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Cytotoxic and apoptosis-inducing effects of bendamustine used alone and in combination with rituximab on chronic lymphocytic leukemia cells *in vitro**

Cytotoksyczny i proapoptotyczny wpływ bendamustyny zastosowanej pojedynczo lub w skojarzeniu z rytuksymabem na komórki przewlekłej białaczki limfocytowej *in vitro*

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Aim: The aim of our study was to compare the cytotoxic effects of bendamustine (BENDA) and rituximab (RIT) used either alone or in combination and to evaluate the influence of the above mentioned drugs on apoptosis measured as changes in mitochondrial transmembrane potential ($\Delta\psi_m$), expression of caspases and selected apoptosis-regulating proteins in freshly isolated peripheral blood mononuclear cells of chronic lymphocytic leukemia (CLL) patients.

Material/Methods: Cytotoxic effect of tested drugs, as well as induction of apoptosis, drop in $\Delta\psi_m$ and expression of selected proteins involved in regulation of apoptosis were assessed in 48 hour cultures containing autologous serum (AS) using flow cytometry. BENDA was used at the concentration of 40 μg/ml and RIT at the concentration of 10 μg/ml. Control cultures were incubated without drugs.

Results: BENDA used either alone or in combination with RIT strongly induced apoptosis as well as enhanced expression of selected apoptotic proteins, especially those involved in the intrinsic apoptotic pathway: P53, PUMA and BAX, which cause mitochondrial transmembrane potential changes leading to activation of caspase-9 and -3.

Conclusions: Our results indicate that both BENDA and RIT participate in the induction of apoptosis of CLL lymphocytes *in vitro* in the presence of AS in the culture medium. The drug-induced apoptosis occurs mainly via intrinsic pathway and activation of P53 and PUMA proteins, however the extrinsic pathway is likely to be involved as well. We also found that the combination of these drugs induces the expression of P53, caspase-8 and -9 more potently than either of them used separately.

Keywords: **chronic lymphocytic leukemia • bendamustine • rituximab • cytotoxicity • mitochondrial potential changes • apoptosis-regulatory proteins**

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Introduction

Chronic lymphocytic leukemia (CLL) is a clonal lymphoproliferative disorder affecting morphologically mature B cells, resulting in the accumulation of cells expressing the surface antigens CD5, CD19 and CD23 in the bone marrow, peripheral blood, lymph nodes and eventually non-lymphoid organs. Leukemic transformation leads to deregulated and uncontrolled proliferation as well as inhibition of apoptosis of circulating leukemic cells. Apoptosis, a programmed cell death, is a physiological process occurring via intrinsic and extrinsic pathways. The expression of appropriate genes responsible for the physiological activation of apoptosis leads to changes in the expression levels of specific apoptosis-regulating proteins [4,9,12].

Despite the undeniable progress that has taken place in the CLL therapy, the disease still remains incurable. In recent years the efficacy of bendamustine (BENDA) in the treatment of hematological diseases, particularly indolent non-Hodgkin lymphomas and CLL has been proven [3,11,14,16,19,28]. Due to its chemical structure, BENDA combines the properties of cytotoxic alkylating agents and anti-metabolites [16,22]. The drug induces DNA damages, which are much larger, more extensive and also more difficult to repair than those caused by other alkylating agents. This leads to inhibition of the expression of genes involved in the cell cycle control and results in the mitotic catastrophe and cell death by necrosis. On the other hand, the presence of the benzimidazole ring is responsible for the induction of apoptosis and attenuation of the drug toxicity [17,22]. In the therapy of CLL, BENDA is widely used either alone or in combination mainly with rituximab (RIT) – an anti-CD20 first class monoclonal antibody, especially in patients in whom fludarabine-based regimens are contraindicated [11,14,18,21,23,25]. The mechanism of action of RIT can be described as multipath phenomenon since it induces complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), exerts the effect of a vaccine involving cytotoxic T-lymphocytes and dendritic cells and additionally the dimeric form of RIT induces apoptosis [8,20,29]. Such mechanism of action of RIT on CLL cells can be fully achieved only in the presence of appropriate cytokines and complement system components which circulate in the serum or have to be added to the *in vitro* culture medium. In our previous study we demonstrated that the type of serum (autologous serum - AS, fetal calf serum – FCS, or human AB serum - ABS) used for the culture is important for the cytotoxic and apoptotic effect of RIT on CLL cells. We found that AS used alone or in combination with FCS significantly enhances the cytotoxic effects of RIT on CLL cells *in vitro* [31].

Only few studies have been devoted to the mechanisms of action of BENDA used in combination with RIT and they were not carried out in the AS-containing culture medium [2,24,26]. Therefore, it seemed justified to undertake a study aimed at evaluating the cytotoxic and apoptotic effect of BENDA used alone or in combination with RIT on the viability of CLL cells *in vitro* and assessing the expression of some apoptosis-involved proteins: P53, PUMA, BAX, BCL-2 and APAF-1 participating in the intrinsic apoptotic pathway, and of FADD involved in the extrinsic pathway, in the culture containing AS.

