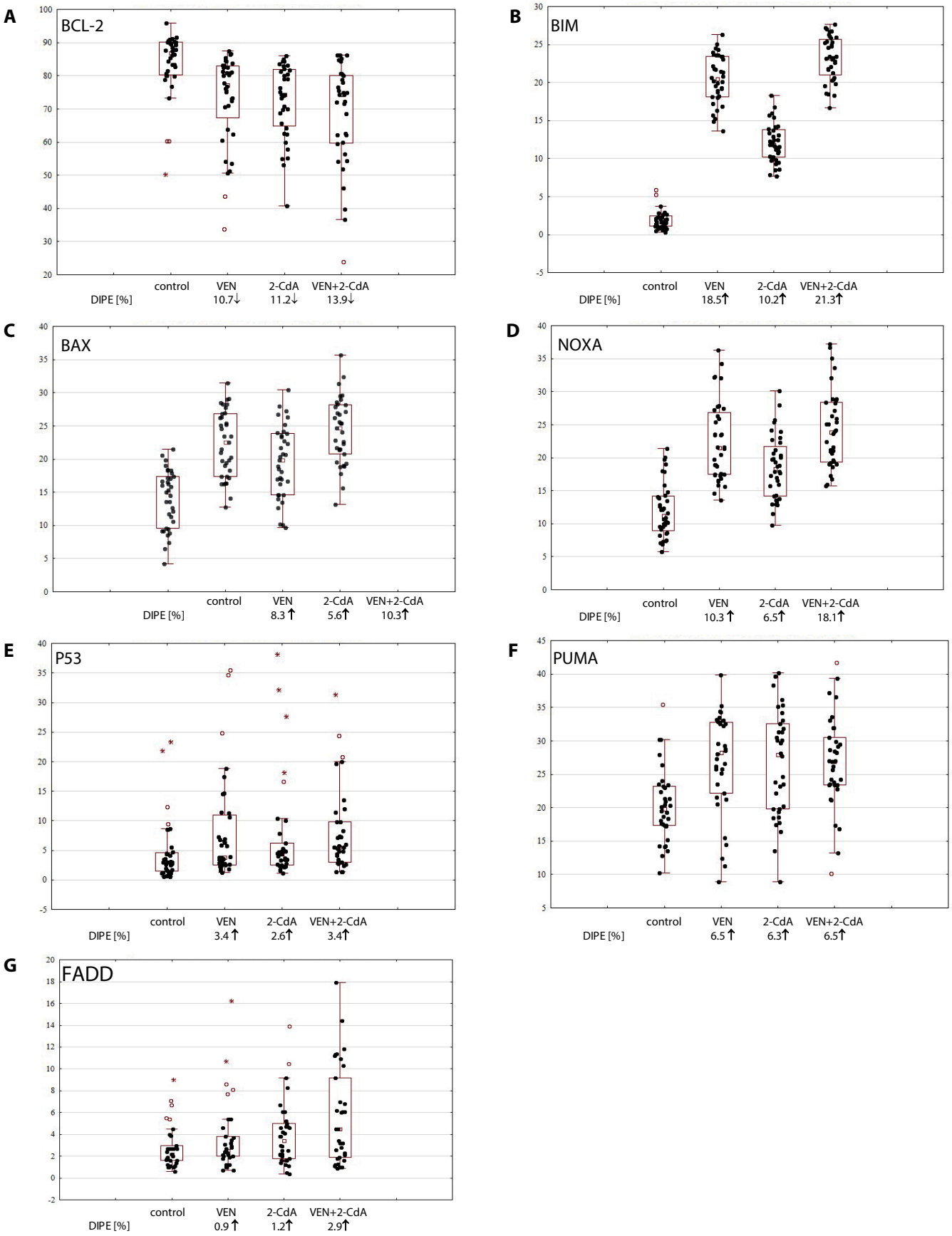

Fig. 3. The influence of 40 nM venetoclax (VEN) and 16 μM cladribine (2-CdA) used alone or in combination on the percentage of chronic lymphocytic leukemia (CLL) cells expressing proteins after 48 h of incubation in vitro. A. BCL-2; B. BIM; C. BAX; D. NOXA; E. P53; F. PUMA; G. FADD

↑ – increase of expression; ↓ – decrease of expression; DIPE – drug-induced protein expression.

