Induction of apoptosis in B-CLL cells by selected histone deacetylase inhibitors

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Abstract

Histone modifications influence the chromatin structure, changing the patterns of gene expression. Histone deacetylases (HDACs) comprise a group of enzymes governing acetylation status of N-terminal lysine residues of four core histones. Acetylation, the result of histone deacetylases inhibition, leads to cell cycle arrest and differentiation or apoptosis in neoplastic cells. The aim of the study was to induce apoptosis in B-cell chronic lymphocytic leukemia cells in vitro using histone deacetylase inhibitors. B-CLL cells isolated from peripheral blood of 30 patients were examined after 24 hour culture with histone deacetylase inhibitors: phenylbutyric acid and sodium butyrate. Control B-CLL cells were cultured either with dexamethasone (positive control of apoptosis) or media alone (negative control of apoptosis). Normal cells were also examined in this study: 6 specimens of B-lymphocytes isolated from tonsils and 6 specimens of peripheral blood lymphocytes isolated from healthy blood donors. All samples were treated in identical conditions. The number of apoptotic cells was assessed in a flow cytometer (BD FACScalibur) with the use of active caspase-3 apoptosis kit. Expression of P21 and HDAC1 genes was analysed using RT-PCR technique and compared to GAPDH gene expression. The level of histone H3 and H4 acetylation was determined with Western-blot analysis. Histone deacetylase inhibitors used in this study (phenylbutyric acid and sodium butyrate) induced apoptosis in B-CLL. In the cells treated with HDAC inhibitors the level of acetylated histones H3 and H4 increase along with P21 gene expression. These findings may be applied in future in vivo tests inducing apoptosis of neoplastic cells in patients with chronic lymphocytic leukemia.

Key words: *histone deacetylases (HDACs), phenylbutyric acid, sodium butyrate, apoptosis, B-CLL.*

(Centr Eur J Immunol 2011; 36 (1): 24-32)

Introduction

Histone deacetylases (HDACs) comprise a group of enzymes governing acetylation status of N-terminal lysine residues of four core histones [1]. Increase in acetylation level corresponds with loosened chromatine structure and increased gene expression, whereas deacetylation leads to more dense chromatin conformation and decreased accessibility for transcription factors [2]. Only 2-9% of genes expression patterns change when histone deacetylase inhibitors are used [3], about 7% of genes are deregulated in the absence of HDAC1 in embryonic mouse stem cells [4].

Histone deacetylase inhibitors constitute a heterogenous group including short chain fatty acids [5], phenylbutyric acid [6, 7], benzamides etc., which have the ability of restoring acetylation of histone tails. This ability is essential in neoplastic cells, in which deacetylation is one of features of malignant phenotype [8]. Acetylation, the result of histone deacetylases inhibition, leads to cell cycle arrest and differentiation or apoptosis [9].

The aim of the study was to induce apoptosis in B-cell chronic lymphocytic leukemia cells *in vitro* using histone deacetylase inhibitors: phenylbutyric acid and sodium butyrate. Expression of *P21* and *HDAC1* gene as well as histone H3 and H4 acetylation status were assesed.

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Material and methods

Cells isolation

B-CLL cells were obtained from peripheral blood of 30 previously untreated patients from Clinic of Hemato-oncology and Bone Marrow Transplantation of Medical University in Lublin (patients characteristic see Table 1). Control lymphocytes were obtained from 6 healthy blood donors and tonsils were derived from 6 patients of ENT Ward of District Railway Hospital in Lublin after routine tonsilectomy.

Culture

After isolation of lymphocytes by gradient density centrifugation on Ficoll, cells from each patient were divided into 4 culture dishes in concentration of 20 million cells for 10 ml of media. The culture media were prepared of 84 ml of RPMI with L-glutamine with 15 ml of bovine serum and 1 ml of antibiotic (1 mln of cristal penicylin and 1 g of streptomycin in 100 ml of PBS). They were incubated in Haereus incubator in 37˚C in 5% CO_2. After 24 hours histone deacetylase inhibitors were added to one of 4 cultures: phenylbutyric acid (Sigma) – 91 μM, sodium butyrate (Merck) – 255 mM as well as dexamethasone 1 mg/ml as a positive control of apoptosis. One of dishes was left as a negative control. After 24 hours of treatment cells from each dish underwent assessment.

