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Marta Karp[1, B–D, F], Kamila Kosior[2, B–D], Agnieszka Karczmarczyk[1, D–F], Małgorzata Zając[1, D–F], Joanna Zaleska[1, D–F], Waldemar Tomczak[2, D–F], Sylwia Chocholska[2, D–F], Marek Hus[2, D–F], Anna Dmoszyńska[2, D–F], Krzysztof Giannopoulos[1, 2, A–F]

Cytotoxic Activity of Valproic Acid on Primary Chronic Lymphocytic Leukemia Cells*

[1] Department of Experimental Hematooncology, Medical University of Lublin, Poland
[2] Department of Hematooncology and Bone Marrow Transplantation, Medical University of Lublin, 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 article; **G** – other

Abstract

Background. Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in western civilization. The accumulation of CD5⁺CD19⁺ B lymphocytes in peripheral blood is due to a defect in the apoptotic pathway rather than excessive proliferation in the bone marrow and lymph nodes. Despite a number of treatments, CLL remains an incurable disease. Valproic acid (VPA) activity, as a histone deacetylase inhibitor, could restore the epigenetic changes underlying the pathogenesis of CLL and thus induce cell death.

Objectives. In the present study we hypothesized that VPA could induce CLL primary cells death through activation of apoptosis.

Material and Methods. Peripheral blood samples were obtained from 53 CLL patients. Peripheral blood mononuclear cells were isolated through density gradient centrifugation and were the subject of a 24-hour cell culture with 10 mM of VPA. The cytotoxic effect of VPA was evaluated with an XTT test and thereafter confirmed using Annexin V-FITC/PI staining and flow cytometry techniques.

Results. In this study, a median VPA cytotoxicity of 13.88% with a range of 0-54.65% was observed. Annexin V/PI staining confirmed that the demonstrated cytotoxicity was caused by increased apoptosis in the VPA treated cells as compared to control cells. Statistical analysis showed that VPA's effect on CLL cells depends on lactate dehydrogenase serum levels, but is independent of all other prognostic markers.

Conclusions. The results of the present experiments found that VPA at a clinically applicable concentration significantly induces apoptosis independently of the disease stage and might be a valuable therapeutic agent for all CLL patients (**Adv Clin Exp Med 2015, 24, 1, 55-62**).

Key words: apoptosis, CLL, chronic lymphocytic leukemia, VPA, valproic acid.

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the Western world. CLL mainly affects older people, the average age at the time of diagnosis is 72 years. It is a lymphoproliferative malignancy characterized by the accumulation of small, mature $CD5^{+}CD19^{+}$ B lymphocytes in the peripheral blood, bone marrow, lymph nodes and spleen [1–3]. It is a very heterogeneous disease – many patients never need treatment due to its indolent course, meanwhile in other cases it is highly aggressive and requires immediate therapy [4]. The clinical heterogeneity of CLL is a reflection of genetic and epigenetic abnormalities and might be predicted by several prognostic markers, including deletion of chromosomes 11q, 17p and 13q, trisomy 12, CD38 and

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zeta chain associated protein 70 (ZAP-70) expression as well as well as the mutational status of immunoglobulin variable heavy chain genes (*IGHV*) [5].

Despite the implementation of new immunochemotherapies, CLL remains an incurable disease and the group of patients with unfavorable prognostic factors responds poorly to treatment. Therefore, the need for the development of novel therapeutic options still exists. Lately, inhibitors of B cell receptor signaling were investigated as a targeted CLL treatment. An inhibitor of Bruton tyrosine kinase and an inhibitor of the PI3K pathway alone or in combination with monoclonal antibodies and/or standard chemotherapy induced a durable response in early clinical trials in CLL patients independent of adverse prognostic factors [6, 7]. Also, immunomodulating agents, including thalidomide and lenalidomide, successfully used in multiple myeloma (MM), have shown promising effects in CLL and are currently being studied in clinical trials [8–10]. In 2009 our team defined a thalidomide-induced molecular signature on CLL cells using gene expression profiling [11]. With the application of the Connectivity Map database, a comparison of the thalidomide signature with other anti-tumor drugs was performed and it revealed that thalidomide targets similar genes as valproic acid (VPA) – a histone deacetylase inhibitor (HDI). This finding provided a rationale to assess the cytotoxicity of VPA on CLL cells.