Material and methods

Patients

Peripheral blood samples were collected from 65 newly diagnosed CLL patients (23 females, 42 males) aged 65 years (range 24-84), as a part of routine diagnostic procedure. The diagnosis was based on the IWCLL criteria [13]. All patients signed a written informed consent to participate in this laboratory protocol, which was approved by the Ethics Committee of the Medical University of Lodz.

Therapeutics

Rituximab (RIT; Mabthera, Roche, Switzerland),commercially available, was used at the final concentration of 10 μg/ml. Bendamustine (BENDA) was kindly supplied by Mundipharma LTD and used at the final concentration of 40μg/ml. BENDA concentration was chosen as per our previous studies [30].

Isolation of PBMNCs and cell culture conditions

Peripheral blood mononuclear cells (PBMNCs) were isolated from heparinized peripheral blood by Histopaque-1077 (Sigma-Aldrich, St. Louis, USA) gradient separation and

centrifuged in a density gradient by 3600 rpm for 20 min. The buffy coat of PBMNCs was collected and washed twice in RPMI 1640 medium (PAA Laboratories GmbH, Austria). Afterwards, PBMNCs at a concentration of $1.0x10^6$ cells/ml were cultured for 48 hours in RPMI 1640 supplemented with 20% of sera combination and antibiotics (streptomycin 50 mg/ml and penicillin 50 IU/ml; Life Technologies, Scotland), at the temperature of 37°C, in the atmosphere of 5% CO_2 and full humidity. As per our previous study, the following sera combination was used: 10% (v/v) heat inactivated FCS (Gibco, Life Technologies, Scotland) and 10% (v/v) AS [19]. Mean B-cell (CD19+) purity was >95% as measured by flow cytometry (FACS).

Assessment of BENDA- and RIT- induced cytotoxicity

At the onset of cell cultures and after 48h incubation, the absolute number of cells per culture was estimated using a hemocytometer. Afterwards, PBMNCs were washed twice with cold PBS and then resuspended in 100 ml of binding buffer containing 10 mg/ml of propidium iodide (PI; Sigma Aldrich, Germany) and incubated for 15 min. at room temperature in the dark. The fluorescence was measured immediately after staining by flow cytometer (FACSCalibur; Becton Dickinson, San Diego, USA) at 490"20 nm using FL1, and at 530"20 nm using FL3 standard fluorescent filters. The control cultures were incubated without drugs.

Drug-induced cytotoxicity (DICy) stands for the mean value of the diferences between the percentage of dead cells after incubation with drug(s) studied and that in the control culture, calculated for each sample.

Assessment of the effect of drugs on apoptosis

The cells were washed twice with cold PBS and then resuspended in 100 ml of binding buffer, containing 2 ml of fluorescein isothiocyanate (FITC; BD Pharmingen, San Diego, CA) conjugated Ann-V (Ann-V; BD Pharmingen, USA). The fluorescence was measured immediately with an FACScalibur, as described above.

Drug-induced apoptosis (DIA) stands for the mean value of the differences between the percentage of cells which underwent apoptosis (AnnV+ cells) in the presence of the drug(s) studied, and the percentage of cells undergoing spontaneous apoptosis in the control cultures, calculated for each sample. Drug-induced necrosis (DIN) stands for the mean value of the differences between the percentage of necrotic cells (AnnV-/PI+ cells) in the presence of BENDA or RIT or BENDA+RIT and the percentage of cells undergoing spontaneous necrosis in the control cultures, calculated for each sample.

Disruption of mitochondrial transmembrane potential changes ($\Delta\psi_m$)

Changes in the mitochondrial transmembrane potential ($\Delta\psi_m$) were evaluated using MitoTracker Red CMX Ros kit (Invitrogen, Australia). After 48 hour incubation of CLL cells in the above described conditions, PBMNCs were centrifuged, and the supernatant above the cell pellet was removed. Afterwards, 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 then repelleted by centrifugation and resuspended in fresh warmed-up medium. Assays were done in duplicate and analyzed by flow cytometry (FACSCalibour, Becton Dickinson, San Diego, USA).

Drug induced $\Delta\psi_m$ ($DI\Delta\psi_m$) stands for the mean value of the differences between the percentage of cells with $\Delta\psi_m$ after incubation with drug(s) and the percentage of cells with $\Delta\psi_m$ in the control culture, calculated for each sample.

Detection of the active forms of caspase-3, caspase-9 and caspase-8 by flow cytometry

The caspase-3 activity was quantitatively assessed by flow cytometry on FACSCalibour (Becton Dickinson, San Diego, USA), using Becton Dickinson Pharmingen PE Active Caspase-3 Apoptosis Kit (San Diego, USA), according to the manufacturer's protocol. Similarly, quantitive analyses of active forms of caspase-8 and caspase-9 were also performed on FACSCalibour 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 done in duplicate.