Significantly higher ΔΨm values were observed after the action of VEN and 2-CdA used separately when compared to controls, and even higher values were noted after VEN+2-CdA. Accordingly, the cultures exposed to the drugs demonstrated higher percentages of cells expressing caspase-3, caspase-9 and caspase-8 than controls. Interestingly, the percentage of cells expressing caspase-8 was not significantly different for VEN and 2-CdA used alone; however, the use of VEN+2-CdA resulted in a significant increase of the percentage of these cells compared to 2-CdA.

The 2nd part of our study examined the influence of both drugs alone and in combination on the expression of some relevant proteins involved in apoptosis. It was found that the percentage of cells with detectable P53 increased under all 3 treatments. This is somewhat surprising, since it is believed that the mechanism of action of VEN is independent of *TP53*. It is possible that VEN may influence P53 protein expression by an unknown mechanism, e.g., by increasing its stability.

Another finding which needs further explanation is the observed decrease of the percentage of BCL-2-positive cells following VEN addition as compared to the control culture, as well as their even more profound decrease under the influence of 2-CdA and the greatest decrease after VEN+2-CdA. As mentioned above, VEN is believed to form a complex with BCL-2, without apparently affecting the expression of the latter. However, it is possible that BCL-2 expression may be inhibited by the aforementioned increase of P53 expression found in cells subjected to both drugs separately and in combination. Indeed, P53 has been found to have an inhibitory effect on the expression of *BCL-2* gene in both human and murine malignant cells.[35,36] On the other hand, it is also possible that the decrease of the percentage of BCL-2-expressing cells may result from the presence of normal lymphocytes among the mononuclear cells (MNCs) used for culture. The mean purity of $CD19^+$ B cells was found to be approx. 95%, as measured using flow cytometry.

The study then assessed the expression of 4 proapoptotic proteins involved in the intrinsic pathway: BIM, BAX, PUMA, and NOXA. Their expression increased under the influence of VEN and/or 2-CdA above the values found in control cells. The highest increase in cells expressing BIM, BAX and NOXA was found in the cells incubated with VEN+2-CdA, a lower rise was observed after VEN only and the lowest after 2-CdA only. All these proteins appear important for the intracellular activity of anticancer drugs, including VEN.

As mentioned above, VEN binds to the BH3-binding groove of BCL-2 and displaces BIM from its binding site, thus allowing activation of the apoptotic effectors BAX and BAK.[37] The BAX activation leads to its oligomerization and then permeabilization of the MOM, manifested in an increase of the mitochondrial transmembrane potential, release of cytochrome c from mitochondria, apoptosome formation, and caspase cascade activation.[26,27] As previously shown in M1 murine leukemia cells, BAX is a target of transcriptional activity of P53.[36] This may account, at least partially, for the increase of BAX protein observed together with the increase of P53.

The NOXA protein is less effective than PUMA in induction of *TP53*-mediated apoptosis. The PUMA can bind to all antiapoptotic BCL-2 family members, whereas NOXA antagonizes MCL-1 and A1 proteins only. However, in some cases of apoptosis caused by DNA damage, PUMA and NOXA can cooperate. It is likely that if the functionality of one of the proteins weakens, it can be enhanced by the action of the other.[38] Additionally, autoactivation of BAX and BAK can occur independently of activator BH3s through downregulation of BCL-2, BCL-XL and MCL-1.[15,39] It is also important to note that various P53 family members such as P63 and P73, also have the potential to induce apoptotic outcomes through the induction of the pro-apoptotic BCL-2 family members, such as PUMA and NOXA.[40] Unlike BIM, BAX and NOXA, the percentage of cells expressing PUMA in our experiments increased to a similar degree after VEN, 2-CdA and VEN+2-CdA addition. Therefore, this factor probably does not play an essential role in the interaction of those drugs in inducing CLL cell apoptosis.

Finally, the study examined whether the drugs induced the extrinsic apoptotic pathway. To our knowledge, no experimental data support such activity of VEN, and the involvement of 2-CdA in the extrinsic apoptotic way is controversial. One element of this pathway is FADD. Together with the adaptor FAS and the initiator procaspase-8 (or procaspase-10), FADD forms the death-inducing signaling complex (DISC), which in turn cleaves and activates the executioner caspase-3 and caspase-7, eventually bringing about apoptosis.[27] Nomura et al. suggested that 2-CdA may stimulate both the intrinsic and extrinsic apoptotic pathways.[20] Our findings indicate that the percentage of cells expressing FADD was significantly higher after 2-CdA and 2-CdA+VEN administration as compared to control cells, which would be in line with the possibility that 2-CdA is involved in the extrinsic pathway. The fact that VEN alone had no significant effect on FADD expression is consistent with the current concept of the mechanism of action of VEN. However, the observed higher expression of FADD after VEN+2-CdA administration compared to VEN or 2-CdA administration alone might suggest that VEN, despite being incapable of triggering the extrinsic pathway, may enhance this potential in 2-CdA. This hypothesis remains to be proven by further studies.