Assessment of apoptosis

The assessment of apoptosis was performed with the use of Anti-Active Caspase-3 FITC Mab Apoptosis Kit (Becton Dickinson) – according to producers instructions.

From each of cultures 1 ml of suspension (approximately 2 mln cells) were taken and after standard producents procedure they underwent assessment in Flow Cytometer FACSCalibur (Becton Dickinson Immunocytometry System), equipped with argon laser 15 mW (488 nm). 1000 events for sample were evaluated. Obtained data were analysed by Cell-Quest software. Population of lymphocytes gated by two-dimensional dot-diagram was analysed in SSC (side scatter)/active caspase-3 system. Cells with active caspase-3 were defined as apoptotic ones. Results were given as percentage of positively dyed cells on the basis of monodimensional diagram (histogram) (Fig. 1).

RNA isolation

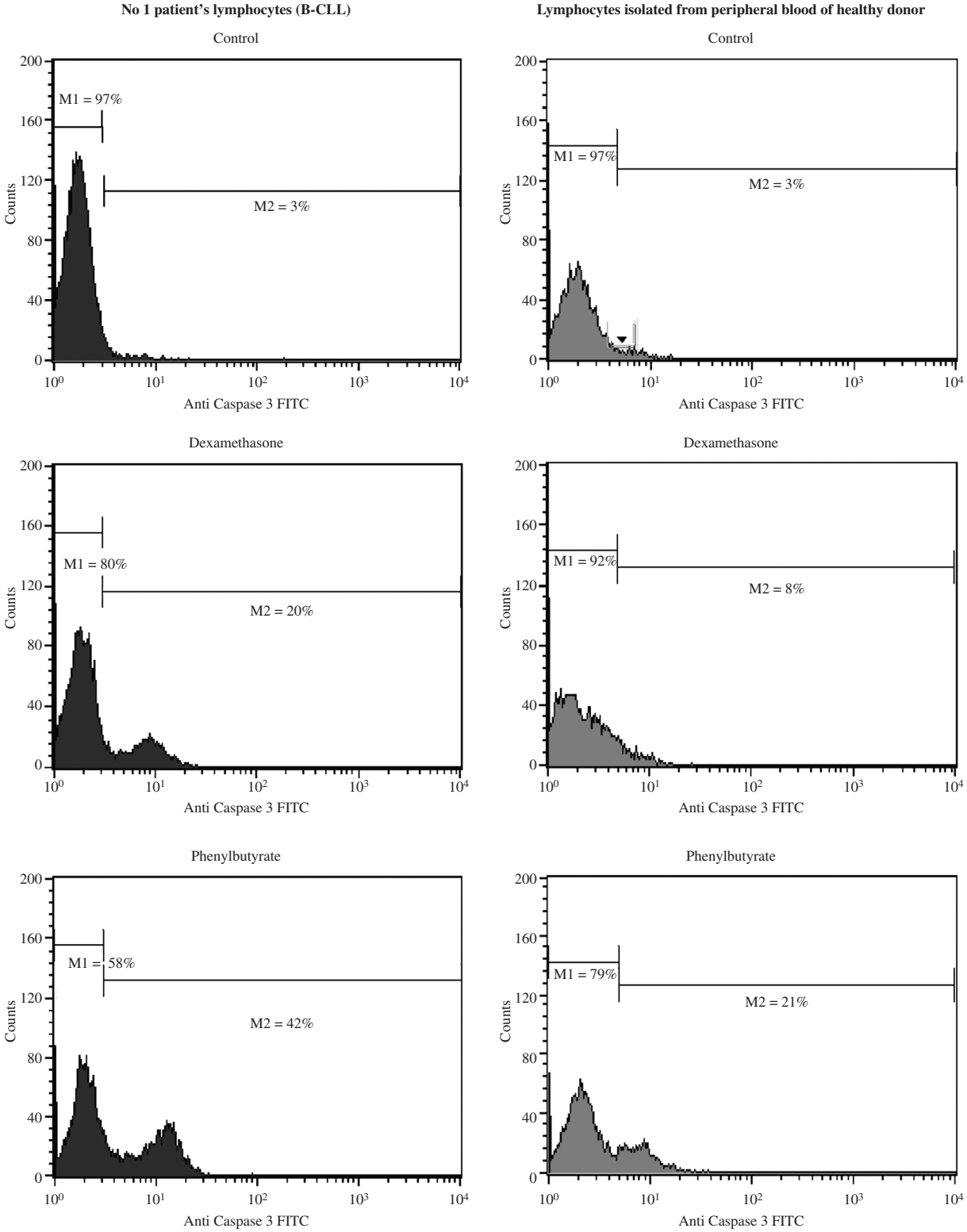
Total RNA isolation from lymphocytes was performed according to Chomczynski-Sacchi method [10], using 5 ml of suspension (approximately 10 mln cells) from each culture. Isolated RNA was stored in 700 μl of 75% Ethanol at –20˚C until PCR was performed. To assess the amount of RNA in each of samples pellets of RNA after centrifugation (14 000 rpm/13 min/4˚C) and collecting alcohol were dried for 10 min in room temperature. Then mRNA was resuspended in 11 μl of Rnase free water and 1 μl of the suspension underwent electrophoresis in 2% agarose gel with ethidium bromide. Quantitative RNA assessment was performed in comparison to pattern dilutions.