VPA (2-propylpentanoic acid) is a short-chain fatty acid commonly used as an anticonvulsant with recently identified HDI activity [12, 13]. Generally, VPA is well tolerated by patients, but might have a teratogenic effect when administered during early pregnancy [14]. The anti-tumor features of VPA have been reported in many types of malignancies, including breast and ovarian cancers, glioblastoma, neuroblastoma, colorectal cancer, prostate cancer, thyroid cancer, liver cancer, melanoma and many more [14, 15]. Microarray analysis of VPA's influence on various neoplastic cells revealed upregulation of many different genes encoding ribosomal proteins, oxidative phosphorylation proteins, adhesion molecules, and also sequences involved in the cell cycle, antigen presentation, apoptosis, PI3K and Wnt signaling and many more. The mechanism of action of the drug on particular neoplastic cells is characteristic and depends on the type of cancer [15]. VPA was also mentioned in hematological malignancies to induce differentiation and inhibit the growth of cells. In MM, VPA treatment induced G1 cell cycle arrest and apoptosis. Moreover, its anti-angiogenic effect on the bone marrow was observed on MM cell lines [16]. In acute myeloid leukemia cells and blasts, VPA inhibited proliferation, induced cell differentiation and apoptosis. On other human leukemic cell lines, including chronic myeloid leukemia and promyelocytic leukemia cells lines. VPA was shown to have anti-proliferative and proapoptotic activity by the stimulation both caspase-dependent and caspase-independent pathways [17, 18]. Taking into consideration that VPA has a similar molecular signature to thalidomide with lower toxicity, in this study we aimed to evaluate the anti-leukemic effect of VPA on CLL cells.

Material and Methods

Patients

Peripheral blood was obtained from 53 chronic lymphocytic leukemia (CLL) patients diagnosed and treated in the Department of Hematooncology and Bone Marrow Transplantation, Medical University of Lublin, Poland. The clinical characteristics of the patients are summarized in Table 1.

Table 1. Clinical characteristics of patients

Characteristic	No. of patients
Median age (years)	65
Range	47–84
Sex	
female	33 (62.26%)
male	20 (37.74%)
Rai stage	
0–I	32 (60.38%)
II–IV	14 (26.41%)
not available	7 (13.21%)
ZAP-70 (cut-off 20%)	
positive	12 (22.64%)
negative	29 (54.72%)
not available	12 (22.64%)
CD38 (cut-off 30%)	
positive	11 (20.75%)
negative	35 (66.04%)
not available	7 (13.21%)
IGHV mutational status	
mutated	6 (11.32%)
unmutated	6 (11.32%)
not available	41 (77.36%)
Cytogenetics	
del11q	2 (3.77%)
del13q	8 (15.09%)
del17p	3 (5.66%)
no changes	12 (22.64%)
not available	31 (58.49%)

Peripheral Blood Samples

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (Biochrom AG, Germany) density gradient centrifugation. The viability of cells was over 95% as determined by trypan blue staining and quantified in a Neubauer chamber (Zeiss, Germany). After isolation, the cells were used for XTT assay.

XTT Assay

The cytotoxic effect of VPA (Sigma-Aldrich Co, USA) was measured using an *in vitro* Toxicology Assay Kit, XTT based (Sigma-Aldrich Co, USA). All cells were suspended in X-VIVO w/o Phenol red and Genatamycin (Lonza, Switzerland). PBMCs were seeded on a 96-well plate at a concentration of 5×10^5 cells/100 µL/well. VPA was added to experimental wells at a final concentration of 10 mM. As a negative control, live cells were used. As a positive control, cells treated with 0.1% Triton X-100 (Sigma-Aldrich Co, USA) were used. 25 µL of XTT was added to all samples. The plates were incubated for 24 h in a humidified atmosphere with 5% CO_2 at 37°C. Optical densities (OD) were measured at 450 nm with a VICTOR3 1420 multi-label counter (PerkinElmer, USA), and a wavelength at 690 nm was used as a background. Each sample was done in triplicate. The cytotoxic effect was calculated as follows:

$$\text{cytotoxicity} = [1 - (OD_s - OD_b)/(OD_c - OD_b)] \times 100\%,$$

where: OD_s is the OD of an assayed sample, OD_b – the OD of a positive control and OD_c is the OD of live cells (negative control).

