Valproic Acid Sensitizes Chronic Lymphocytic Leukemia Cells to Apoptosis and Restores the Balance Between Pro- and Antiapoptotic Proteins

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Chronic lymphocytic leukemia (CLL) is one of the most common leukemias in adults in the developed world. Despite significant advances in the treatment of cancer, CLL remains incurable. The main feature of the disease is the generation of circulating B-cells with prolonged survival caused by aberrant apoptosis. In this study, we observe that valproic acid (VPA), a well-established histone deacetylase (HDAC) inhibitor, mediates apoptosis in CLL cells ex vivo through caspase activation via both the extrinsic and the intrinsic apoptosis pathways, as indicated by the activation of the caspase proteins 8 and 9, and cleavage of the proapoptotic protein BID. The Bcl-2/Bax ratio was decreased as a consequence of decreased bcl-2 mRNA levels in response to treatment with VPA. With the results presented in this study, we have identified the HDAC inhibitor VPA as restoring the apoptotic pathways in CLL cells and thus their ability to undergo apoptosis.

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INTRODUCTION

B-cell chronic lymphocytic leukemia (CLL) is one of the most common leukemias in adults. The disease is characterized by the accumulation of monoclonal $CD5^+$ B lymphocytes arrested in the G_0/G_1 phases of the cell cycle. The primary pathogenic event that causes the generation of aberrant B cells remains to be determined, but impaired apoptosis results in increased survival of circulating CLL cells. In contrast to most other hematological malignancies, CLL cells have a negligibly low fraction of growing cells. The clinical course of the disease is highly variable and ranges from indolent disease requiring no treatment over years to highly aggressive disease that needs immediate therapy. So far, all accepted treatment regimens for CLL are neither curative nor associated with prolonged survival (1). CLL therefore presents unique challenges to the development of new treatment strategies.

Apoptosis can be induced via two main pathways: interference with mitochondrial function, also called the “intrinsic pathway,” or triggering of cell surface death receptors, also called “the extrinsic pathway” (2,3). The intrinsic pathway is usually triggered by stress or chemotherapeutic agents and is regulated by the balance of pro- and antiapoptotic members of the Bcl-2 family of proteins. The extrinsic or receptor-dependent pathway is induced by members of the tumor necrosis factor (TNF) family of proteins, where a ligand binds to one of the death receptors that subsequently recruit the death-inducing signaling complex (DISC) and the apical caspase 8 (4,5). Activation of apical caspases then leads to cleavage and activation of the effector caspases 3, 6, and 7 and subsequently to apoptosis (6).

Some cell types produce insufficient amounts of active caspase 8 at the receptor level. Thus, the induction of apoptosis via the extrinsic pathway requires additional cleavage of the proapoptotic Bcl-2 family member BID through caspase 8, which activates the intrinsic pathway and subsequently apoptosis (type II cell mechanism) (7,8).

For decades, chemotherapy was considered to be the only approach in the treatment of patients with advanced or accelerated CLL. Nonspecific cytotoxic drugs such as alkylating agents or purine analogs have commonly been used to induce apoptosis via the intrinsic pathway. The use of chemotherapy in CLL is limited, however, because of rapidly developing resistance and partial unresponsiveness of patients with p53 abnormalities (9,10). Therefore, the implementation of novel, highly selective, and less toxic agents gains more and more importance in the treatment of CLL.

Valproic acid (VPA) is a short-chain fatty acid that belongs to a relatively new class of agents used for anticancer

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Table 1. Patient characteristics and individual response in vitro.

Patient	Age, years	Sex	Karyotype[a]	Prognosis[b]	Previous therapy[c]	Leukocytes/ nL	Spontaneous apoptosis[d]	VPA effect[e]
1	75	M	Normal	p	+	44	+	0
2	71	F	13q-	g	-	16	0	++
3	79	F	NA	g	-	122	0	+
4	56	F	13q-	g	-	146	+	++
5	74	M	NA	g	-	16	0	++
6	75	M	13q-	p	+	261	+	++
7	67	M	Normal	g	-	79	++	++
8	75	M	13q-	p	+	128	+	+
9	72	M	Normal	p	-	58	+	+
10	64	M	13q-, 17p-	p	+	83	+	+
11	68	F	Normal	p	+	98	+	++
12	75	M	+12	g	-	27	0	0
13	57	M	Normal	g	+	278	0	++
14	71	M	NA	p	+	11	+	++
15	72	F	13q-	g	-	19	+	++
16	81	F	NA	p	-	385	++	++
17	59	F	13q-	g	-	43	++	++
18	68	F	NA	g	-	50	0	++
19	54	M	Normal	g	-	53	0	+
20	66	M	NA	p	-	61	++	++
21	65	M	+12	g	-	35	++	++
22	67	M	+12, 17p-	p	-	81	0	+
23	67	M	+12	p	-	43	+	++
24	66	M	+12	g	-	45	0	++
25	66	M	13q-, 11q-	g	-	188	0	++
26	73	M	17p-	p	-	87	+	++
27	73	M	13q-	g	-	55	0	+
28	41	M	Normal	p	-	68	++	++
29	65	F	Normal	g	-	12	+	++
30	75	M	13q-, 11q-	g	-	39	+	+

Fifty patients were assessed (34 male, 16 female, median age 67.4 years, age range 41 to 81 years); 34 patients never had any form of CLL-specific treatment. In 16 patients, the last CLL treatment course was at least 3 months before this analysis. In 30 patients, annexin V/PI staining was done. NA = not available.