Reverse transcription

Reverse transcription was performed with the use of ImProm II Reverse Transcription System according to producers instructions. 3 μl of RNA solution after digestion with DNase and 1 μl of oligo dT starters and 1 μl RNase

Table 1. Characteristics of B-CLL patients

No.	Age	Sex	Rai stage	Lymphocytosis [10^3/mm^3]
1	48	F	2	87.8
2	77	F	2	87.1
3	54	M	2	11.4
4	69	M	0	26.3
5	64	F	1	–
6	74	F	2	–
7	80	M	3	51.8
8	74	M	1	88.288
9	66	M	3	176.76
10	70	M	1	22.6
11	64	M	0	46.4
12	52	M	1	23.9
13	48	F	2	80.1
14	57	M	2	83.2
15	63	F	2	8.49
16	44	M	0	12.02
17	65	M	1	32.4
18	64	M	2	–
19	55	M	2	92.6
20	80	M	1	45.8
21	35	M	1	11.3
22	70	M	3	12.8
23	70	M	0	15.4
24	54	M	2	21.3
25	76	F	2	92
26	69	M	2	37.89
27	71	F	1	42.2
28	61	M	2	262
29	58	M	–	177
30	57	M	1	14.1



X axe presents intencity of fluorescence, Y axe number of cells, M1 – population of active caspase-3 negative cells, M2 – population of active caspase-3 positive cells (apoptotic cells)

Fig. 1. Examplary histograms of cytometer gated populations of leukaemic and normal lymphocytes from different cultures: control, dexamethasone, phenylbutyrate and natrium butyrate

