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Comparison of the effects of valproic acid and levetiracetam on apoptosis in the human ovarian cancer cell line OVCAR-3

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Abstract:
Background: We have previously shown that due to its cytotoxic and cytostatic activities, valproic acid (VPA), but not levetiracetam (LEV), may have potential as a drug for treating human ovarian cancer. In the present study, we compare apoptotic mechanisms including gene and protein expression in the human ovarian cancer cell line, OVCAR-3, following exposure to VPA and LEV.
Methods: Cells were cultured with VPA or LEV at concentrations between 0.1 mM and 10 mM. Apoptosis was assessed by DNA fragmentation assay and expression of apoptosis-regulatory genes determined by real-time PCR and confirmed by western blotting. Time-dependent effects of VPA and LEV on activity of caspases (-3, -8 and -9) activity were evaluated by fluorescent assay and western blotting.
Results: Exposure to VPA at concentrations above 5 mM resulted in an increase in DNA fragmentation, modulated expression of genes and proteins associated with apoptosis and activated caspases ca scade. Exposure to LEV, however, did not affect DNA fragmentation and modulation of the mechanisms of apoptosis was not observed in LEV-treated cells at all doses used.
Conclusions: Exposure to high concentrations of VPA significantly stimulated apoptosis, by modulating the expression of genes and proteins responsible for cell death and also by activation of caspases cascade. Such effects were not observed with LEV. These data suggest that VPA should be seriously evaluated as an anti-cancer drug for ovarian cancer.

Key words:
valproic acid, levetiracetam, apoptotic gene and protein expression, caspases activity, ovarian cancer cell line OVCAR-3

Introduction

Epithelial ovarian cancer is the most common type of ovarian cancer and usually has a poor prognosis [17]. Current treatment of ovarian cancer is based on the integration of surgery and chemotherapy, with cytotoxic chemotherapy playing a major role. Several drugs have been developed for the treatment of ovarian cancer in recent decades, but novel, more effective agents are still needed.

Valproic acid (VPA) is one of the most widely prescribed anti-epileptic drugs (AEDs) in the world [34], used in the treatment of epilepsy, migraine, cluster headaches, and bipolar psychiatric disorders [18]. Levetiracetam (LEV) is a relatively new AED that has been extensively used during recent years [29]. Accumulating evidence indicates that VPA has anti-cancer activity [for review see ref. 2], inducing differentiation, growth arrest, and/or apoptosis in a broad spectrum of tumor cells through histone deacetylase

(HDAC) inhibition [23]. LEV has also been reported to demonstrate anti-tumor effects in glioblastoma cells [3] and prostate cancer [37]. Our previous paper [24] also suggested that VPA may have potential as an anti-cancer drug, particularly for human ovarian cancer, due to observations of cytotoxic and cytostatic activities in an ovarian epithelial carcinoma cell line. In contrast, the actions of LEV on ovarian cancer development are highly uncertain, with reported results varying from a pronounced apoptotic effect in ovarian granulosa cells [39] to an absence of apparent effect in ovarian epithelial carcinoma cells [24]. Thus, the aim of the work described here was to investigate the effects of both VPA and LEV on apoptotic mechanisms.

In this study, we first investigated the action of VPA and LEV on DNA fragmentation, the last step of apoptosis. Secondly, we performed real-time PCR on a panel of selected apoptosis-regulatory genes and verified the results obtained by western blot. Finally, the actions of VPA and LEV on caspase-8, -9 and -3 activity and protein expression were evaluated by fluorometric activity assay and western blot analyses.

Materials and Methods

Reagents

Trypsin, Ac-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), Ac-Ile-Glu-Thr-Asp--7-amido-4-methylcoumarin(Ac-IETD-AMC), Ac-Leu-Glu-His-Asp-7-amido-4-trifluoromethylcoumarin (Ac-LEHD-AFC), HEPES, CHAPS, EDTA, glycerol, DTT and DMSO were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI1640 medium without phenol red, fetal bovine serum (FBS, heat inactivated), phosphate-buffered saline (PBS) and penicillin/streptomycin solution (penicillin 10000 Units/ml, streptomycin 10 mg/ml) were obtained from Cytogen GmbH (Bienenweg, Germany). VPA was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in sterile water. LEV was purchased from UCB Pharma, Belgium (Keppra 100 mg/ml®, for intravenous infusion). The solutions were then further diluted in culture medium.