[a]Determined by classic cytogenetics or FISH; normal = no aberrations detected.

[b]g, favorable prognosis (Binet A, Rai 0/I); p, unfavorable prognosis (Binet B/C, Rai II/III/IV).

[c]Previous CLL-specific treatment with purine analogs, alkylating agents, or antibodies.

[d]FACS analysis after 4 days in the absence of treatment; 0, negative control: <30% apoptotic cells; +, 30%-50% apoptotic cells; ++, >50% apoptotic cells.

[e]FACS analysis after 4 days of treatment with 1 mM VPA.

therapy, the histone deacetylase inhibitors (HDAC-I). VPA has been used as an anticonvulsant and mood-stabilizing drug for decades. Even when taken over a long time, VPA is usually well-tolerated, although it is contraindicated during pregnancy because of its teratogenic effects (11). HDAC-Is exert pleiotropic antitumor effects by inducing growth arrest, differentiation, and apoptosis, both in vitro and in vivo (12). Growth inhibition goes along with cell cycle arrest as a consequence of transcriptional activation of p21WAF1/CIP1 and p27KIP1 and/or inhibition of cyclin A, cyclin D, and thymidylate synthetase (13). In malignant cells, HDACIs induce apoptosis by upregulation of proapoptotic and repression of antiapoptotic genes (14-16). In this study, we investigated the effect of VPA on CLL cells ex vivo. It was our goal to further elucidate the effects of VPA on apoptosis in these cells.

MATERIALS AND METHODS

Patients and Control Group

Peripheral blood samples were obtained from 50 patients (34 men, 16 women, median age 67.4 years) with B-CLL and from 5 healthy donors (2 men, 3 women, median age 32.2 years). Informed consent was given. The patient characteristics are given in Table 1.

Isolation of PBMCs and Cell Culture

PBMCs were isolated from heparinized peripheral blood through density gradient centrifugation (Biocoll Separating Solution, Biochrom, Berlin, Germany) and washed in PBS without Ca and Mg (Cambrex, Verviers, Belgium). Cells were then incubated at a density of 1 to 4 × 10^6/mL in RPMI 1640 (2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% FBS) at 37°C, 5% CO_2. VPA (Orfiril; Destin Arzneimittel, Hamburg, Germany) was added in a single dose immediately after resuspension of the cells.

Flow Cytometry

Cells were washed in PBS (+10 % FBS) and stained with anti-CD3 (BD Pharmingen), anti-CD19 (DAKO), annexin V-Fluos (Invitrogen, Karlsruhe, Germany), or propidium iodine according to the manufacturer's instructions. FACS analyses were carried out on a FACScan (Becton Dickinson, Heidelberg, Germany).

Histone Acetylation Assessment

Histones were isolated by acidic extraction from CLL cells after 72 h of cultivation as indicated. SDS-PAGE and immunoblotting were carried out according to standard protocols. Histone acetylation was assessed by anti-acetylhistone H3 and anti-acetylhistone H4 antibodies (both Upstate/Millipore, Lake Placid, NY, USA).

Western Blot

Cells were lysed in no-salt buffer (50 mM HEPES, pH 7, 1% NP40, 100 μg/mL PMSF) and stored at -80°C until usage. Total protein in each sample was quantified with Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA). SDS-PAGE and immunoblotting were performed according to standard protocols. Caspase 8 and 9 antibodies were obtained from Santa Cruz (SC-6134 and SC-17784; Santa Cruz, Heidelberg, Germany). The anti-BID antibody was from Cell Signaling Technology (Danvers, MA, USA). For the detection of BCL-2, BAX, and BAK, we used the Apoptopak Miniature Set (Upstate/Millipore). The quantification of Western bands was done with the TotalLab software (Phoretix); the relative protein amount was normalized to actin.