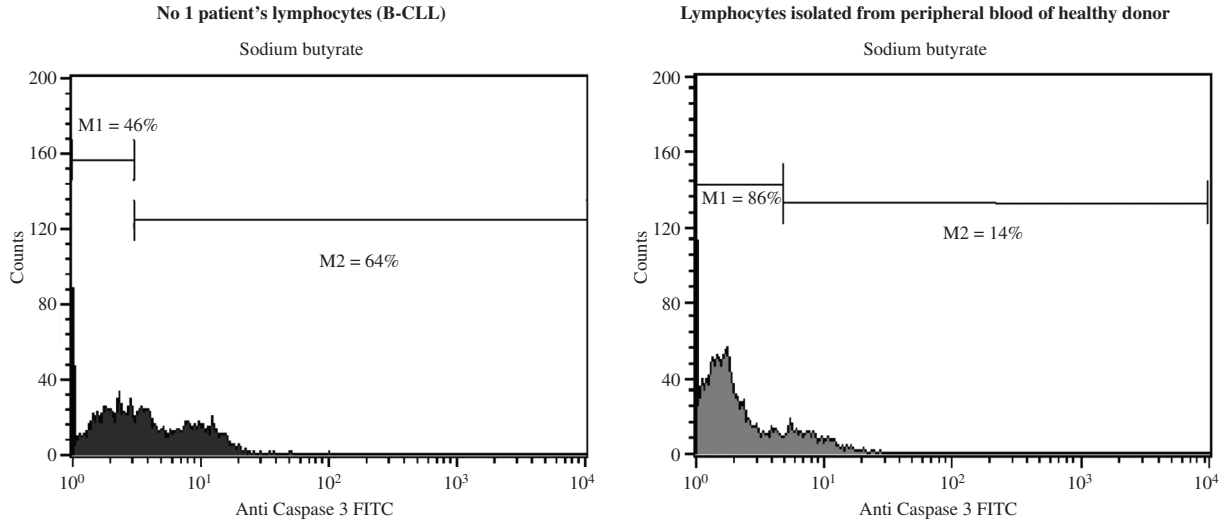


Fig. 1. cont.

free water were incubated in cycler for 5 min in 70°C and immediately cooled on ice. 4 µl of 5× concentraded reaction buffer, 3.2 µl $MgCl_2$ (25 mM), 1 µl 10 mM mixture of dATP, dGTP, dCTP, dTTP, 1 µl Rnasine 40 U/µl, 1 µl of reverse transcriptase 20 U/µl were added to the mixture and filled with Rnase free water up to 20 µl. Samples were placed in cycler in 25°C for 5 min, 42°C for 60 min and 70°C for 15 min. cDNA was stored at –20°C.

Polymerase Chain Reaction: Amplification of DNA was performed in 25 µl volume. 5 µl cDNA, 2.5 µl 10× concentrated PCR reaction buffer, 0.5 µl 10 mM of each deoxynucleotides: dATP, dGTP, dTTP, dCTP, 1.5 µl of each oligonucleotide starters (forward and reverse of *HDAC1*, *P21*, *GAPDH*; Table 2) in 50 pM/µl concentration, 0.5 µl 25 mM $MgCl_2$, 0.25 µl of 10 u/µl Taq DNA polymerase were used to this reaction. The samples were filled with Rnase free water up to 25 µl. Amplification was performed in Perkin Elmer Cycler in conditions presented in Table 3.

PCR products electrophoresis: after amplification PCR products were tested by electrophoresis in 2% agarose TBE gel. Amplified products were stained with ethidium bromide. Size marker pUC19MspI (Fermentas) was used as size of DNA fragments marker. Pictures of electrophoresis products were taken, scanned and intensity of fluorescence of each of strip was measured

Table 2. Starters used in the study

Gene	Type of starter	Starters' sequence	Reaction products' length [nt]
HDAC1	Forward	5' – AGA TAA CAT GTC GGA GTA CAG – 3'	264
	Reverse	5' – CTC TGG TGA TAC TTT AGC AG – 3'	
P21	Forward	5' – CCA CTG GAG GGT GAC TTC – 3'	284
	Reverse	5' – TGG TAG AAA TCT GTC ATG CTG – 3'	
GAPDH	Forward	5' – TGG TAG AAA TCT GTC ATG CTG – 3'	222
	Reverse	5' – GGA TCT CGC TCC TGG AAG – 3'	

Table 3. Amplification conditions

Gene	Denaturation	Starters attachment	Synthesis	No of cycles	Final elongation
HDAC1	94°C, 30 s	55°C, 30 s	72°C, 30 s	30	72°C, 10 min
p21	94°C, 30 s	57°C, 30 s	72°C, 60 s	29	
GAPDH	94°C, 30 s	58°C, 30 s	72°C, 40 s	30	