Drug-induced expression (DIE) of active forms of caspases stands for the mean value of the differences between the percentage of cells with expression of caspase after incubation with drug(s) and the percentage of cells with expression of caspase in the control culture, calculated for each sample.

Detection of apoptosis-regulatory proteins

After 48 hour incubation the number of cells per culture was determined by hemocytometer. Cells from each culture flask were collected and washed with PBS. Protein expression was assessed in fixed, permeabilized cells by direct staining of specific labeled antibodies.

The following proteins were evaluated: P53 (FITC Mouse Anti-Human P53 Set, BD Pharmingen, San Diego, USA), BAX (Rabbit Anti-BAX antibody, Abcam, United Kingdom), BAK (Rabbit Purified Mouse Anti-Human Monoclonal BAK, BD Pharmingen, San Diego, USA), APAF-1 (Rabbit Anti-APAF1 Antibody, Abcam, United Kingdom), PUMA (Rabbit Anti-PUMA Antibody, Abcam, United Kingdom), FADD (Rabbit Anti-FADD Antibody, Abcam, United Kingdom) and BCL-2 (Rabbit Purified Mouse Anti-Human BCL-2, BD Pharmingen, San Diego, USA).

All investigated proteins but one (P53 – FITC-conjugated) were used with appropriate secondary antibody (Goat polyclonal Antibody to Rabbit IgG (FITC

coniugated), Abcam, United Kingdom) and with appropriate isotype controls. The fluorescence was measured by flow cytometer (FACSCalibour, Becton Dickinson, San Diego, USA), using standard emission green filter (FL1).

Drug induced expression (DIE) of protein-expressing CLL cells stands for the mean value of the differences between the percentage of cells expressing protein after 48 hour incubation with drug(s) and the percentage of cells expressing protein in the control culture, calculated for each sample.

Statistical analysis

Statistica STATSoft version 10.0 was used to carry out the computations for all analyses. Statistical differences between experimental and control cultures were evaluated by Wilcoxon signed-rank test, and the p value less than 0.05 was considered as statistically significant.

Results

The influence of BENDA and RIT on the cytotoxicity of CLL cells

We demonstrated that BENDA used alone showed higher cytotoxic effect on CLL cells in 48 hour cultures than RIT ($p<0.01$) (Table 1). Furthermore, DICy calculated for the combination of both drugs was significantly higher than for BENDA used alone (23.4% vs 18.3%), whereas DICy for RIT was 5.5% only (Fig. 1A).

Induction of apoptosis and necrosis of CLL cells by studied drugs

We showed a significant predominance of apoptosis over necrosis, especially under BENDA ($p=0,015$) (Table 2). DIA were 12.9% and 16.1%, and DIN were 7.5% and 11.2% for BENDA and BENDA+RIT; respectively (Fig. 1B-C).

Evaluation of mitochondrial transmembrane potential changes ($\Delta\psi_m$)

A statistically significant decrease in $\Delta\psi_m$ was shown for both drugs and their combination as compared to the control culture ($p<0.001$) (Table 3). The strongest changes of $\Delta\psi_m$ were induced by BENDA or the combination of BENDA with RIT, with DI$\Delta\psi_m$ of 24.6%, 21.0% and 5.3%; respectively for BENDA, BENDA+RIT and RIT (Fig. 2A).

The influence of drugs on expression of active forms of caspase-9, caspase-3 and caspase-8

We demonstrated a statistically significant increase of expression of active forms of caspases (-9, -3 and -8) under the studied drugs or their combination as compared to the control culture (Table 3). The highest percentage of CLL cells with expression of the above mentioned caspases was observed after incubation with BENDA or BENDA + RIT compared to either controls or incubation with RIT alone. Additionally, BENDA and RIT used in combination statistically significantly increased the rates of cells expressing all caspases activity as compared to the effect of BENDA used alone (47.39% vs 43.96% for caspase-9; 46.79% vs 45.83% for casase-3 and 46.99% vs 43.73% for casase-8) (Table 4)(Fig. 2B).

The influence of drugs on the expression of apoptosis-regulating proteins

The percentage of cells expressing P53 protein after incubation with the drugs used alone or in combination was significantly higher as compared to the results obtained in the control culture. However, the percentage of P53+ cells after incubation with RIT was significantly lower as compared to the percentage of P53+ cells after incubation with BENDA used alone or BENDA with RIT (3.91%, 5.97% and 9.02%; $p<0.001$) (Table 5).