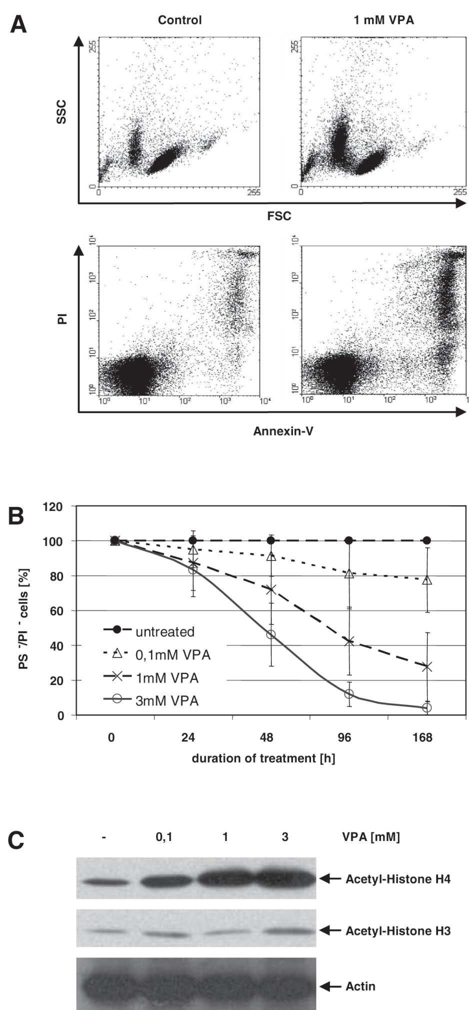

Figure 1. VPA-mediated cell death in CLL cells. Mononuclear cells isolated from peripheral blood of CLL patients were treated as indicated and analyzed by FACS. (A) Dot blot analyses are shown for FSC/SSC and for annexin V/PI for a representative patient analyzed after 48 h of culture. (B) Cell viability was monitored for 30 patients over 7 days at different concentrations of VPA. The graphs display the decrease in viable cells (annexin V^-/PI^-) under VPA treatment normalized to untreated cells. VPA-treated CLL cells show increased histone acetylation. (C) PBMCs from a CLL patient were left untreated (-) or were cultivated in the presence of different VPA concentrations (0.1, 1, 3 mM) for 48 h. Histone extracts were subjected to Western blot analysis using acetylation-specific antibodies for histones H4 and H3. Equal loading of gels was documented with anti-actin antibody.

Real-Time PCR

Total RNA was prepared with the RNeasy Mini Kit (Qiagen). Up to 10 μg total RNA was applied in the high-capacity cDNA archive kit (Applied Biosystems) for the generation of cDNA. mRNA expression of *bcl-2*, *bid*, and *bax* was measured with the TaqMan Gene Expression Assays (Applied Biosystems). The thermal cycling conditions were 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Real-time PCR was performed in triplicate on 96-well plates

using the ABI Prism 7700 Sequence Detector (Applied Biosystems). Results were then analyzed with the ABI Prism 7700 sequence detection system software (Applied Biosystems). Each reaction was normalized to *gapdh* expression, and relative expression was calculated with the ΔCt method.

Assessment of Caspase Activity

CLL-PBMCs were isolated and cultured in the presence or absence of VPA and the inhibitors of caspase-8/FLICE, Z-IETD-FMK; caspase-9/mch6, Z-LEHD-FMK (all BioVision, Mountain View, CA, USA) for 2 days. Inhibitors were diluted in DMSO, so negative control with DMSO alone was included; concentrations in culture were 50 µM for Z-IETD-FMK and Z-LEHD-FMK and 150 µM for Z-VAD-FMK. Caspase activity was determined with Caspase-Glo 3/7 Assay (Promega, Heidelberg, Germany) according to the manufacturer's instructions. Identical counts of intact cells were ensured by counting of resuspended cells with Trypan blue using a Neubauer hemocytometer.

Statistical Analysis

The amount of viable cells was determined by FACS, and mRNA levels were detected by real-time PCR and compared by Student *t* test (two-sided). Significant differences were analyzed between either absolute fractions of untreated versus VPA treated Annexin$^-$/PI$^-$ cells or corresponding Ct values normalized to *gapdh*.

RESULTS

VPA Mediates Cell Death in CLL Cells

To determine the in vitro effect of VPA on CLL cell viability, cells from 30 patients were incubated with different concentrations of VPA for up to 7 days. Cell viability was determined after 24 h, 48 h, 4 d, and 7 d by annexin V/PI FACS analysis. Figure 1a shows cells from a representative patient after culture for 48 h (± 1 mM VPA). Under both culture conditions, increased externalization of phosphatidylserine (PS) was observed