Table 4. Buffers, media and gels used in the study

Buffers' name	Buffers' ingredients
Cell culture	RPMI with glycine 84 ml
	Bovine serum 15 ml
	Antibiotic (1 000 000 units of cristal penicyline, 1 g and streptomycine in 100 ml PBS) 1 ml
TBE 5x	900 mM boric acid
	2 mM EDTA Na_2
	900 Mm Tris HCL, pH 8.0
PBS	136 mM NaCl, 3 mM KCl
Sample buffer for electrophoresis in SDS (sodium dodecyl sulphate)	0.5 M Tris HCl pH 6.8
	glicerol 2 ml
	10% SDS
	0.1% bromophenol blue 0.5 ml
	deionized water up to 6 ml
Electrode buffer	3.03 g Trisma Base
	18.7 g glycine
	10 ml 10% SDS
	destilled water up to 1000 ml
Transfer buffer	2.21 g CAPS
	62 mg DDT (DL-Dithiotreitol) – pH = 10.4
	100 ml methanol
	destilled water up to 1000 ml
Densifying gel (SDS)	Pro-Sieve 250 µl gel solution
	deionized water – 1.9 µl
	buffer pH 6.8 – 320 µl: 0.5 M Trisma base pH 6.8 0.4% SDS deionized water up to 100 ml
	Temed – 6 µl, APS (Ammonium peroxodisulfat) – 30 µl
Separating gel (SDS)	Pro-Sieve gel solution – 2.9 ml,
	Deionized water – 6 ml
	buffer pH 8.8 – 3 ml: 1.5 M Trisma base 0.4% SDS deionized water up to 100 ml Temed – 16 µl, APS (Ammonium peroxodisulfat) – 160 µl
Destaining solution for SDS gel	10 ml methanol
	10 ml acetic acid
	distilled water up to 100 ml

by TotalLab software. All results for *HDAC1* and *P21* genes were compared to relative GAPDH results. It allowed for semiquantitative assessment of analysed genes expression.

Western blot

Protein isolation – with the use of CelLytic M (Sigma) kit: From each of cultures 4 ml of suspension (approximately 8 mln cells) were taken. Protein isolation was performed according to producers instructions. The protein concentration in each sample was assessed by nefelometry in Specol 220. In order to do that 10 µl of protein supernatant was added to 1.5 ml of destilled water with 100 µl phosphoric acid solution with methanole (Bio-Rad Protein Assay). Protein concentration was measured at 597 nm wave length, with 1 mg/1 ml albumine solution extinction as a reference.

From each of samples 20 µg of protein volume was taken and mixed with 1/3 of volume of sample buffer with mercaptoethanol (20 : 1). Before electrophoresis the samples were denaturated for 3 min in boiling water (100˚C) and immediately cooled on ice. Gels were placed in Minipol device (Krzysztof Kucharczyk, Techniki Elektroforetyczne) in electrode buffer (Table 4). Marginal wells of the gels were filled with 10 µl of 1 mg/1 ml albumine solution in PBS and 5 µl of size marker (Page Ruler Prestained Ladder) respectively. Examined samples were put into remaining wells. Electrophoresis was performed with constant voltage 11 V/cm in densifying gel and 16.25 V/cm in separating gel at 4˚C.

After finished electrophoresis the gels were soaked in transfer buffer for 40 min. Membranes with pore size of 0.45 µm (Immobilon P Transfer Membrane) were cut to suit gel size (6 × 9 cm), soaked in methanol for 15 s, washed in deionized water for 2 min and soaked in transfer buffer for 10 min, as well as Blotting paper and sponges. Gels were than placed on membranes, covered with blotting papers and sponges from both sides and put in electrotransfer device (Minitrans Krzysztof Kucharczyk, Techniki Elektroforetyczne) in transfer buffer. Electrotransfer was performed for 15 hours at 4˚C with 30 V. After finished transfer gels were soaked in Coomasie blue solution (Brilliant Blue-G Concentrate) for 1 hour to asses the amount of protein left on gels after transfer and then put in destain solution to obtain strips pattern. The quality of transfer was estimated with the use of Ponceau solution, after cutting off fragments with transferred marker from the membranes. After dying marginal parts of the membranes with albumine were cut off, the remaining parts of membranes were cut according to needs. Membranes were destained in 0.1 M NaOH solution and washed for 5 min in running deionized water. They were dried for 2 hours at room temperature.