Cell culture

The human ovarian epithelial carcinoma cell line, OVCAR-3, was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were routinely cultured in RPMI1640 medium without phenol red, supplemented with 20% heat-inactivated FBS, 50 IU/ml of penicillin and 50 μg/ml of streptomycin. The medium was changed every two days. Cells were grown in 75 cm^2 tissue culture flasks in a 37°C incubator with a humidified mixture of 5% CO_2 and 95% air. For individual experiments, cells were cultured in RPMI 1640 without phenol red supplemented with 5% heat inactivated FBS.

DNA fragmentation assay

The dose-dependent influence of VPA and LEV on cell apoptosis was measured by the detection of DNA fragments. DNA fragmentation was determined using the Cellular DNA Fragmentation ELISA kit (Roche Applied Science, Mannheim, Germany). This assay is based on the quantitative detection of bromodeoxyuridine (BrdU)-labeled DNA fragments.

After exposure to BrdU for 18 h, cells were reseeded into 96-well culture plates at a density of 1.5×10^4 cells/well and treated for 24 h with VPA or LEV, at concentrations ranging from 0.1 mM to 10 mM. Culture medium was used as a control. After 24 h, DNA fragmentation was determined according to the manufacturer's instructions. Absorbance values were measured spectrophotometrically at 450 nm using an ELISA reader ELx808 (BioTek, Winooski, VT, USA). Six replicates of each sample were run in the same assay.

Real-time PCR analysis

The cells were seeded into 96-well culture plates at a density of 2×10^4 cells/well. The next day, the medium was changed and cells were treated for 24 h with 5 mM VPA or 10 mM LEV (doses selected on the basis of our previously published data [24]). Culture medium was used as a control. Total RNA isolation and cDNA synthesis was performed using the TaqMan Gene Expression Cell-to-C_T Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol. The purity and the quantity of the RNA and cDNA were determined by spectrophotometry at optical densities of 260 nm and 280 nm, respectively. Amplifications were performed in dupli-

cate using the StepOnePlus system (Applied Biosystems, Carlsbad, CA, USA), the TaqMan Array Human Apoptosis, Fast 96-Well Plate (Cat. No. 4418717) and the TaqMan Array Human Cellular Apoptosis Pathway (Cat. No. 4418762), in combination with TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instruction. PCR was performed in a final volume of 10 μl including 50 ng/reaction cDNA. The PCR conditions were as follows: pre-incubation for 2 min at 50°C and 10 min at 95°C, amplification for 40 cycles (15 s at 95°C and 1 min at 60°C). The relative expression of genes was normalized against the endogenous reference gene, GAPDH.

Western blot analysis

Cells were plated at a density of 1.5×10^5 per well in 12-well plates. The next day, the medium was changed and cells were treated with 5 mM VPA or 10 mM LEV for 3, 6, 12, 24, 48, and 72 h. Culture medium was used as a control. Media were changed every day, with new media and new fresh test compounds added. At the end of the incubation period, the cells were washed with ice-cold PBS and lysed with lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet NP-40 and 0.5% SDS), stored on ice for 30 min. The lysed cells were then scraped, transferred to microtubes, and stored at –70°C until analysis.