Apoptosis Analysis

For apoptosis analysis, an annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich Co, USA) was used according to the manufacturer's instructions. PBMCs of 3 exemplary CLL patients were treated with 10 mM of VPA, and live cells were used as a negative control. After 24-h incubation, the PBMCs were washed with PBS (Biochrom AG, Germany), suspended in a binding buffer provided and stained with 5 µL of annexin V-FITC and 10 µL of propidium iodide. The PBMCs were incubated for 10 min in darkness and immediately analyzed with a FACSCalibur (BD, USA).

Statistical Analysis

All results are presented as median values with range. The *U* Mann-Whitney test was used to evaluate the differences between subgroups of patients. Correlations of variables were calculated with the Spearman rank-correlation coefficient.

Results

VPA Inhibits Proliferation of CLL Cells

In order to evaluate the cytotoxic effect of VPA on CLL cells, an XTT test was performed on the freshly isolated PBMCs of 53 CLL patients. After a 24-h incubation of PBMCs with 10 mM of VPA, a median cytotoxicity of 13.88% was observed and it varied between individual cases (range: 0–54.65%). In 11 patients (20.75%), the obtained VPA cytotoxicity was over 30%. A cytotoxicity

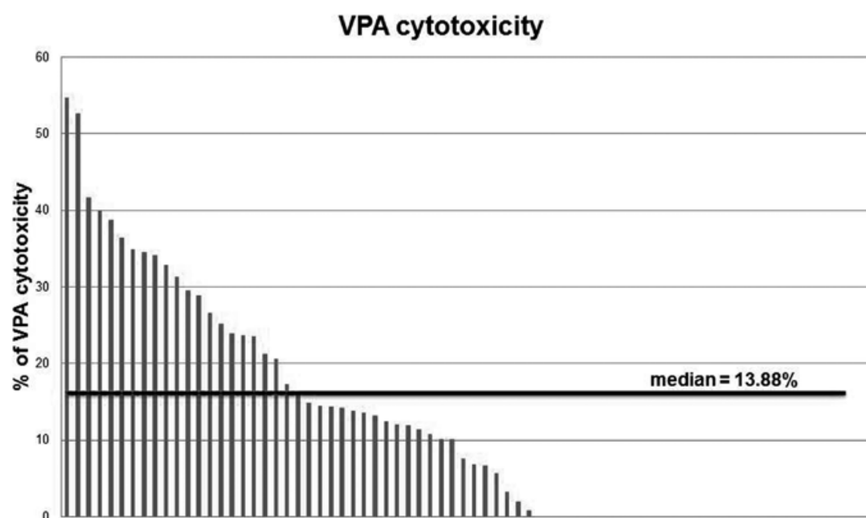

Fig. 1. VPA cytotoxicity after 24-hour treatment of PBMCs assessed with XTT test

between 20–30% was found in 9 patients (16.98%). In 16 patients (30.19%), the cytotoxicity ranged from 10% to 20%. In 17 patients (32.08%), the cytotoxicity observed was less than 10% (Fig. 1).

VPA Cytotoxicity Correlates with LDH Levels

To identify the patients who will most benefit from VPA treatment, we correlated the HDI response with prognostic factors. Statistical analysis showed a strong positive correlation of VPA cytotoxicity with lactate dehydrogenase (LDH) serum levels (p = 0.012; Table 2). There was no correlation between cytotoxicity and age, white blood cell count or β2-microglobulin levels (Table 2).