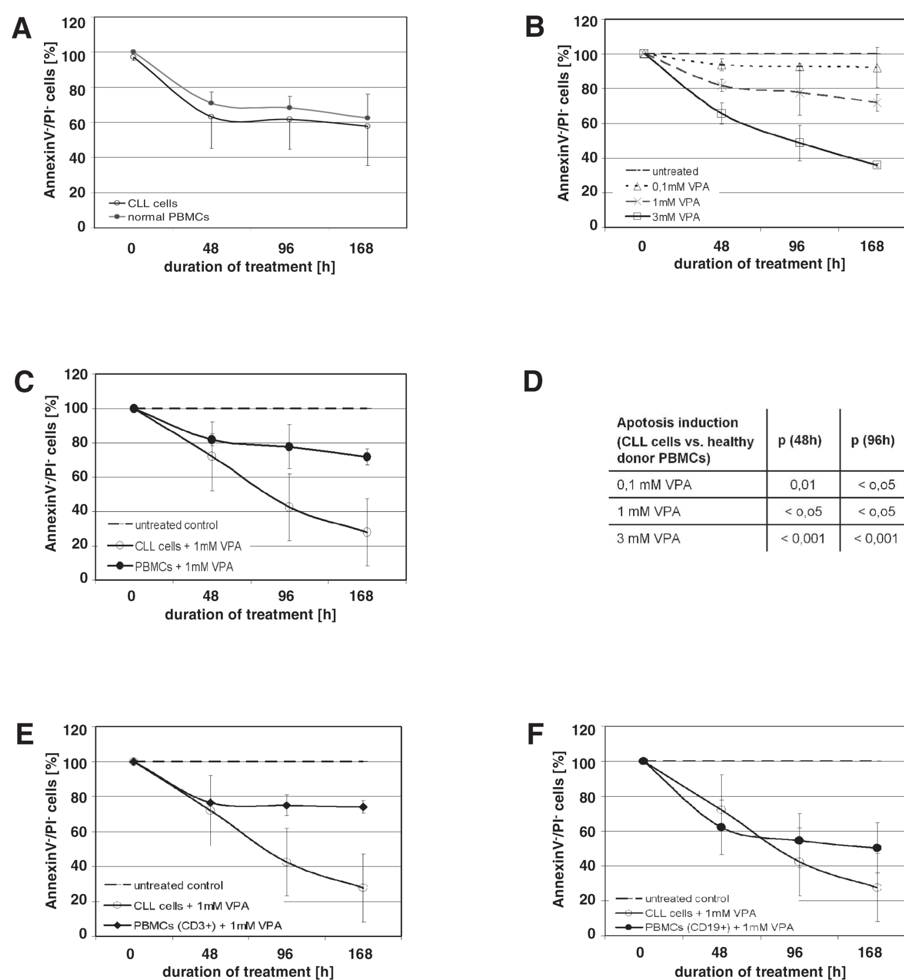

Figure 2. Impact of VPA on the viability of PBMCs from healthy donors. (A) PBMCs from 5 healthy donors and from CLL patients were isolated, and spontaneous apoptosis was assessed by FACS. The diagram shows the relative amounts of viable cells (annexin V$^-$/PI$^-$) monitored over 7 days. (B) The graphs display the decrease in viable cells (annexin V$^-$/PI$^-$) from healthy donors monitored over 7 days of treatment in the presence of absence of different VPA concentrations normalized to untreated cells. (C) VPA-dependent apoptosis at 1 mM VPA was compared in PBMCs from CLL patients and healthy donors. The diagram presents the relative amounts of viable cells (annexin V$^-$/PI$^-$) normalized to untreated cells. (D) The table shows the significances (*P* values) determined for VPA-dependent apoptosis induction in CLL cells versus PBMCs from healthy donors. The viability of CD3$^+$ cells (E) and CD19$^+$ cells (F) from healthy donors during VPA treatment was compared with similarly treated CLL cells.

due to spontaneous apoptosis; however, the number of PS$^+$ cells was significantly higher under VPA treatment. In a time course assay, the number of viable cells (PS$^-$/PI$^-$) under VPA treatment was calculated after subtraction of the number of cells that underwent spontaneous apoptosis in the untreated control. The first signs of increased numbers of apoptotic cells under VPA were seen as soon as 24 h. After 48 h of VPA treatment, we identified increased numbers of apoptosis, with 72% viable cells at 1 mM and 46% viable cells at 3 mM. After 96 h, the differences in cell viability were even more obvious, with 42.4% viable cells at