We demonstrated an increase of percentage of CLL cells with PUMA expression under the influence of BENDA

Table 1. The cytotoxic effect of bendamustine used alone or in combination with rituximab on CLL cells in culture *in vitro* after 48 hours cultures

Drug	Control	BENDA 40 µg/ml	RIT 10 µg/ml	BENDA 40 µg/ml + RIT 10 µg/ml	Statistical analysis (p)
	1	2	3	4	
n	63	63	63	63	1v2<0.001 1v3<0.001 1v4<0.001 2v3<0.001 2v4<0.001 3v4<0.001
X	18.65	37.34	23.89	42.41	
SD	10.41	17.61	12.62	17.15	
Range	3.5-54.7	8.1-79.3	5.93-68.1	11.2-79.7	
DICy (%)		18.25	5.52	23.41	

n- number of samples; x- mean percentage of dead cells (PI+); PI- propodium iodide; SD- standard deviation; DICy- drug induced cytotoxicity; BENDA- bendamustine; RIT – rituximab; v- versus

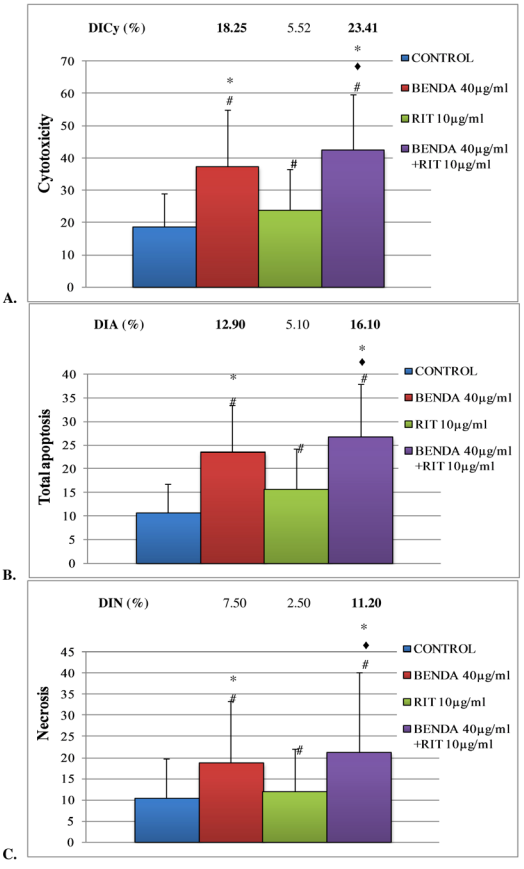


Fig. 1. The influence of BENDA used alone or together with RIT on cytotoxicity (A), apoptosis (B) and necrosis (C) of CLL cells in vitro
- difference statistically significant vs CONTROL; * - difference statistically significant vs RIT; ◆ - difference statistically significant vs BENDA; DICy- drug induced cytotoxicity, DIA – drug induced apoptosis, DIN – drug induced necrosis

used alone and BENDA+RIT. However, statistically significant differences were observed only for BENDA used alone (p=0.01) (Table 5). Furthermore, we observed that the drugs used alone significantly increased the percentage of BAX positive cells as compared to the control. Similarly, the drugs used in combination significantly increased the percentage of these cells as compared either to the control or RIT used alone (22.9%, 14.99% and 17.88%; respectively) (Table 5).

We observed a non-significant decrease in the percentage of BCL-2 expressing cells and also a non-significant increase in APAF-1 and FADD expressing cells under the influence of all the drugs tested as compared to the control (Fig. 3D-F).

Discussion

The observations of recent years indicate that BENDA, bifunctional chemotherapeutic drug with a unique mechanism of action, is highly effective in the therapy of patients with relapsed-refractory indolent non-Hodgkin lymphoma and CLL. In particular, BENDA is more and more widely used in the treatment of both front-line and relapsed-refractory cases, especially in aged and physically less fit patients, in whom fludarabine-based regimens cannot be administered. The pivotal phase III study of Knauf et al. [14] showed superiority of BENDA used in monotherapy over chlorambucil. However, in current clinical practice BENDA is frequently used in CLL in association with RIT [11], although a superiority of BENDA+RIT over BENDA in monotherapy has not been directly demonstrated. Therefore, it is justified to carry on studies aimed at proving, if addition of RIT to BENDA enhances antileukemic properties of the latter drug *in vitro*. It was demonstrated, that the cytotoxic response to benda-

Table 2. The influence of bendamustine used alone or in combination with rituximab on apoptosis and necrosis of CLL cells in vitro after 48 hours cultures