Reaction with antibodies: Dried membranes were placed in 1% albumine with 0.05% Tween in PBS solution with respective antibodies: polyclonal Rabbit IgG anti-acetyl-histone H3 and H4 at concentration 1 : 3000 and anti BCL-2

at concentration 1 : 50 as the control of the method. They were incubated for 1 hour at room temperature on shaker. After 3 times washing in PBS (15 s each) membranes were placed in secondary antibody against rabbit, mouse and goat conjugated with biotine at concentration 1 : 50 for 45 min, then washed (like previously) and put in streptavidine marked with alkalic phosphatase at concentration 1 : 50 for another 45 min. After another washing membranes were soaked in BCIP/NBT (alkalic phosphatase substrate) for 5-10 min. After strips appeared membranes were washed for 10 min in deionized water and then dried for 24 hours in room temperature.

Statistics software

Obtained data were statistically analyzed with Stastistica. The levels of examined features were characterized by median, minimum and maximum. The influence of apoptosis stimulators used in the experiment on examined cells was assessed by Wilcoxon's test.

Results

The number of apoptotic cells (active caspase-3 positive cells) was significantly higher in cultures with histone deacetylase inhibitors than in negative control according to Wilcoxon test ($p < 0.01$). Exemplary histograms of B-CLL and healthy donor cells after culture examined in cytometer using active caspase-3 antibody are presented on Figure 1. Median percentage of apoptotic cells in B-CLL cell cultures with phenylbutyric acid and butyric acid was 40.56% and 61.74%, respectively in comparison to 6.76% in control cultures without HDAC inhibitors. Median, minimum and maximum percentage of apoptotic cells in samples examined are presented in Table 5. B-CLL cells were more prone to apoptosis induced this way than normal cells. There were no significant differences in the number of apoptotic cells between samples from patients differing with respect to Rai stage or lymphocytosis.

Expression of *P21* gene increased following HDAC inhibitors treatment (Table 6) according to Wilcoxon test

Table 5. Medians of apoptotic cells percentage with minimum and maximum in examined cultures

Type of cultured cells	Parameter	Percentage of apoptotic cells			
		Culture type			
		Control	Dexamethazone	Phenylbutyric acid	Sodium butyrate
B-CLL lymphocytes	minimum	2.11	3.61	10.24	9.83
	median	**6.76**	**39.40**	**40.56**	**61.74**
	maximum	21.81	84.95	81.54	89.99
Tonsil lymphocytes	minimum	1.96	18.15	12.58	11.91
	median	**5.12**	**24.50**	**26.30**	**23.27**
	maximum	8.32	31.94	40.84	29.98
Peripheral blood lymphocytes	minimum	0.61	3.08	5.39	14.16
	median	**2.37**	**12.24**	**11.03**	**16.44**
	maximum	5.46	18.24	20.91	56.93

Table 6. Median of *P21* expression level as a percentage of *GAPDH* expression level with minimum and maximum

Type of cultured cells	Parameter	Percentage of *P21* expression level in relation to *GAPDH* [%]		
		Culture type		
		Control	Phenylbutyric acid	Sodium butyrate
B-CLL lymphocytes	minimum	5.2	14.3	15
	median	**48.8**	**68.6**	**74.5**
	maximum	100	146	143
Tonsil lymphocytes	minimum	28.5	38	81.25
	median	**46**	**78.5**	**96.5**
	maximum	62	195	112

Table 7. Median of *P21* expression level as a percentage of *GAPDH* expression level with minimum and maximum

Type of cultured cells	Parameter	Percentage of *HDAC1* expression level in relation to *GAPDH* [%]		
		Culture type		
		Control	Phenylbutyric acid	Sodium butyrate
B-CLL lymphocytes	minimum	15	23	10.3
	median	**67.9**	**60.5**	**61.9**
	maximum	107	106	106
Tonsil lymphocytes	minimum	70	47.6	69
	median	**105**	**87.5**	**90.4**
	maximum	121	157	105