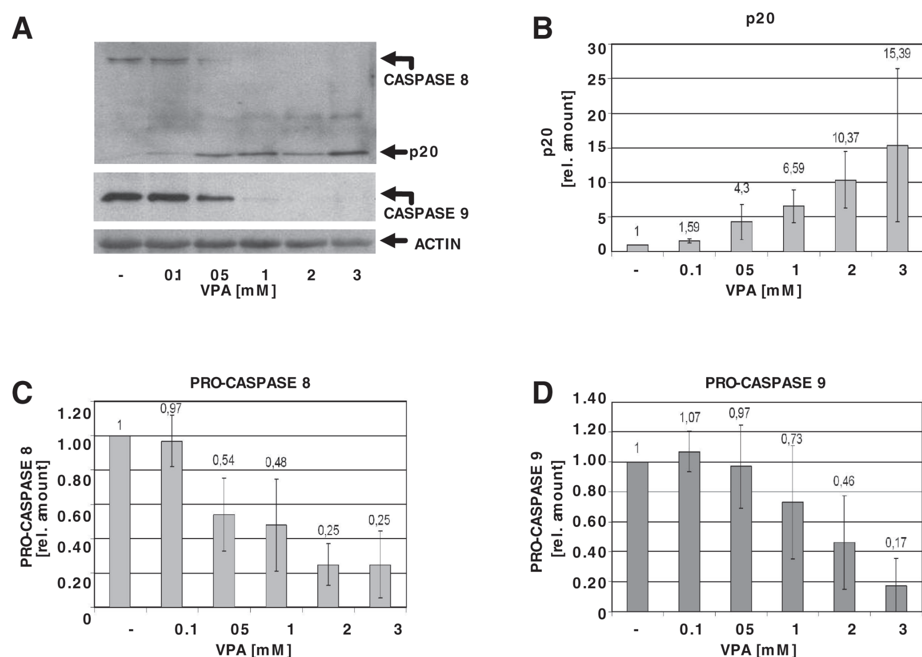

Figure 3. VPA-induced apoptosis is caspase dependent. (A) PBMCs from a representative CLL patient were cultured either in the presence or absence (-) of VPA (0.1, 0.5, 1, 2, 3 mM) for 3 days. Levels of caspases 8 and 9 were assessed from cellular lysates by Western blot. Equal loading of gels was documented with anti-actin antibody. Relative intensities of protein bands for p20 (activated caspase 8) (B) pro-caspase 8 (C), and pro-caspase 9 (D) were quantified with the TotalLab software and normalized to actin. Quantifications include data from at least 5 independent experiments.

1 mM VPA and only 12% at 3 mM VPA (Figure 1b).

VPA Leads to Increased Histone Acetylation in CLL Cells

To determine the effect of VPA treatment on HDAC activity in CLL cells, we determined the acetylation status of histone proteins. Cells were cultured for 48 h at different concentrations of VPA, and histone proteins were isolated by acid extraction. Western blot analysis revealed a dose-dependent accumulation of hyperacetylated histone proteins H3 and H4 in CLL cells after VPA treatment. In all tested patient cells, the effect was more pronounced on H4 histone proteins.

PBMCs from Healthy Donors Are Less Sensitive to Apoptosis Induction by VPA than CLL Cells

To compare the effect of VPA on apoptosis between PBMCs from healthy donors and from CLL patients, we quantified the number of apoptotic cells under identical culture conditions. As shown in Figure 2a, the CLL cells from 25 patients show a slightly higher rate of spontaneous apoptosis compared with PBMCs from healthy donors. However, the variation among the single patients was remarkable, and thus, the standard deviation in the patient group was greater than in the control group. Under treatment with VPA, we also found decreasing numbers of viable cells (PS^-/PI^-) in the healthy control group in a time- and dose-dependent manner after subtraction of the cells that had undergone spontaneous apoptosis in the untreated control: with 1 mM VPA the number of living cells was 81.8% after 48 h and 67.7% after 96 h. After treatment with 3 mM VPA, the number of living cells was 65.8% after 48 h and 48.7% after 96 h in the healthy donor group. For the lowest VPA concentration of 0.1 mM, a significant increase in the rate of apoptosis was not discernible (Figure 2b). In conclusion, VPA also reduces the amount of viable cells in normal PBMCs in a time- and dose-dependent manner.

As shown in Figure 2c, we found PBMCs from healthy donors to be by far less sensitive to apoptosis induction by VPA than cells from CLL patients. This difference becomes even more prominent for higher concentrations and later time points (Figure 2d). The direct comparison of healthy donor PBMCs to primary cells from CLL patients revealed that PBMCs from healthy donors are by far less sensitive to VPA-mediated apoptosis than CLL cells in a time- and dose-dependent manner (Figures 2c and 2d). To further elucidate the effects of VPA on different cell types, we separately analyzed healthy donor $CD3^+$ and $CD19^+$ PBMCs after treatment with VPA. Figure 2e shows that healthy donor $CD3^+$ T cells were significantly less sensitive to VPA than primary CLL cells. By contrast, healthy donor $CD19^+$ B cells were more susceptible to VPA than healthy donor $CD3^+$ T cells but revealed higher viability after VPA treatment compared with CLL cells (Figure 2f).