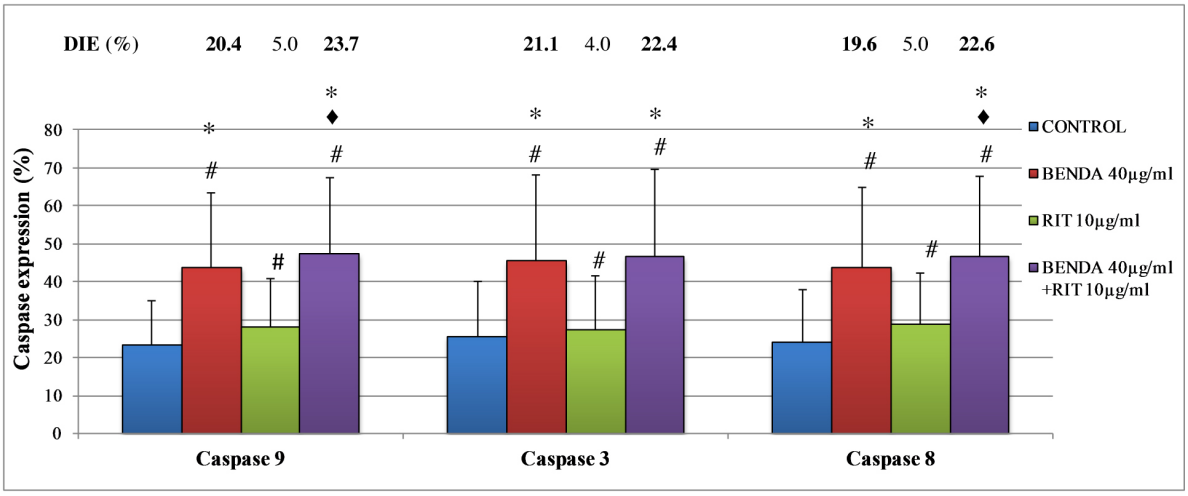
Drug	Control		BENDA 40 µg/ml		RIT 10 µg/ml		BENDA 40 µg/ml + RIT 10 µg/ml		Statistical analysis (p)
	Apoptosis A(+)/ PI(-/+)	Necrosis A(-)/PI(+)	Apoptosis A(+)/ PI(-/+)	Necrosis A(-)/PI(+)	Apoptosis A(+)/ PI(-/+)	Necrosis A(-)/PI(+)	Apoptosis A(+)/ PI(-/+)	Necrosis A(-)/PI(+)	
	1	2	3	4	5	6	7	8	
n	63	63	63	63	63	63	63	63	1v3<0.001; 1v5<0.001; 1v7<0.001; 2v4<0.001; 2v6=0.003; 2v8<0.001; 3v4=0.015; 3v5<0,001; 3v7<0.001; 4v6<0.001; 4v8<0.001; 5v6=0.044; 5v7<0.001; 6v8<0.001; 7v8=0.046;
X	10.78	10.41	23.57	18.93	15.68	12.06	26.78	21.41	
SD	5.95	9.39	10.00	14.52	8.60	10.08	11.18	18.78	
Ranges	0.20-29.95	1.10-45.00	5.60-52.30	0.79-63.60	5.00-45.71	0.77-49.20	6.86-56.84	0.79-68.60	
DIA/DIN (%)			12.93	7.49	5.11	2.48	16.13	11.21	

n- number of samples; x- mean percentage of cells ; A- annexin V; PI- propodium iodide; SD- standard deviation; BENDA- bendamustine; P- statistical analysis; v- versus; DIA- drug induced apoptosis, DIN- drug induced necrosis

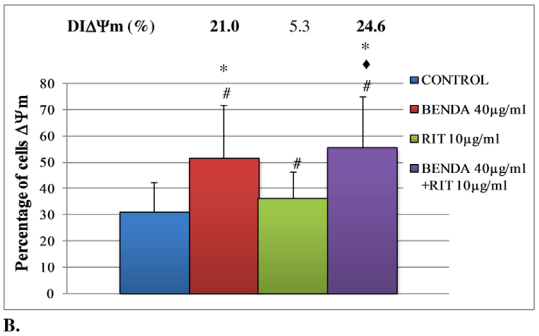
Table 3. The influence of bendamustine used alone or combination with rituximab of the both drugs on mitochondrial potential ($\Delta\psi_m$ low) of CLL cells *in vitro* after 48 hours incubation

Drug	Control	BENDA 40 µg/ml	RIT 10 µg/ml	BENDA 40 µg/ml + RIT 10 µg/ml	Statistical analysis (p)
	1	2	3	4	
n	63	63	63	63	
X	31.04	51.77	36.22	55.75	1v2<0.001
SD	11.28	19.91	12.70	19.46	1v3<0.001
Ranges	2.91-58.20	5.33-91.17	5.15-66.60	10.66-91.70	1v4<0.001 2v3<0.001 2v4<0.001 3v4<0.001
DI$\Delta\psi_m$ (%)		20.98	5.26	24.59	

n- number of samples; x- mean percentage of $\Delta\psi_m$ low cells ; SD- standard deviation; BENDA- bendamustine; RIT – rituximab; v- versus; DI$\Delta\psi_m$ – mitochondrial potential changes under influence of drug



A.



B.