Limitations of the study

The only limitation of our study is the quantitative method of assessment of proteins studied, i.e., as a percentage of cells in which protein expression was detectable. It would be interesting to measure the protein expression

semi-qualitatively as the mean fluorescence intensity. However, we felt that intensity of fluorescence in cells after the culture would not have been correlated strongly enough with the actual intracellular concentration of proteins. In particular, we feared that the cell membrane would have been altered by the culture, so that its sensibility to permeabilization during the staining would have been somewhat different as compared to fresh lymphocytes. As a results, the penetration of antibodies into the cytoplasm would have been not strictly comparable to cells before and after culture. Therefore, we preferred to restrict our measures to qualitative assessment of the presence of the protein studied.

It might be objected that the status of *TP53* gene in patients enrolled into the study was not indicated. As a matter of fact, the del17p/mut*TP53* might have influenced the in vitro response of CLL lymphocytes to cladribine. However, the cytogenetic analysis is not included in the initial diagnostic workup of CLL patients unless the initiation of the treatment is planned immediately. Nevertheless, the incidence of del17p/mut*TP53* in treatment-naïve patients is above 10%, so it would probably have been less than 10 patients in our series. Such small group of cases with *TP53* aberrations would not have allowed for any reliable statistical analysis. The possible presence of such patients in our population would not distort the results we have obtained.

Conclusions

To conclude, it is important to note that the regulation of apoptosis of CLL cells in vitro is different than in vivo. The CLL lymphocytes cultured without the support provided by circulating cytokines and factors provided by microenvironmental cells die much quicker than circulating ones. However, the abovementioned findings provide a rationale to extend the investigations on the mechanism of action of VEN in order to clarify the role of P53 and related antiproliferative agents in the mechanism of its action. It must also be noted that although VEN is efficacious in patients with mutated *TP53* and/or deletion of 17p, it cannot fully abolish the negative prognostic significance of those cytogenetic/molecular abnormalities. Moreover, our results confirm the involvement of both VEN and 2-CdA in the intrinsic apoptotic pathway, and demonstrate the synergistic effect of both drugs on CLL cells in vitro. Our findings justify further molecular studies aimed at assessing the feasibility of VEN+2-CdA application in clinical practice.

ORCID iDs

Aleksandra Beata Kubiak https://orcid.org/0000-0001-5650-1312
Ewelina Izabela Ziółkowska https://orcid.org/0000-0002-4427-9811
Anna Barbara Korycka-Wołowiec https://orcid.org/0000-0001-9256-0328
Tadeusz Robak https://orcid.org/0000-0002-3411-6357
Dariusz Wołowiec https://orcid.org/0000-0003-4081-5397