Apoptosis Induction by VPA Is Caspase-Dependent

To further distinguish whether VPA-mediated apoptosis was driven through the extrinsic or the intrinsic pathway, we assessed the activation status of caspases 8 and 9 by immunoblotting. Protein lysates from all patients evaluated showed a dose-dependent cleavage and thus an activation of both precursor caspases (Figure 3a). As shown in Figure 3c, about 50% of pro-caspase 8 were cleaved after 3 days of cultivation in the presence of 0.5 or 1 mM VPA. In CLL cell samples that were exposed to 2 and 3 mM VPA, approximately 75% pro-caspase 8 were cleaved. The activation of caspase 8 was also associated with the appearance of p20, the active form of caspase 8 (Figure 3b). Caspase 9 activation was

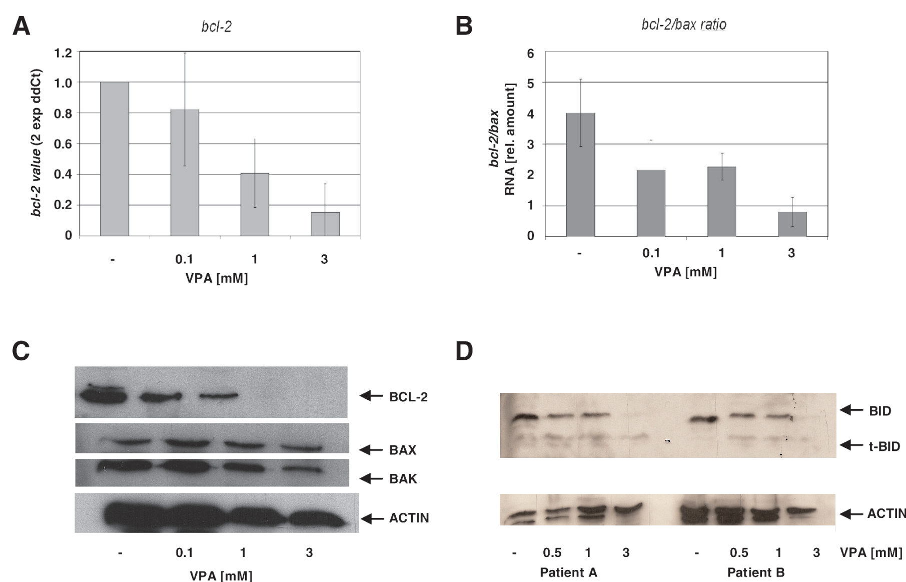

Figure 4. VPA modifies the expression of pro- and antiapoptotic CLL proteins. mRNA expression was measured by real-time PCR in 10 patients after 24 h of culture in the presence or absence of VPA. (A) *bcl-2* expression of treated cells is shown in relation to untreated control cells. Student's *t* test values reveal significant values for all tested VPA concentrations: 0.1 mM ($P < 0.05$), 1 mM ($P < 0.001$), and 3 mM ($P < 0.001$). (B) Gene expression values were normalized to *gapdh,* and the *bcl-2/bax* ratio was calculated. (C) *bcl-2*, *bax*, and *bak* levels were analyzed by Western blotting of total cellular extracts of CLL cells from 1 representative patient after culture for 3 days. (D) VPA-dependent apoptosis is accompanied by cleavage of BID. CLL cellular extracts were obtained after a 3-day culture in the presence or absence of VPA (2 representative patients are shown). BID and truncated BID (t-BID) were detected by Western blotting.

assessed on the basis of the disappearance of pro-caspase 9. After 3 days, >25% were cleaved in the presence of 1 mM VPA and >80% in the presence of 3 mM VPA (Figure 3d).

VPA Decreases the *bcl-2/bax* Ratio

To further characterize the apoptotic mechanisms involved, we determined whether VPA altered the expression of Bcl-2 family genes. The mRNA levels of *bcl-2*, *bax*, and *bid* were analyzed after 24 h by real-time PCR. We found that VPA consistently decreased the levels of *bcl-2*, as shown in Figure 4a. The levels of *bax* and *bid* were slightly decreased (data not shown). Consequently, the *bcl-2/bax* ratio was reduced in treated cells, which leads from a clearly antiapoptotic (range 2.3 to 7.7 for control) to a proapoptotic (range 0.3 to 1.4 for 3 mM VPA) signal, as shown in Figure 4b.

In addition, we validated the VPA concentration-dependent decrease of BCL-2 by immunoblotting. Figure 4c confirms decreased levels of BCL-2 and constant levels of BAX and BAK after 3 days of treatment.

CASPASE 8 Activation Causes BID Cleavage in CLL Cells

The involvement of BID as a connection between the extrinsic and the intrinsic apoptotic pathways is well described. Activated caspase 8 cleaves BID, and truncated BID triggers the mitochondria-dependent pathway by activating BAX and BAK, and in turn, caspase 9. To explain the activation of both apical caspases of the main apoptotic pathways, we analyzed the cleavage of BID by immunoblotting. As illustrated in Figure 4d, VPA treatment results in a concentration-dependent cleavage of BID.