Fig. 2. Caspases activity (A) and mitochondrial potential changes (B) under influence of BENDA used alone and in combination with RIT # - difference statistically significant vs CONTROL; * - difference statistically significant vs RIT; ♦ - difference statistically significant vs BENDA; DIE- drug induced expression, DI$\Delta\psi_m$ – drug induced mitochondrial potential changes

mustine is caspase- and P53-independent and results in induction of PUMA and NOXA expression without influencing the levels of BCL-2 and BAX [22,26]. So far, only few studies have been undertaken to understand the apoptotic pathways and complex mechanisms of action of BENDA combined with RIT on CLL cells *in vitro*. Studies of Chow et al. [5,6] and Rummel et al. [23] demonstrated that addition of RIT to the culture medium allows the reduction of the concentration of BENDA required to induce apoptosis in *ex-vivo* CLL cells. The authors suggested that this drug used with RIT shows synergistic effect against various leukemia and lymphoma cells and this effect does not depend on the presence of a complement. All these studies, however, were performed with FCS only used as a culture medium. Nevertheless, it is well known that apoptosis of CLL cells *in vivo* depends not only on the intrinsic abnormalities of leukemic cells, but also on the microenvironmental cellular and humoral factors, among them circulating cytokines. The effect of such factors on the viability of cultured cells may be mimicked by addition of AS to the culture medium. As a matter of fact, our previous studies addressing the effect of RIT on CLL

Table 4. The influence of bendamustine used alone or in combination with rituximab on caspase activity/expression *in vitro* after 48 hours cultures

Drug	Control			BENDA 40 µg/ml			RIT 10 µg/ml			BENDA 40 µg/ml + RIT 10 µg/ml			Statistical analysis (p)
	Caspase 9	Caspase 3	Caspase 8	Caspase 9	Caspase 3	Caspase 8	Caspase 9	Caspase 3	Caspase 8	Caspase 9	Caspase 3	Caspase 8	
	1	2	3	4	5	6	7	8	9	10	11	12	
n	65	65	65	65	65	65	65	65	65	65	65	65	1v4<0.001; 1v7<0.001; 1v10<0.001; 2v5<0.001; 2v8<0.001; 2v11<0.001; 3v6<0.001; 3v9<0.001; 3v12<0.001; 4v7<0.001; 4v10<0.001; 5v8<0.001; 6v9<0.001; 6v12<0.001; 7v10<0.001; 8v11<0.001; 9v12<0,001;
X	23.62	25.69	24.39	43.96	45.83	43.73	28.39	27.65	28.90	47.39	46.79	46.99	
SD	11.43	14.42	13.55	19.78	22.53	21.20	12.52	13.94	13.41	20.31	22.89	21.14	
Ranges	6.39-54.00	2.66-60.70	5.43-58.00	7.04-83.70	2.38-88.50	5.74-81.90	9.60-60.90	4.46-67.20	7.68-59.50	9.79-82.80	5.05-86.10	10.69-85.00	
DIE (%)				20.41	21.15	19.57	1.78	3.96	4.97	23.71	22.37	22.63	

n- number of samples; x- mean percentage of cells; A- annexin V; PI- propodium iodide; SD- standard deviation; DIE- drug induced expression; BENDA- bendamustine; RIT – rituximab; v- versus

Table 5. The influence of bendamustine used alone or in combination with rituximab on the percentage of CLL cells with P53, PUMA, BAX, BCL-2, APAF-1 and FADD proteins expression after 48 hours incubation

Drug 1		Control	BENDA 40 µg/ml	RIT 10 µg/ml	BENDA 40 µg/ml + RIT 10 µg/ml	Statistical analysis (p)
		2	3	4		
P53	n	48	46	45	51	1v2<0.001 1v3=0.001 1v4<0.001 2v3<0.001 2v4=0.013 3v4<0.001
	X	3.13	5.97	3.91	9.02	
	SD	3.11	5.57	3.45	8.32	
PUMA	n	27	27	28	28	1v2=0.01
	X	12.06	20.19	15.82	19.07	
	SD	8.83	16.78	13.47	14.09	
BAX	n	55	55	55	55	1v2<0.001 1v3=0.015 1v4<0.001 2v3<0.001 3v4<0.001
	X	14.99	21.17	17.88	22.88	
	SD	19.72	23.78	23.99	24.97	
BCL-2	n	45	50	49	47	NS
	X	34.86	32.94	30.78	32.82	
	SD	22.62	24.51	24.92	23.28	
APAF-1	n	27	28	29	29	NS
	X	11.29	15.15	13.68	15.10	
	SD	10.19	12.42	12.30	12.65	
FADD	n	30	30	30	30	NS
	X	16.64	18.31	22.11	22.11	
	SD	21.99	21.72	27.13	26.05	

n- number of samples; x- mean percentage of cells ; SD- standard deviation; BENDA- bendamustine; RIT - rituximab; NS – not significant, v- versus