References

1. Plati J, Bucur O, Khosravi-Far R. Apoptotic cell signaling in cancer progression and therapy. *Integr Biol (Camb)*. 2011;3(4):279–296. doi:10.1039/c0ib00144a
2. Roberts A, Huang D. Targeting BCL2 with BH3 mimetics: Basic science and clinical application of venetoclax in chronic lymphocytic leukemia and related B cell malignancies. *Clin Pharmacol Ther*. 2017;101(1):89–98. doi:10.1002/cpt.553
3. Stilgenbauer S, Eichhorst B, Schetelig J, et al. Venetoclax in relapsed or refractory chronic lymphocytic leukaemia with 17p deletion: A multicentre, open-label, phase 2 study. *Lancet Oncol*. 2016;17(6):768–778. doi:10.1016/S1470-2045(16)30019-5
4. Vogler M, Dinsdale D, Dyer MJS, Cohen GM. ABT-199 selectively inhibits BCL2 but not BCL2L1 and efficiently induces apoptosis of chronic lymphocytic leukaemic cells but not platelets. *Br J Haematol*. 2013;163(1):139–142. doi:10.1111/bjh.12457
5. Roberts AW, Davids MS, Pagel JM, et al. Targeting BCL2 with venetoclax in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2016;374(4):311–322. doi:10.1056/NEJMoa1513257
6. Adams JM, Cory S. The BCL-2 arbiters of apoptosis and their growing role as cancer targets. *Cell Death Differ*. 2018;25(1):27–36. doi:10.1038/cdd.2017.161
7. Dai H, Meng XW, Kaufmann SH. Mitochondrial apoptosis and BH3 mimetics. *F1000Res*. 2016;5:2804. doi:10.12688/f1000research.9629.1
8. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell*. 2002;2(3):183–192. doi:10.1016/S1535-6108(02)00127-7
9. Gentile M, Petrungaro A, Uccello G, et al. Venetoclax for the treatment of chronic lymphocytic leukemia. *Expert Opin Investig Drugs*. 2017;26(11):1307–1316. doi:10.1080/13543784.2017.1386173
10. Lew TE, Anderson MA, Lin VS, et al. Undetectable peripheral blood MRD should be the goal of venetoclax in CLL, but attainment plateaus after 24 months. *Blood Adv*. 2020;4(1):165–173. doi:10.1182/bloodadvances.2019000864
11. Schieber M, Ma S. The expanding role of venetoclax in chronic lymphocytic leukemia and small lymphocytic lymphoma. *Blood Lymphat Cancer*. 2019;9:9–17. doi:10.2147/BLCTT.S177009
12. Juárez-Salcedo LM, Desai V, Dalia S. Venetoclax: Evidence to date and clinical potential. *Drugs Context*. 2019;8:1–13. doi:10.7573/dic.212574
13. Herling CD, Abedpour N, Weiss J, et al. Clonal dynamics towards the development of venetoclax resistance in chronic lymphocytic leukemia. *Nat Commun*. 2018;9(1):727. doi:10.1038/s41467-018-03170-7
14. Dreger P, Ghia P, Schetelig J, et al. High-risk chronic lymphocytic leukemia in the era of pathway inhibitors: Integrating molecular and cellular therapies. *Blood*. 2018;132(9):892–902. doi:10.1182/blood-2018-01-826008
15. Korycka-Wolowiec A, Wolowiec D, Kubiak-Mlonka A, Robak T. Venetoclax in the treatment of chronic lymphocytic leukemia. *Expert Opin Drug Metab Toxicol*. 2019;15(5):353–366. doi:10.1080/17425255.2019.1606211
16. Robak T, Błoński J, Skotnicki AB, et al. Rituximab, cladribine, and cyclophosphamide (RCC) induction with rituximab maintenance in chronic lymphocytic leukemia: PALG – CLL4 (ML21283) trial. *Eur J Haematol*. 2018;100(5):465–474. doi:10.1111/ejh.13042
17. Robak T, Jamroziak K, Gora-Tybor J, et al. Comparison of cladribine plus cyclophosphamide with fludarabine plus cyclophosphamide as first-line therapy for chronic lymphocytic leukemia: A phase III randomized study by the Polish Adult Leukemia Group (PALG-CLL3 Study). *J Clin Oncol*. 2010;28(11):1863–1869. doi:10.1200/JCO.2009.25.9630
18. Robak T, Korycka A, Lech-Maranda E, Robak P. Current status of older and new purine nucleoside analogues in the treatment of lymphoproliferative diseases. *Molecules*. 2009;14(3):1183–1226. doi:10.3390/molecules14031183
19. Marzo I, Pérez-Galán P, Giraldo P, Rubio-Félix D, Anel A, Naval J. Cladribine induces apoptosis in human leukaemia cells by caspase-dependent and -independent pathways acting on mitochondria. *Biochem J*. 2001;359(3):537–546. doi:10.1042/bj3590537
20. Nomura Y, Inanami O, Takahashi K, Matsuda A, Kuwabara M. 2-Chloro-2′-deoxyadenosine induces apoptosis through the Fas/Fas ligand pathway in human leukemia cell line MOLT-4. *Leukemia*. 2000;14(2):299–306. doi:10.1038/sj.leu.2401649