Furthermore, we separated patients into two subgroups depending on the stage of disease according to Rai classification. In the first subgroup, patients in Rai stage 0 and I were qualified (n = 32), in the second subgroup, patients in the advanced stages were enrolled – Rai stage II, III or IV (n = 14). Median cytotoxicity in the first analyzed group was 14.14% (range: 0–54.65%) and in the second group – 18.87% (range: 0–34.46%). There were no significant differences in the VPA cytotoxicity observed between the groups (p = 0.821; Fig. 2A). The VPA effect was shown to be independent of ZAP-70 expression. ZAP-70 positive patients (n = 12) had a median VPA cytotoxicity of 15.85% (range: 0–36.46%), meanwhile ZAP-70 negative patients (n = 29) had 13.22% (range: 0–54.65%; p = 0.547; Fig. 2B). No correlation

Table 2. Spearman rank-correlation between VPA cytotoxicity, age and blood parameters

Parameter	R Spearman	p value
LDH	0.363	0.012
β2-microglobulin	0.087	0.594
Age	0.039	0.792
WBC	0.017	0.910

WBC – white blood cells, LDH – lactate dehydrogenase.

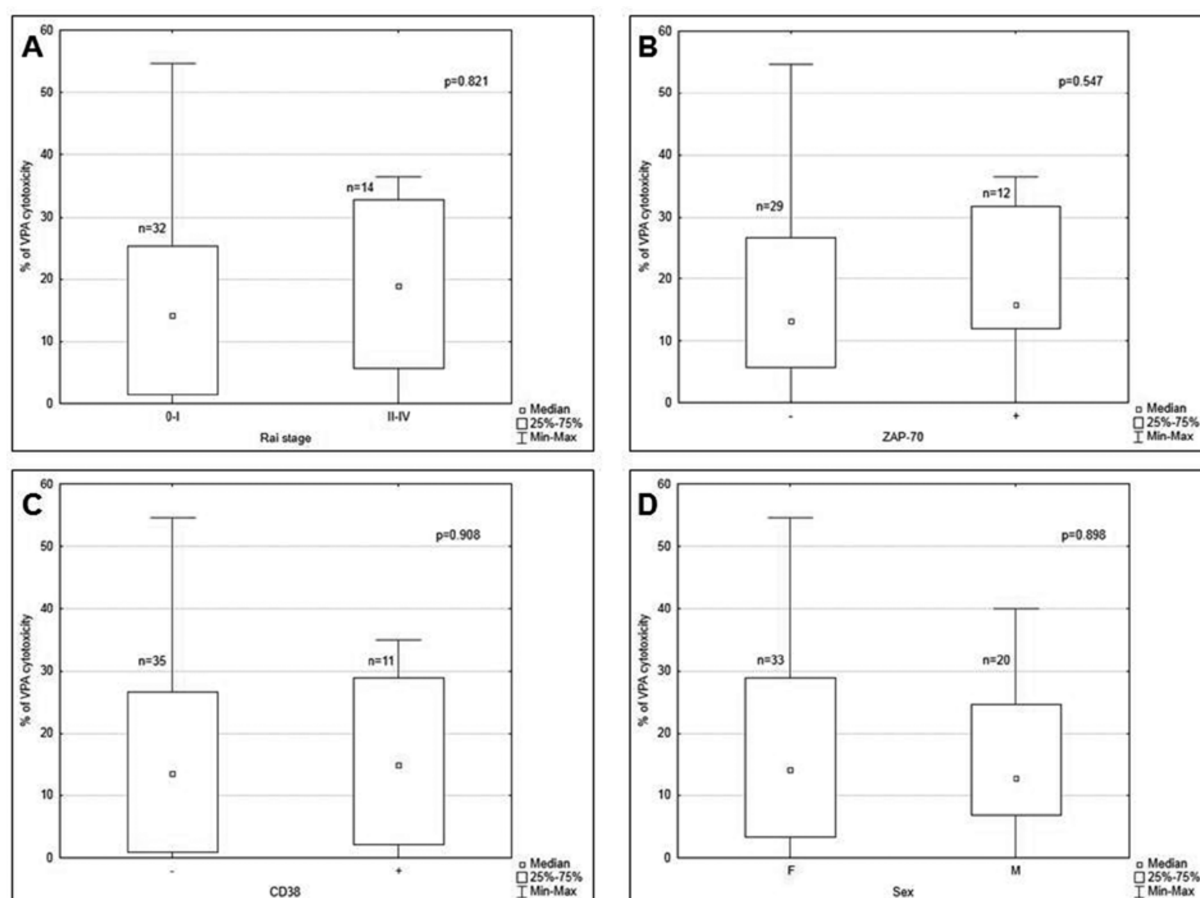

Fig. 2. The VPA response in different subgroups of patients evaluated with *U* Mann-Whitney test. The graphs show differences in VPA cytotoxicity (A) between early (0–I) and advanced (II–IV) groups of patients according to Rai classification; (B) among patients with positive (+) and negative (–) ZAP-70 expression; (C) between subgroups with positive (+) and negative (–) CD38 molecule expression; (D) depending on sex of patients; F – female; M – men