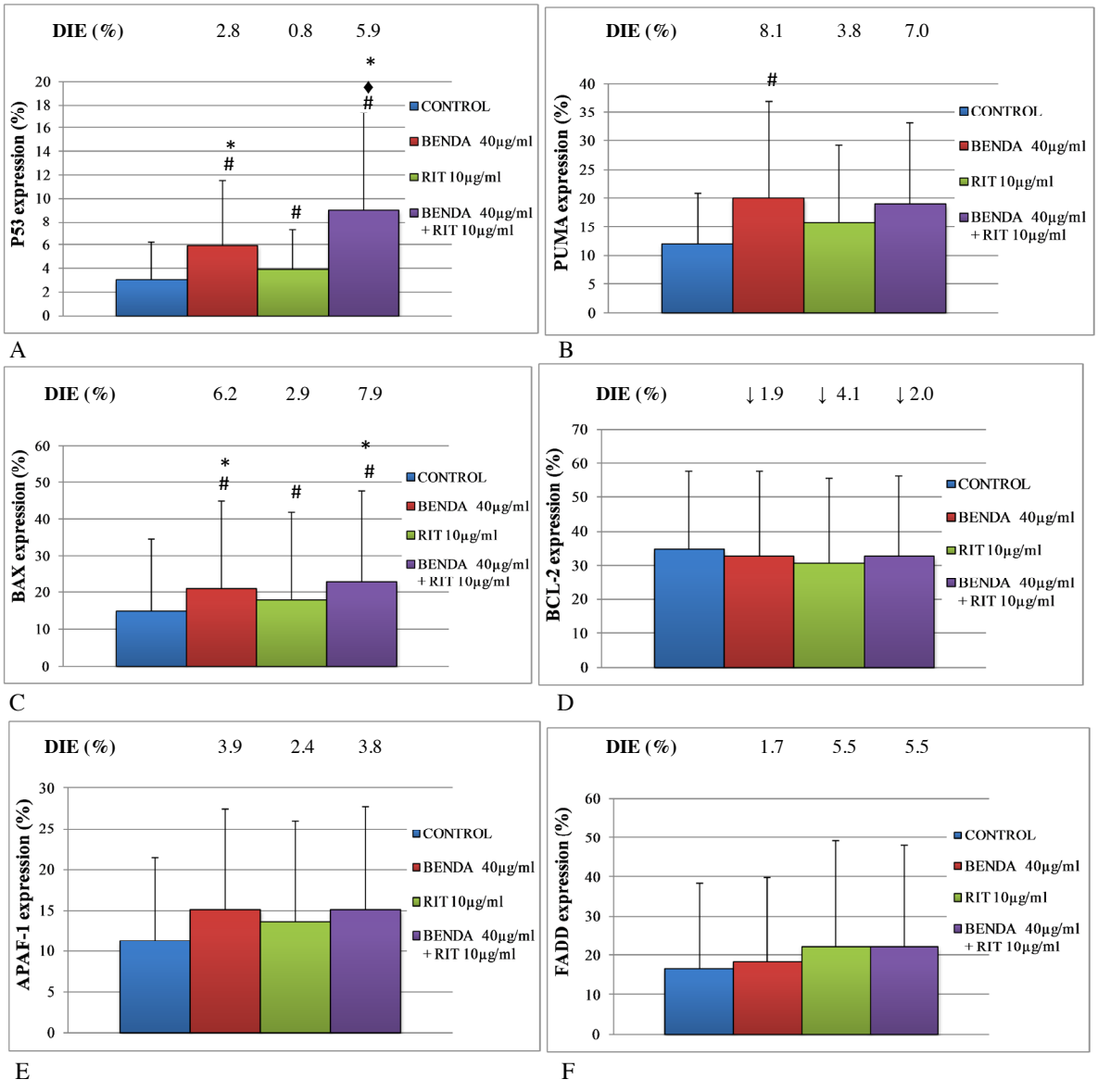


Fig. 3. Percentage of CLL cells expressing P53 (A), PUMA (B), BAX (C), BCL-2 (D), APAF-1 (E) or FADD (F) under influence of BENDA used alone and in combination with RIT *in vitro*

- difference statistically significant vs CONTROL; * - difference statistically significant vs RIT; ◆ - difference statistically significant vs BENDA; ↓ - decrease of protein expression

cells demonstrated an increase of viability *in vitro* of CLL lymphocytes cultured with AS, as compared to the FCS, and suggested that the effect of RIT on leukemic cells is dependent on some endogenous substances circulating in the patient's serum [31].

We then set out to determine the influence of BENDA, RIT and these drugs used in combination on apoptosis, necrosis and expression of several apoptosis-involved proteins in order to determine if the addition of RIT to BENDA enhances the proapoptotic effect of the latter drug in the presence of AS, i.e. under the influence of humoral factors which are likely to play a role in leukemic cells viability *in vivo*. As we showed in our previous study, a mixture 1:1 of AS and FCS influences the viability parameters in a similar way as the pure AS; therefore we choose for our experiments a combination of AS and FCS in order to reduce the quantity of serum to be obtained from patients' blood.