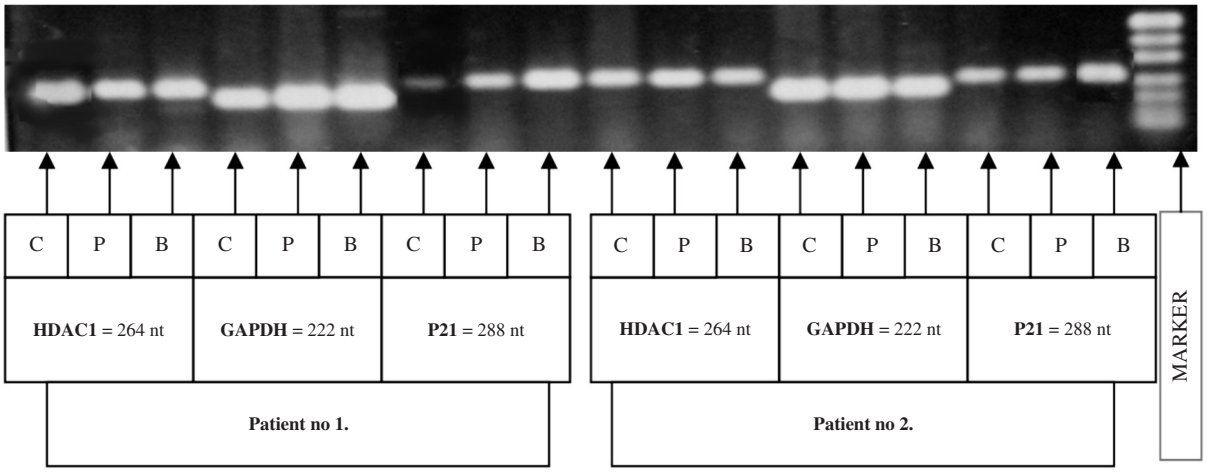


Fig. 2. RT-PCR product electrophoresis of B-CLL products after culture-control (C), and with histone deacetylase inhibitors (P – phenylbutyric acid, B – butyric acid). Products of HDAC1 and P21 in comparison to GAPDH amplification producs were presented

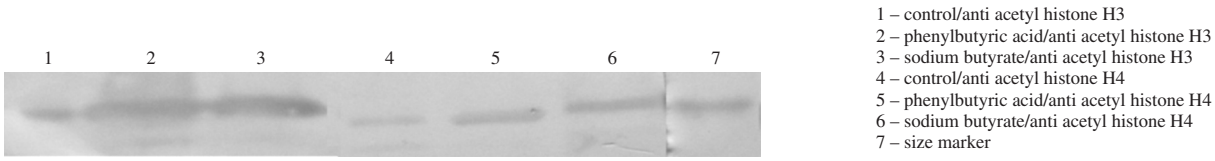


Fig. 3. Western blot analysis of protein isolated from PBL-B cells after culture with anti acetyl histone H3 and anti acetyl histone H4 antibody

at $p < 0.01$, these differences were statisticaly relevant. *P21* gene expression level reached 48.8% of GAPDH expression level in control cells and 68.6% and 74.5% in cultures with phenylbutyrate and sodium butyrate, respectively.

HDAC1 gene expression showed no statistically significant changes (Table 7) in cultures with HDAC inhibitor as compared with control ones.

Electrophoresis of exemplary product of RT-PCR of *P21*, *HDAC1* as well as *GAPDH* are shown on Figure 2.

Histone acetylation level of histones H3 and H4 was higher in cultures with phenylbutyric acid and sodium butyrate than in negative control (Fig. 3). This phenomenon was observed in both neoplastic and normal cells examined.

Discussion

B-CLL used to be considered to be a disease of immature, immune-incompetent, minimally self-renewing B cells, which accumulate because of a faulty apoptotic mechanism. Now B-CLL is viewed as two related entities, both originating from antigen-stimulated mature B lymphocytes, which either avoid death through the intercession of external signals or die by apoptosis, only to be replaced by proliferating precursor cells [11]. B-CLL cells are equipped with all elements of apoptotic pathways, but inproper caspase activation in these cells may be the reason for avoiding programmed cell death [12].