between VPA influence and expression of the CD38 molecule was found. Patients who were CD38 positive (n = 11) had a median cytotoxicity of 14.93% (range: 0–34.95%), similarly to CD38 negative patients (n = 5) with a median of 13.59% (range: 0–54.65%; p = 0.908, Fig. 2C). No differences in VPA response in regard to the mutational status of *IGHV* genes were observed. In the unmutated group (n = 6), median VPA cytotoxicity was 28.37% (range: 12.42–34.95%), meanwhile patients with mutated *IGHV* genes (n = 6) had a median of 14.45% (range: 0–38.78%; p = 0.200). Also, VPA response in the subgroups of patients with different cytogenetic abnormalities was evaluated. Mean cytotoxicity in patients with del11q (n = 2) was 6.61%, and in the case of del17p (n = 3), median VPA cytotoxicity was 16.46%. In the groups of patients with favorable cytogenetics, median cytotoxicity was as follows: del13q (n = 8) – 11.86%, normal karyotype (n = 12) – 16.11% of VPA cytotoxicity. The differences in the VPA cytotoxic effect in regard to patient sex were also calculated. In the group of women (n = 33), median cytotoxicity was 14.20% (range: 0–54.65%), meanwhile in men (n = 20) – 12.88% (range: 0–40.01%; p = 0.898, Fig. 2 D).

VPA Induces Apoptosis of CLL Cells

To confirm the results obtained in the XTT test, the PBMCs of 3 exemplary patients after 24-hour VPA treatment were analyzed using annexin V/PI staining by flow cytometry. The percentage of live cells (R3) in the treated cells was decreased in comparison to control cells in all analyzed examples: 34.83% vs 62.22% in patient #1, 63.93% vs 86.92% in patient #2 and 45.74% vs 86.55% in patient #3, respectively (Fig. 3). The percentage of apoptotic cells (R2 + R4) before and after incubation with VPA was respectively 1.09% vs 41.09% in patient #1, 8.47% vs 21.60% in patient #2 and 3.55% vs 36.82% in patient #3 (Fig. 3).

Discussion

The effectiveness of VPA as a HDI has already been reported in several solid tumors and hematologic malignancies [14–18]. In the present study, we found that VPA induces apoptosis at a concentration of 10 mM independent of the disease stage according to the Rai system, ZAP-70 and CD38 expression, *IGHV* status and cytogenetics, but it depends on LDH serum levels. This indicates that