We demonstrated that the cytotoxic and proapoptotic effect of BENDA or RIT on CLL cells in 48 hour culture were higher than in the control culture. Moreover, the cytotoxic effect of BENDA was higher than of RIT, and BENDA+RIT significantly more potently induced cytotoxicity of CLL cells than BENDA used alone. In particular, we stated that the combination of BENDA+RIT increases both early (decrease in $\Delta\psi_m$) and late apoptosis (Ann-V binding), as well as necrosis of CLL cells. The proapop-

totic properties of BENDA and RIT, as well as the mutual enhancement of their proapoptotic action have already been reported by others authors [24,26]. In particular, Rummel et al. [23] reported that the introduction of RIT 10 μg/ml into the culture allows to reduce the concentration of BENDA to 20 μg/ml for the cell lines and 5 μg/ml for the isolated CLL cells. However, the novelty of our study as compared to reports published so far is that we were able to confirm, that the enhancement of proapoptotic action of BENDA and RIT takes place also in cultures supplemented by AS which contains humoral factors influencing the viability of leukemic cells in vivo.

We also showed that BENDA used either alone or in combination with RIT strongly induces the expression of several apoptosis-involved proteins, especially those participating in the intrinsic pathway of programmed cell death. This pathway consists in the participation of proapoptotic molecules such as P53, PUMA and BAX, the interaction of which leads to the mitochondrial transmembrane potential changes and to the release and activation of initiator caspase-9 and executioner caspase-3. Although RIT used alone induced significant changes in the percentage of $\Delta\psi_m$ cells and caspases expression as compared to control, BENDA and BENDA+RIT led to stronger than RIT and control $\Delta\psi_m$ changes and higher expression of caspases-9 and -3. It is of note that the expression of caspase 9 was induced in a significantly stronger degree by BENDA+RIT than BENDA alone. Thus intrinsic pathway of apoptosis seems to be more enhanced by this drug combination as compared to those agents used separately.

Bendamustine exposure leads to apoptosis via activation of P53-dependent and -independent pathways. Some studies demonstrated the induction of P53 protein and the activation of caspase-3 under BENDA and the synergistic effect of BENDA and RIT, whereas other authors suggested the P53-independent pathway [1,7,10,17,22,27]. Our results confirm the activation of P53, PUMA and BAX proteins as well as an increase of the activity of the caspases studied. The mean percentage of cells expressing P53 was the highest under the influence of BENDA+RIT, lower in the presence of BENDA and RIT alone and the lowest in control cultures. These results suggest that the combination of BENDA and RIT stimulate the P53 expression more strongly that those drugs used separately. The mean percentage of cells expressing PUMA was also higher in the cultures containing BENDA and BENDA+RIT as compared to RIT used separately but we did not observe any difference between BENDA and BENDA+RIT.

Studies on the expression of apoptosis-regulating proteins in CLL lymphocytes *ex vivo* and incubated for 48 hour with 2 μg/ml of BENDA were carried out by Schwanen et al. [26]. The authors found no significant changes in the expression of BCL-2 and BAX proteins under the influence of BENDA. They failed to demonstrate any differences in the expression of proteins tested probably due to insufficient concentration of BENDA. In our study BENDA alone and in combination with RIT induced BAX expression but we failed to show any modification of BCL-2 protein under the influence of these drugs. BAX activity leads to mitochondrial potential changes resulting in the release of cytochrome c from mitochondria to cytosol and its binding with apoptotic protease activating factor (APAF-1) and procaspase-9, which leads to formation of apoptosome. Although, as mentioned above, we demonstrated the induction of caspase-9 by BENDA and especially by BENDA+RIT, we did not observe any influence of these drugs on APAF-1.

Chow et al. [6] evaluated the influence of cytokines, complement, and caspases on the cytotoxic effect of RIT (10 μg/ml) and BENDA (0.1-200 μg/ml) on CLL cells. They found that RIT in the presence of complement sensitizes CLL cells to BENDA by the induction of expression of caspase-7 and -8, but not by P53 protein nor by caspase-3. RIT used alone induced the apoptosis only moderately, whereas its combination with BENDA in the presence of complement significantly potentiated this process. In contrast, in our study RIT significantly increased the percentage of cells expressing P53 protein as well as caspases -9, -3, and -8. It is probably due to different conditions of cultures, in particular to the presence of survival-regulating cytokines in the AS we used for our experiments.

We also evaluated the influence of drugs studied on the extrinsic apoptotic-pathway via active form of caspase-8 and FADD expression. We demonstrated statistically significant induction of caspase-8 activity under the influence of drugs without any influence on FADD expression. The induction of caspase-8 was significantly stronger under the influence of BENDA+RIT than these drugs used alone. Therefore, combination of BENDA and RIT seems to stimulate also the extrinsic pathway of apoptosis, but the mechanism of their involvement in this pathway remains obscure.

Conclusions

Our results indicate that both BENDA and RIT participate in the induction of cell death of CLL lymphocytes *in vitro* also in the presence of AS in the culture medium. The drug-induced apoptosis occurs mainly via intrinsic pathway, and via P53 and PUMA induction, however the extrinsic pathway is likely to be involved as well. We found that the association of these drugs induces the expression of P53, caspase-8 and -9 more potently than either of them used separately. This finding provides an element for understanding the mechanism of action of the combination of BENDA and RIT in the treatment of CLL, and justifies further studies on the molecular mechanisms underlying its effectiveness.

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