Before analysis, samples were sonicated and centrifuged at 4°C for 30 min at 15,000 × g and the clear supernatant was used in electrophoresis. Equal amounts of protein (20 μg) from each treatment group were separated by SDS-PAGE and transferred to PVDF membranes using a Bio-Rad Mini-Protean 3 apparatus (Bio-Rad Laboratories, Inc., USA). The blots were blocked for 2 h in 5% dry milk with 0.1% Tween-20 in 0.02 M TBS buffer. Blots were incubated overnight with antibodies specific to p53 (9282), Bid (2002), Bcl-xl (2762), caspase-8 (4790), cleaved caspase-8 (9496), caspase-9 (9502) and caspase-3 (9662) (Cell Signaling Technology Inc., Beverly, MA, USA) (1:1000 dilution). After incubation with the primary antibody, the membranes were washed three times with 0.1% Tween-20 in 0.02 M TBS buffer and incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody (7074; Cell Signaling Technology Inc., Beverly, MA, USA) (1:2000 dilution). For reprobing in order to determine β-actin as an internal loading control, membranes were washed for 30 min in stripping buffer (0.25 M glycine, 1% SDS, pH 2) and reprobed. Blots were incubated overnight with antibodies specific to β-actin (A5316; Sigma Chemical Co., St. Louis, MO, USA) (1:3000 dilution) and for 1 h with a horseradish peroxidase-conjugated secondary antibody (P-0447; Dako, Glostrup, Denmark) (1:5000 dilution).

Immunopositive bands were visualized using Western Blotting Luminol Reagent (Santa Cruz Biotechnology Inc., CA, USA) and ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Inc., USA). In order to obtain quantitative results, the bands representing each data point were densitometrically scanned using Image LaB™ 2.0 Software (Bio-Rad Laboratories, Inc., USA). Quantitative analysis was performed for three separately repeated experiments from each control and experimental group. The protein level within a control group was arbitrarily set as 1, against which statistical significance was analyzed.

Activity of caspases

The activities of caspase-8, -9 and -3 were measured according to Nicholson et al. [32] method as described previously [25] using fluorescent substrates (Ac-IETD-AMC, Ac-LEHD-AFC, Ac-DEVD-AMC, respectively).

Cells were plated at a density of 2×10^4 per well in 96-well plates. The next day, the media were changed, and cells were treated with 5 mM VPA or 10 mM LEV for 3, 6, 12, 24, 48 h and 72 h. Culture media were used as a control. Media were changed every day, and new medium and new fresh test compounds added. At the end of the incubation period, culture media were replaced with caspase assay buffer containing 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, and 10 mM DTT (pH 7.4), and the cells were incubated on ice for 10 min. The cell lysate was then incubated at 37°C with the appropriate caspase substrate at a final concentration of 10 μM. The amounts of fluorescent products were monitored every 30 min until 5 h using a fluorescence microplate reader (FLx800 Bio-Tek Instruments, USA) at excitation wavelength of 360 nm and emission wavelength of 460 nm, for caspase-3 and caspase-8, and at excitation wavelength of 400 nm and emission wavelength of 505 nm, for caspase-9. Culture medium alone was used as a control for non-specific binding. All the samples were run in quadruplicate in the same assay. Lysate protein levels were