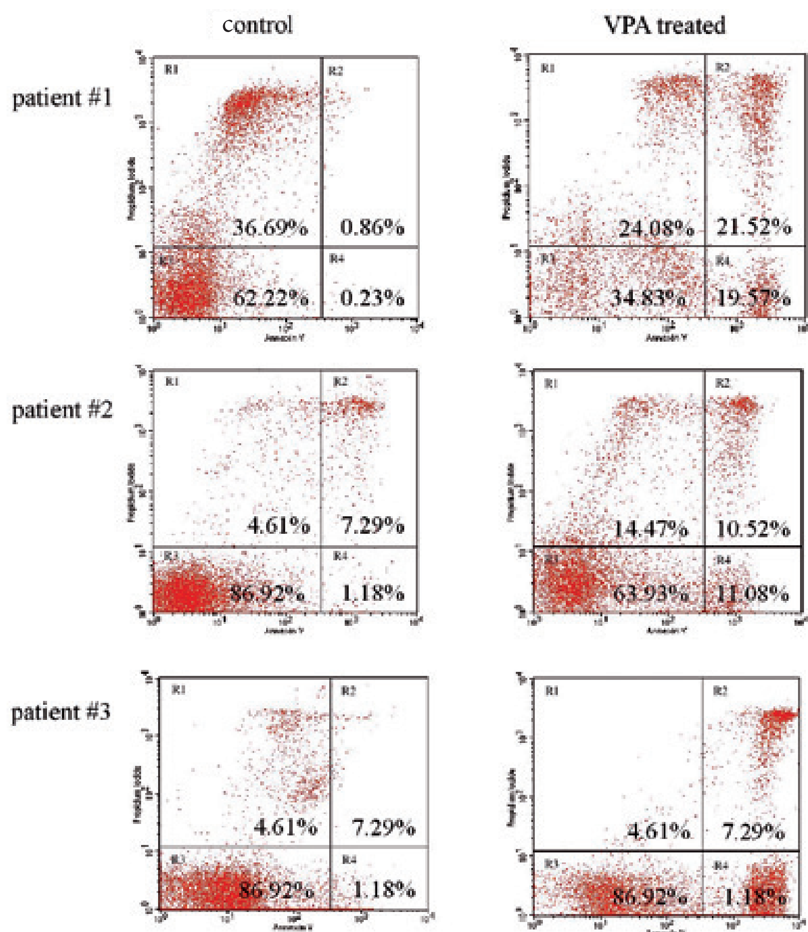

Fig. 3. Apoptosis analysis with annexin V/PI staining of 3 exemplary CLL patients. Cells were treated with 10 mM of VPA for 24 h, after incubation period flow cytometric analysis of annexin V/PI double-stained was performed. In each graph, the lower left quadrant (R3) indicates viable cells, the upper left quadrant (R1) represents necrotic cells, the lower right quadrant (R4) shows early apoptotic cells, and the upper right quadrant (R2) represents late apoptotic cells

VPA might be used in clinical practice for the treatment of a wide variety of CLL patients regardless of adverse prognostic factors and resistance to other therapies.

It has already been shown that VPA increases acetylation of histones H3 and H4 [19]. Kawagoe et al. [17] demonstrated that in a B-myeloid cell line, VPA activates two different apoptotic pathways – a caspase-dependent pathway causing nuclear apoptotic changes and a caspase-independent pathway [17]. Apoptosis might also be caused by surface receptors through an intrinsic pathway induced by a member of the tumor necrosis factor family – TRAIL (TNF-related apoptosis inducing ligand). Although many studies have shown that CLL cells are resistant to TRAIL induced apoptosis, Lagneaux et al. [12] demonstrated that 1 mM of VPA sensitizes CLL cells to TRAIL activated apoptosis. At first it was thought that the TRAIL resistance of leukemic B cells was caused by a low expression of caspase 8 mRNA. Now it is known that the resistance is more complex. VPA-induced upregulation of caspase 8 mRNA levels leading to activation of an extrinsic apoptosis pathway has already been highlighted [12, 19]. In VPA treated cells, there is downregulation of *C-FLIP* gene encoding cFLIP (cellular FLICE (FADD-like IL-1β--converting enzyme)-inhibitory protein), one of the major antiapoptotic regulator proteins, and this underexpression might lead to sensitization of CLL cells to TRAIL induced apoptosis [12, 20].

Apart from the above-mentioned *C-FLIP* downregulation and caspase 8 mRNA upregulation, VPA has been proven to deregulate the expression of other genes involved in tumorigenesis. Microarray analysis of leukemic cells after VPA treatment showed significant downregulation of *BCL2, BCL-XL, XIAP, AVEN cIAP, AKT* and *ATM*, whereas *BCLA1, APAF1* and *TP53* as well as genes encoding caspases 8, 2, 3, 6, 9 and 10 were overexpressed. It has been speculated that VPA-induced restoration of the normal expression of these key pro- and antiapoptotic genes renews apoptosis in CLL cells via extrinsic and intrinsic pathways [21, 22]. This explains the apoptosis after VPA therapy regardless of the prognostic factors shown in this study.

The VPA cytotoxic effect reported here appears to be caused not only by an extrinsic pathway mediated by caspase 8, but as proven by Bokelmann and Mahlknecht [13], additionally by a caspase 9 intrinsic pathway. According to their findings, caspase 8 activation was the first occurrence of VPA impact that led to the activation of a mitochondrial pathway by BID protein, then caspase 9 and finally induced apoptosis of CLL cells [13]. Engagement of caspase 9 into VPA-induced apoptosis clarifies the overexpression demonstrated by Stamatopoulos et al. of mRNA sequences encoding caspase 9 [21].