VPA-Mediated Apoptosis in CLL Cells Is Caspase 8-Dependent and Requires a Mitochondrial Amplification Loop

The achievement of apoptosis is dependent on caspase activity. Extrinsic or intrinsic stimuli start the caspase cascade with activation of the apical caspases (8 or 9), which leads to activation of the downstream caspases, 3, 6, and 7 (11,17,18). Finally, we compared the effects of the caspase proteins 8 and 9 on downstream caspase activation in CLL cells. With a luminescence assay, we assessed the degree of VPA-dependent caspase 3/7 activation after 48 h, in the presence and absence of specific caspase inhibitors (caspase 8 inhibitor, Z-IETD-FMK (C8-I); caspase 9 inhibitor, Z-LEHD-FMK (C9-I); pan-caspase inhibitor, Z-VAD-FMK). The downstream caspase activity increased in a concentration-dependent manner in cells that were exposed to VPA compared with untreated cells that were defined to have a caspase 3/7 activity of 1. Whereas C8-I was able to completely block caspase 3/7 activation in cells that were cultured in the presence or absence of 2 mM VPA, C9-I decreased spontaneous and VPA-dependent caspase 3/7 activation by 10-fold (Figure 5a). In cells that were cultured in the presence of the pan-caspase inhibitor, caspase activity was not measurable (data not shown). Immunoblot analysis of lysed cells confirmed the VPA-dependent cleavage of caspases 8 and 9 and BID. Inhibition of caspase 8 prevented cleavage of BID and caspase 9. In addition, inhibition of caspase 9 had no effect on the cleavage of caspase 8 or BID (Figure 5b). Taken together, our findings suggest that caspase 8 is the first caspase activated under VPA and is required for BID cleavage and caspase 9 activation, which seems to amplify the apoptotic signal strength in CLL cells.

DISCUSSION

In this report we demonstrate that the HDAC inhibitor VPA reestablishes the apoptotic pathways in CLL cells and thus their ability to undergo apoptosis

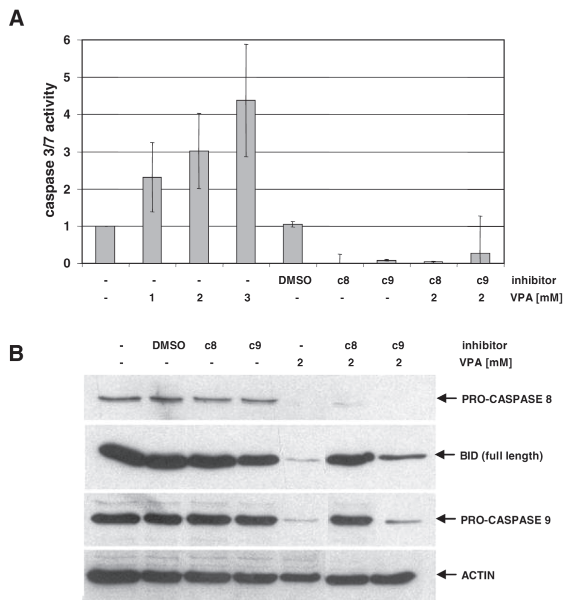

Figure 5. VPA-mediated apoptosis in CLL cells involves both apical caspases. Primary CLL cells were cultured for 48 h in the presence or absence of 1, 2, or 3 mM VPA, the inhibitor solvent DMSO, caspase 8 inhibitor (c8), caspase 9 inhibitor (c9), or a combination of caspase inhibitors and 2 mM VPA. (A) Caspase 3 and 7 activities were analyzed and are presented relative to the untreated control (n = 5). (B) Western blot analysis of total cellular extracts was done for caspase 8 and 9 and BID; equal loading was ensured by reprobing with the actin antibody.

by a type II cell mechanism. Although a number of studies have examined the activity of VPA in tumor cell line models, our report characterizes its molecular effects on primary CLL cells. We have observed that almost 30% of CLL cells cultured in the presence of 1 mM VPA for 48 h became apoptotic. At the same time, <20% PBMCs from healthy donors went into apoptosis when cultured under identical conditions, demonstrating a considerably higher sensitivity of CLL cells to apoptosis. These results are opposed to a report by MacFarlane et al. (18) in which no apoptosis was seen when CLL cells were treated for as long as 16 h and assayed after 20 h of culture. We detected apoptotic changes as soon as after 24 h of treatment and continued our analyses for a total of 7 days to further determine the differences between cells that were exposed to VPA compared with untreated cells.

The mechanisms by which apoptosis is induced in leukemic cells through HDAC inhibitors seems to depend on both the underlying disease entity and the inhibitor used. LAQ824 and SAHA have been reported to preferentially induce the intrinsic apoptotic pathway in human acute myeloid leukemia cell lines (19,20), but other HDAC inhibitors seem to sensitize various cells to apoptosis via either the extrinsic pathway or to apoptosis activation through both apoptotic pathways simultaneously (18,21). It needs to be kept in mind that the cell lines used for most studies in the literature were mitotically and metabolically active and thus characterized by profound differences compared with the nondividing, quiescent CLL patient cells used in this study.