The mechanism of apoptosis activated by HDACs inhibitors may be dependent on the type of cell as well as the type of HDACs inhibitor. Thus it is so important to examine various types of these substances and their influence on neoplastic cells. Generally, it is assumed that HDACs inhibitors act through caspase activation [5, 9, 12-15], but apoptosis level is decreased, although not completely stopped by the use of caspase inhibitor zVAD-FMK, which may indicate, that there is an additional apoptotic pathway, probably dependent on mitochondrial apoptosis stimulating factor (AIF) [16].

In this study the percentage of apoptotic cells were significantly higher in cultures of B-CLL cells treated with HDACs inhibitors – sodium butyrate and phenylbutyric acid than in control ones. Relatively wide range of data (Table 6) may be explained by heterogeneity of B-CLL cell population. Additional analysis, in which samples were divided into subgroups according to Rai stage or lymphocytosis of B-CLL patients was performed, but no significant difference in apoptotic cells number was observed in these groups, what was previously described [17].

The proapoptotic properties of sodium butyrate and phenylbutyric acid on neoplastic cells were previously revealed [5, 18-20]. Similar tests on B-CLL cells with monosaccharide butyrate derivatives were performed by Santini *et al.* [21], with significantly lower concentration of butyrates required to induce apoptosis. The difference between our studies might come from the shorter period of culture in our experiment (24 hours), whereas Santini incubated B-CLL cells for 96 hours. The effective concentration of sodium butyrate in our study was set empirically and short term of culture was due to the need of histone acetylation status assessment.

The acetylation status of cells treated with HDACs inhibitors in this study was also analyzed. It was significantly higher in these cultures in comparison to non-treated ones. This effect was previously described in CEM-CSF cell line treated with sodium butyrate and TSA (trichostatin A) [5, 9], breast cancer cells line (MCF-7) [22], human myeloma cell line (MM1S) [23], Jurkat and HL-60 cell lines [24].

As it was already mentioned in introduction HDACs inhibitors influence expression of 2-9% of cellular genes [3]. While both HDAC inhibitors treatment and individual class I HDAC knock down produce significant transcriptional effects, three-times higher for HDAC inhibitors, the gene-expression profiles of class I HDAC KD compared with that obtained by HDACi treatment exhibited less than 4% of altered genes in common between the two modes of inhibition in HeLa cells [25].

In this study expression of *P21* and *HDAC1* was analyzed, showing significant increase of *P21* expression after treatment with both examined HDACs inhibitors. This gene product is a cycline dependent kinase inhibitor and it is able to stop the cell cycle. Similar results were obtained in Colo 320 and SW 1116 colon cancer cells lines [26], HT-29 [27], HepG2 hepatocellular carcinoma cell line [28] treated with sodium butyrate. Phenylbutyrate revealed similar effects on pulmonar epithelium with CFTR gene defect [29]. Chen *et al.* [30] proved increased acetylation level of H3 and H4 in the transcription start site of *P21* after treating colon cancer cell lines with sodium butyrate and TSA. In another study one of HDAC inhibitors – SAHA, induced, among others, increased expression of *P21* and acetylation of H3 in human prostate cancer cell lines LNCaP, DU145, PC3, and CWR22R [31].

In the absence of HDAC1, mouse embryonic fibroblasts scarcely undergo spontaneous immortalization and display increased *P21* expression. Chromatin immunoprecipitation assays demonstrate a direct regulation of the *P21* gene by HDAC1 in mouse embryonic fibroblasts [4].

In contrast to *P21* no significant changes in *HDAC1* expression occurred in the study. There were two factors increasing HDAC1 gene expression described in previous studies on Swis3T3 mouse cell lines: IL-2 and TSA, HDACs inhibitor [31]. Changes in expression of this gene may vary in different cells and thus need further analyses.

Further investigations should be performed on different genes expression as well as on apoptotic pathways induced by HDACs inhibitors. Their unique ability of selective induction of apoptosis in malignant cells with relatively low influence on normal ones may be a great advance in future tests *in vivo.*

Acknowledgments

Chair and Clinic of Hematooncology and Bone Marrow Transplantation of Medical University in Lublin as well as ENT Ward of District Railway Hospital in Lublin are gratefuly acknowledged for providing cellular material to the study.

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