21. Klöpfer A, Hasenjäger A, Belka C, Schulze-Osthoff K, Dörken B, Daniel PT. Adenine deoxynucleotides fludarabine and cladribine induce apoptosis in a CD95/Fas receptor, FADD and caspase-8-independent manner by activation of the mitochondrial cell death pathway. *Oncogene*. 2004;23(58):9408–9418. doi:10.1038/sj.onc.1207975
22. Hallek M, Cheson BD, Catovsky D, et al. iwCLL guidelines for diagnosis, indications for treatment, response assessment, and supportive management of CLL. *Blood*. 2018;131(25):2745–2760. doi:10.1182/blood-2017-09-806398
23. Smolewski P, Szmigielska-Kaplon A, Cebula B, et al. Proapoptotic activity of alemtuzumab alone and in combination with rituximab or purine nucleoside analogues in chronic lymphocytic leukemia cells. *Leuk Lymphoma*. 2005;46(1):87–100. doi:10.1080/13693780400007151
24. Zaborowska A, Cebula-Obrzut B, Franiak-Pietryga I, et al. Wpływ bendamustyny zastosowanej pojedynczo lub w skojarzeniu z analogami nukleozydów purynowych na przeżywalność oraz apoptozę komórek przewlekłej białaczki limfocytowej w hodowli in vitro. *Acta Heamatol Pol*. 2009;40(4):887–898.
25. Chou TC. The mass-action law based algorithm for cost-effective approach for cancer drug discovery and development. *Am J Cancer Res*. 2011;1(7):925-954. PMID:22016837, PMCID:PMC3196289.
26. Kalkavan H, Green DR. MOMP, cell suicide as a BCL-2 family business. *Cell Death Differ*. 2018;25(1):46–55. doi:10.1038/cdd.2017.179
27. Bose P, Gandhi V, Konopleva M. Pathways and mechanisms of venetoclax resistance. *Leuk Lymphoma*. 2017;58(9):2026–2039. doi:10.1080/10428194.2017.1283032
28. Seymour JF, Kipps TJ, Eichhorst B, et al. Venetoclax-rituximab in relapsed or refractory chronic lymphocytic leukemia. *N Engl J Med*. 2018;378(12):1107–1120. doi:10.1056/NEJMoa1713976
29. Seymour JF, Ma S, Brander DM, et al. Venetoclax plus rituximab in relapsed or refractory chronic lymphocytic leukaemia: A phase 1b study. *Lancet Oncol*. 2017;18(2):230–240. doi:10.1016/S1470-2045(17)30012-8
30. Cramer P, von Tresckow J, Bahlo J, et al. Bendamustine followed by obinutuzumab and venetoclax in chronic lymphocytic leukaemia (CLL2-BAG): Primary endpoint analysis of a multicentre, open-label, phase 2 trial. *Lancet Oncol*. 2018;19(9):1215–1228. doi:10.1016/S1470-2045(18)30414-5
31. Stilgenbauer S, Morschhauser F, Wendtner CM, et al. Phase Ib study (GO28440) of venetoclax with bendamustine/rituximab or bendamustine/obinutuzumab in patients with relapsed/refractory or previously untreated chronic lymphocytic leukemia. *Blood*. 2016;128(22):4393–4393. doi:10.1182/blood.V128.22.4393.4393
32. Stilgenbauer S, Morschhauser F, Wendtner CM, et al. Venetoclax plus bendamustine-rituximab or bendamustine-obinutuzumab in chronic lymphocytic leukemia: Final results of a phase 1b study (GO28440). *Haematologica*. 2021;106(11):2834–2844. doi:10.3324/haematol.2020.261107
33. Robak T, Korycka A, Kasznicki M, Wrzesien-Kus A, Smolewski P. Purine nucleoside analogues for the treatment of hematological malignancies: Pharmacology and clinical applications. *Curr Cancer Drug Targets*. 2005;5(6):421–444. doi:10.2174/1568009054863618
34. Robak T, Lech-Maranda E, Korycka A, Robak E. Purine nucleoside analogs as immunosuppressive and antineoplastic agents: Mechanism of action and clinical activity. *Curr Med Chem*. 2006;13(26):3165–3189. doi:10.2174/092986706778742918
35. Miyashita T, Harigai M, Hanada M, Reed JC. Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res*. 1994;54(12):3131–3135. PMID:8205530.
36. Miyashita T, Krajewski S, Krajewska M, et al. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene*. 1994;9(6):1799-1805. PMID:8183579.
37. Klanova M, Klener P. BCL-2 proteins in pathogenesis and therapy of B-cell non-Hodgkin lymphomas. *Cancers*. 2020;12(4):938. doi:10.3390/cancers12040938
38. Zhang LN, Li JY, Xu W. A review of the role of Puma, Noxa and Bim in the tumorigenesis, therapy and drug resistance of chronic lymphocytic leukemia. *Cancer Gene Ther*. 2013;20(1):1–7. doi:10.1038/cgt.2012.84
39. Chen HC, Kanai M, Inoue-Yamauchi A, et al. An interconnected hierarchical model of cell death regulation by the BCL-2 family. *Nat Cell Biol*. 2015;17(10):1270–1281. doi:10.1038/ncb3236
40. Yu J, Zhang L. PUMA, a potent killer with or without p53. *Oncogene*. 2008;27(S1):S71–S83. doi:10.1038/onc.2009.45