More recently, in a few reports the effects of HDAC inhibitors on CLL cells have been investigated: whereas Depsipeptide (FR901228, FK228) was reported to induce apoptosis via the extrinsic apoptosis pathway (22), MS-275 was shown to trigger apoptosis through activation of both apoptosis pathways (23).

To further elucidate the mechanisms involved in VPA-mediated apoptosis, we analyzed the major factors of both classic

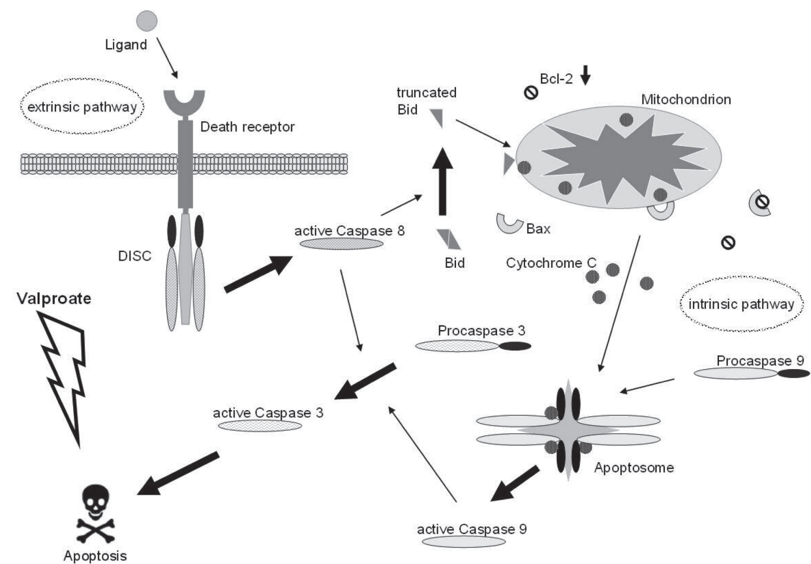

Figure 6. A model for the valproate mediated apoptosis sensitization in CLL cells. In order to futher analyze our observation that valproate promotes cell death in cultured CLL cells, we analyzed the single steps of apoptosis induction and indentified an activation of both classical apoptosis pathways. Following experiments indicated that CASPASE 8, the activator caspase of the extrinsic pathway, was activated first. The cleavage of BID provided a connection to the intrinsic pathway and the activation of CASPASE 9. This is further supported by the downregulation of the antiapoptotic protein Bcl-2, which leads to increased sensitivity towards the induction of apoptosis.

apoptosis pathways (Figure 6). First, we demonstrated by Western blot that both apical caspase proteins, the caspases 8 and 9, are involved in VPA-mediated apoptosis in CLL cells. We then demonstrated that caspase 8 inhibition completely prevents spontaneous and VPA-dependent apoptosis in CLL cells, whereas inhibition of caspase 9 reduced the activation of downstream caspase proteins by ten-fold. Caspase 8 activation was therefore supposed to be the first VPA-mediated event along the caspase cascade in CLL cells, which then leads to cleavage of BID and activation of the intrinsic pathway via caspase 9. Once caspase 8 becomes activated, subsequent events may follow two pathways: in the first scenario (type I cells), caspase 8 directly activates effector caspases and finally apoptosis; in type II cells, caspase 8 engages the mitochondrial pathway through activation of BID and caspase 9 before induction and activation of effector caspases and finally apoptosis.

The involvement of caspase 9 in receptor-mediated apoptosis is therefore part of a signal amplification loop compensating for either low DISC formation or insufficient caspase 8 levels. According to our results, VPA appears to (re)activate both apoptotic pathways in CLL cells, which seem to be unable to complete receptor-mediated apoptosis without signal amplification via the mitochondrial apoptosis pathway; we therefore propose that CLL cells are type II cells.

Analysis of *bcl-2* and *bax* by real time PCR and immunoblotting subsequent to exposure to VPA identified a downregulation of *bcl-2* expression at both mRNA and protein levels and thus, a decrease in the *bcl-2*/*bax* ratio. The *bcl-2*/*bax* ratio is an important cellular survival marker that correlates with responsiveness to drug therapy in vivo and in vitro (8,24). Accordingly, resistance to death receptor-induced apoptosis has been described to correlate with an overexpression of antiapoptotic proteins such as the Bcl-2 family (25). Therefore, our results contribute to a better understanding as to how HDAC inhibitors sensitize cells to receptor-induced apoptosis (26,27).

In conclusion, our study provides new insights into the pathogenesis of VPA-mediated apoptosis in CLL cells. VPA is a drug with a mild toxicity profile that is safe if taken over a long period of time. The agent might therefore be of benefit in the treatment of CLL either as a single agent or complementary and in combination with other drugs.

ACKNOWLEDGMENTS

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