Although VPA *in vitro* induced apoptosis, as proven in this paper, clinical trials have shown that VPA used in monotherapy is well tolerated, but not highly effective. Therefore, the synergistic effect of VPA with other commonly used drugs is under investigation. At present, a combination of VPA with fludarabine, cladribine, bortezomib, flavopiridol, thalidomide and lenalidomide has been found to significantly induce CLL cell death [19, 21–23]. According to Bouzar's et al. [19] findings, VPA together with fludarabine or cladribine, both purine nucleoside analogues (PNA) commonly used in CLL therapy, stimulated both intrinsic and extrinsic apoptotic pathways and increased production of reactive oxygen species (ROS). The correlation between ROS production, DNA damage and apoptosis has already been observed in several other cancers [19]. The synergistic effect of VPA and fludarabine has been demonstrated to depend on ROS accumulation, independent of death receptors. This interaction partially involves AKT and ATM pathways. Reduced AKT phosphorylation has led to downregulation of prosurvival pathways, and at the same time, a reduction of ATM has been shown to increase DNA damage and more effective cytotoxicity of PNA [21, 22]. Recently, Yoon et al. [23] reported upregulated cathepsin B levels after *in vitro* and *in vivo* VPA treatment. Cathepsin B is a protease known to mediate in lysosome membrane permeabilization (LMP)-associated cell death that is a phenomenon induced by several anticancer drugs. As was already proven, CLL cell death induced by VPA alone or in combination with fludarabine involved apoptosis, therefore the degradation of antiapoptotic proteins through cathepsin B might be a novel therapeutic strategy for CLL patients that will make it possible to reduce chemoresistance [23]. All these observations, together with the present study, support VPA's effectiveness on CLL cells.

In conclusion, our report proves that VPA at a well-tolerated concentration of 10 mM has a cytotoxic effect on CLL primary cells as it induces apoptosis and inhibits cell proliferation. We have shown that VPA induces apoptosis irrespective of the aggressiveness of the disease. Therefore, VPA should be considered as a therapeutic single agent or in combination with other drugs for a wide variety of CLL patients.

References

[1] **Ghia P, Ferreri AJ, Caligaris-Cappio F:** Chronic lymphocytic leukemia. Crit Rev Oncol Hematol 2007, 64, 234–246.

[2] **Giannopoulos K:** Biologia i rokowanie w przewlekłej białaczce limfocytowej. Acta Hematol Pol 2010, 41, 433–440.

[3] **Robak T:** Czynniki prognostyczne w przewlekłej białaczce limfatycznej. Acta Hematol Pol 2003, 34, 395–405.

[4] **Abbot BL:** Chronic lymphocytic leukemia: recent advances in diagnosis and treatment. Oncologist 2006, 11, 21–30.

[5] **Zenz T, Mertens D, Dohner H, Stilgenbauer S:** Importance of genetics in chronic lymphocytic leukemia. Blood Rev 2011, 25, 131–137.

[6] **Robak T, Robak P:** BCR signaling in chronic lymphocytic leukemia and related inhibitors currently in clinical studies. Int Rev Immunol 2013, 32, 358–376.

[7] **Kharfan-Dabaja MA, Wierda WG, Cooper LJN:** Immunotherapy for chronic lymphocytic leukemia in the era of BTK inhibitors. Leukemia 2014, 28, 507–517.

[8] **Giannopoulos K, Mertens D, Stilgenbauer S:** Targeting chronic lymphocytic leukemia with thalidomide and lenalidomide. Expert Opin Pharmacother 2011, 12, 2857–2864.

[9] **Chen CI:** Lenalidomide alone and in combination for chronic lymphocytic leukemia. Curr Hematol Malig Rep 2013, 8, 7–13.

[10] **Skórka K, Bhattacharya N, Własiuk P, Kowal M, Mertens D, Dmoszyńska A, Giannopoulos K:** Thalidomide regulation of NF-κB proteins limits Tregs activity in chronic lymphocytic leukemia. Adv Clin Exp Med 2014, 23, 25–32.