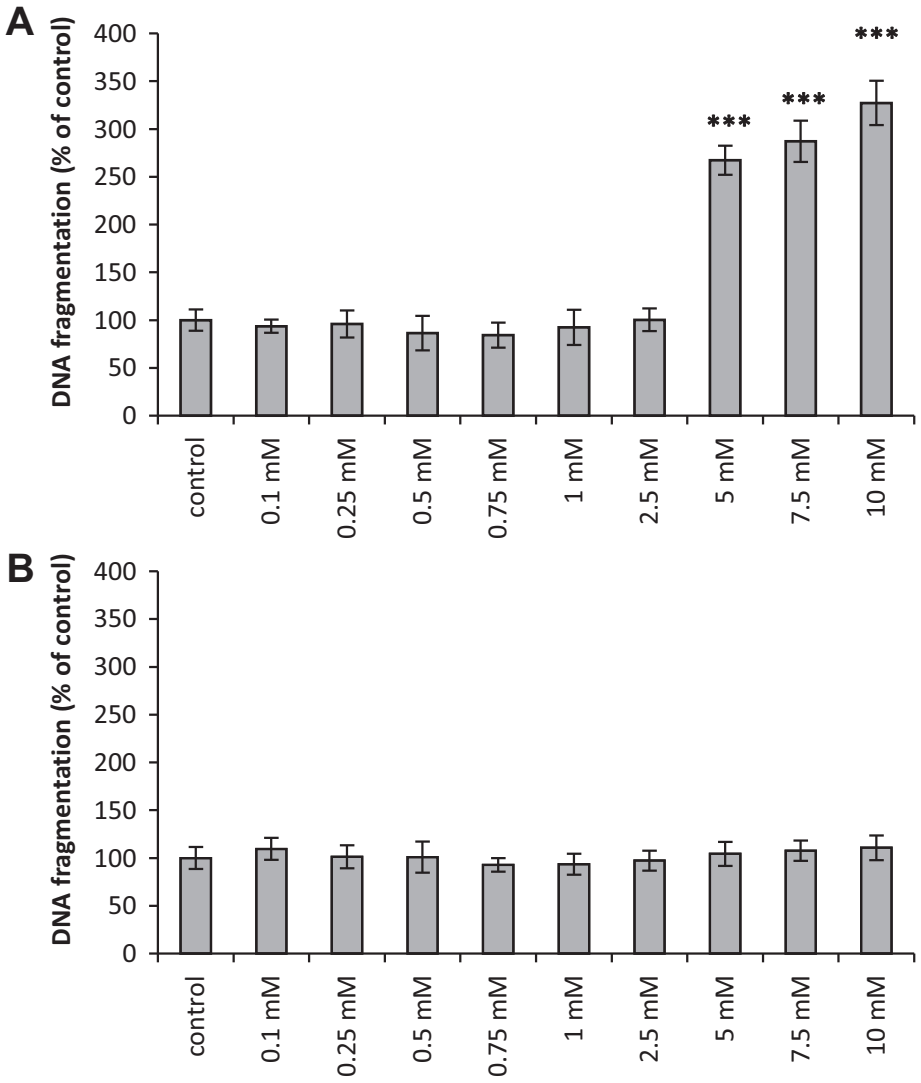

Fig. 1. The effects of VPA (**A**) and LEV (**B**) on cell apoptosis using the DNA fragmentation assay. Each point represents the mean ± SEM of results from three independent experiments with six replicates per treatment group. All means marked with *** ($p < 0.001$) are significantly different from control values

measured by the Bradford method, as described in the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., CA, USA) with bovine serum albumin as standard.

Statistical analysis

Each experiment was repeated three times (n = 3). All results were expressed as the means ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., CA, USA). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) multiple range test. Groups that are significantly different from control are indicated in the figures as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

Determination of cell apoptosis using DNA fragmentation assay

DNA fragmentation assay was performed after 24 h exposure to VPA or LEV at doses from 0.1 mM to 10 mM. VPA at concentrations from 5 mM to 10 mM resulted in a significant increase in DNA fragmentation

Tab. 1. Relative expression of selected genes involved in apoptosis regulation in OVCAR-3 cells exposed to 5 mM VPA or 10 mM LEV for 24 h. All values marked with * ($p < 0.05$), ** ($p < 0.01$) are significantly different from control