[11] **Giannopoulos K, Dmoszyńska A, Kowal M, Wąsik-Szczepanek E, Bojarska-Junak A, Roliński J, Dohner H, Stilgenbauer S, Bullinger L**: Thalidomide exerts distinct molecular antileukemic effects and combined thalidomide/fludarabine therapy is clinically effective in high-risk chronic lymphocytic leukemia. Leukemia 2009, 23, 1771–1778.

[12] **Lagneaux L, Gillet N, Stamatopoulos B, Delforge A, Dejeneffe M, Massy M, Meuleman N, Kentos A, Martiat P, Willems L, Bron D:** Valproic acid induces apoptosis in chronic lymphcytic leukemia cells trough activation of the death receptor pathway and potentiates TRAIL response. Exp Hematol 2007, 35, 1527–1537.

[13] **Bokelmann I, Mahlknecht U:** Valproic acid sensitizes chronic lymphocytic leukemia cells to apoptosis and restores the balance between pro- and antiapoptotic proteins. Mol Med 2008, 14, 20–27.

[14] **Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG, Heinzel T:** Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 2001, 24, 6969–6978.

[15] **Duenas-Gonzales A, Candelaria M, Perez-Plascencia C, Perez-Cardenas E, de la Cruz-Hernandez E, Herrera LA:** Valproic acid as epigenetic cancer drug: preclinical, clinical and transcriptional effects on solid tumors. Cancer Treat Rev 2008, 34, 206–222.

[16] **Kaiser M, Zavrski I, Sterz J, Jakob C, Fleissner C, Kloetzel PM, Sexer O, Heider U:** The effects of the histone deacetylase inhibitor valproic acid on cell cycle, growth suspension and apoptosis in multiple myeloma. Haematologica 2006, 91, 248–251.

[17] **Kawagoe R, Kawagoe H, Sano K:** Valproic acid induces apoptosis in human leukemia cells by stimulationg both caspase-dependent and -independent apoptotic signaling pathways. Leuk Res 2002, 26, 495–502.

[18] **Tang R, Faussat AM, Majdak P, Perrot JY, Chaoui D, Legrand O, Marie JP:** Valproic acid inhibits proliferation and induces apoptosis in acute myeloid leukemia cells expressing P-gp and MRP1. Leukemia 2004, 18, 1246–1251.

[19] **Bouzar AB, Boxus M, Defoiche J, Berchem G, Macallan D, Pettengell R, Willis F, Burny A, Lagneaux L, Bron D, Chatelain B, Chatelain C, Willems L:** Valproate synergizes with purine nucleoside analogues to induce apoptosis of B-chronic lymphocytic leukaemia cells. Br J Haematol 2008, 144, 41–52.

[20] **MacFarlane M, Inoue S, Kohlhaas SL, Majid A, Harper N, Kennedy DBJ, Dyer MJS, Cohen GM:** Chronic lymphocytic leukemic cells exhibit apoptotic signaling via TRAIL-R1. Cell Death Differ 2005, 12, 773–782.

[21] **Stamatopoulos B, Meuleman N, De Bruyn C, Mineur P, Martiat P, Bron D, Lagneaux L:** Antileukemiac activity of valproic acid in chronic lymphocytic leukemia B cells defined by microarray analysis. Leukemia 2009, 23, 2281–2289.

[22] **Yoon JY, Ishdorj G, Graham BA, Johnston JB, Gibson SB:** Valproic acid enhances fludarabine-induced apoptosis mediated by ROS and involving decreased AKT and ATM activation of B-cell-lymphoid neoplastic cells. Apoptosis 2014, 19, 191–200.

[23] **Yoon JY, Szwajcer D, Ishdorj G, Benjaminson P, Xiao W, Johnston JB, Gibson SB**: Synergistic apoptotic response between valproic acid and fludarabine in chronic lymphocytic leukaemia (CLL) cells involves the lysosomal protease cathepsin B. Blood Cancer J 2013, 3, 153.

Address for correspondence:

Department of Experimental Hematooncology
Medical University of Lublin
Chodzki 4a
20-093 Lublin
Poland

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