Assay ID	Description	Gene symbol (Alias)	Fold change	
			VPA	LEV
Extrinsic pathway				
Hs00174128_m1	Tumor necrosis factor (TNF)	*TNF*	1.00	1.00
Hs00234356_m1	TNF (ligand) superfamily, member 10	*TNFSF10*	1.07	1.14
Hs00533560_m1	TNF receptor superfamily, member 1A	*TNFRSF1A (TNFR1)*	1.00	0.84
Hs00269492_m1	TNF receptor superfamily, member 10A	*TNFRSF10A*	1.00	1.00
Hs00366272_m1	TNF receptor superfamily, member 10B	*TNFRSF10B*	1.00	1.00
Hs00205419_m1	TNF receptor superfamily, member 21	*TNFRSF21*	0.84	1.17
Hs00980365_g1	TNF receptor superfamily, member 25	*TNFRSF25*	1.00	1.00
Hs00182558_m1	TNF receptor-1-associated death domain protein	*TRADD*	0.87	0.87
Hs00169407_m1	Receptor-interacting serine-threonine kinase 1	*RIPK1*	1.01	0.87
Hs01572688_m1	Receptor-interacting serine-threonine kinase 2	*RIPK2*	0.85	1.00
Hs00184192_m1	TNF receptor-associated factor 2	*TRAF2*	1.17	1.00
Hs00531110_m1	TNF receptor superfamily, member 6	*FAS*	1.07	1.00
Hs00538709_m1	FAS-associated death domain protein	*FADD*	1.07	1.00
Hs01116280_m1	CASP8 and FADD-like apoptosis regulator (FLICE-like inhibitory protein)	*CFLAR (FLIP)*	1.07	0.93
Hs00354836_m1	Caspase-1	*CASP1*	1.00	1.02
Hs00892481_m1	Caspase-2	*CASP2*	1.00	0.86
Hs01031947_m1	Caspase-4	*CASP4*	0.86	0.86
Hs01018151_m1	Caspase-8	*CASP8*	1.00	1.00
Hs01017902_m1	Caspase-10	*CASP10*	1.00	1.00
Intrinsic pathway				
Pro-survival				
Hs99999018_m1	Apoptosis regulator Bcl-2 (B-cell lymphoma 2)	*BCL2*	1.00	1.00
Hs00236329_m1	Apoptosis regulator Bcl-xL (Bcl-2-like 1 protein)	*BCL2L1 (BCL-XL)*	1.37 *	0.83
Hs00187848_m1	Apoptosis regulator Bcl-w (Bcl-2-like 2 protein)	*BCL2L2 (BCL-W)*	1.17	1.00
Hs00197982_m1	Apoptosis regulator Bcl-2-like protein 11	*BCL2L11*	1.00	1.00
Hs99999008_m1	Murine double minute 2 p53 binding protein	*MDM2*	1.00	1.00
Pro-apoptotic				
Hs00188930_m1	Apoptosis regulator Bad (Bcl2 antagonist of cell death)	*BAD*	1.00	1.00
Hs00832876_g1	Apoptosis regulator Bak (Bcl-2 homologous antagonist/killer)	*BAK1*	1.00	1.00
Hs00180269_m1	Apoptosis regulator Bax (Bcl-2-associated X protein)	*BAX*	1.00	0.92

Tab. 1 – *continued on the next page*

Tab. 1. Relative expression of selected genes involved in apoptosis regulation in OVCAR-3 cells exposed to 5 mM VPA or 10 mM LEV for 24 h. All values marked with * ($p < 0.05$), ** ($p < 0.01$) are significantly different from control *(continued from the previous page)*

Assay ID	Description	Gene symbol (Alias)	Fold change	
			VPA	LEV
Hs00609632_m1	Apoptosis regulator Bid (BH3-interacting domain death agonist)	*BID*	1.36*	0.91
Hs00154189_m1	Apoptosis regulator Bik (Bcl-2-interacting killer)	*BIK*	0.88	1.00
Hs00261296_m1	Apoptosis regulator Bok (Bcl-2-related ovarian killer protein)	*BOK*	1.14	1.00
Hs00219876_m1	Direct IAP-binding protein with low pI (Second mitochondria-derived activator of caspase)	*DIABLO (SMAC)*	0.90	0.90
Hs01847653_s1	Baculoviral IAP repeat-containing protein 1	*BIRC1 (NAIP)*	1.00	1.00
Hs01112284_m1	Baculoviral IAP repeat-containing protein 2	*BIRC2 (CIAP1)*	1.00	1.00
Hs00745222_s1	Baculoviral IAP repeat-containing protein 4	*BIRC4 (XIAP)*	1.00	1.00
Hs01588973_m1	Cytochrome C	*CYCS*	1.00	1.00
Hs00559441_m1	Apoptotic protease-activating factor 1 (Apaf-1)	*APAF1*	0.94	1.00
Hs00154260_m1	Caspase-9	*CASP9*	0.80	0.90
Hs00234387_m1	Caspase-3	*CASP3*	0.84	0.93
	Caspase-6	*CASP6*	1.00	1.00
Hs00169152_m1	Caspase-7	*CASP7*	1.00	1.00
Hs00189336_m1	DNA fragmentation factor subunit α (Inhibitor of caspase-activated DNAse)	*DFFA (ICAD)*	1.00	1.00
Hs00237077_m1	DNA fragmentation factor subunit β (Caspase-activated DNAse)	*DFFB (CAD)*	1.00	0.87
Hs00242302_m1	Poly (ADP-ribose) polymerase 1	*PARP1*	1.00	1.00
Hs00193931_m1	Poly (ADP-ribose) polymerase 2	*PARP2*	1.00	1.00
Hs00193946_m1	Poly (ADP-ribose) polymerase 3	*PARP3*	1.00	1.00
Hs00173105_m1	Poly (ADP-ribose) polymerase 4	*PARP4*	1.17	1.00
Hs00377585_m1	Apoptosis-inducing factor mitochondrion-associated 1	*AIFM1*	1.00	1.00
Hs00172770_m1	Endonuclease G	*ENDOG*	1.00	1.00
Hs01112307_m1	Ataxia telangiectasia mutated	*ATM*	1.00	1.14
Hs00354807_m1	Ataxia telangiectasia and Rad3 related	*ATR*	0.86	0.86
Hs00200485_m1	Checkpoint kinase 2	*CHEK2 (CHK2)*	1.00	1.00
Hs01034249_m1	Tumor protein p53	*TP53*	1.50**	1.00
Hs00248075_m1	Bcl-2 binding component 3	*BBC3 (PUMA)*	1.00	1.00
Hs00560402_m1	PMA-induced protein 1	*PMAIP1 (NOXA)*	1.00	1.00

(Fig. 1A). Exposure to LEV at all concentrations used did not have any effect on DNA fragmentation (Fig. 1B).

Measurement of apoptosis-regulatory genes expression

In order to characterize the apoptotic mechanisms involved, we determined the expression of selected apoptosis-regulatory genes in cells exposed to VPA or LEV. Using real-time PCR, mRNA levels were analyzed in VPA or LEV-treated cells after 24 h and these compared with the levels of mRNA in untreated cells. The relative expressions of selected genes involved in the regulation of apoptosis are shown in Table 1. Exposure of cells to VPA resulted in up-regulation of *BCL2L1* (pro-survival apoptosis regulator Bcl-xl gene), *BID* (pro-apoptotic apoptosis regulator Bid gene) and *TP53* (tumor protein p53 gene). Changes in the expression of the other genes investigated were not observed. Exposure to LEV had no effect on the expression of any of the genes investigated.

Measurement of apoptosis-regulatory proteins expression

Based on the results obtained by real-time PCR, we analyzed p53, Bcl-xl, and Bid protein expression by western blot in VPA- or LEV-treated cells and compared these results with the levels of proteins in untreated cells (Fig. 2). No changes in the expression of p53 and Bid proteins were observed, while there was a decrease in Bcl-xl protein expression after 48 and 72 h of incubation with VPA. There was no statistical difference between LEV-treated and untreated cells.

Measurement of caspase activity and protein expression

In VPA-treated cells, no changes in activities of caspase-8, -9 and -3 were noted following from 3 to 12 h of exposure. However, after 24 h to 72 h of incubation with VPA, an increase in activity of all caspases investigated was observed, with maximum activity following 24 h of exposure (Fig. 3A–C). LEV had no effect on the activities of caspase-8, -9 and -3 at any of the time points (Fig. 3A–C).

To show if VPA, besides its effects on caspases activity, also have regulatory action on caspases protein expression, we performed western blot analysis. Western blot analysis of full length pro-caspases and active (cleaved) forms of caspase-8, -9 and -3 level in VPA-treated cells from 24 h of incubation resulted in an appearance of active forms of all caspases, compared with the levels of protein in untreated cells (Fig. 4), which is in line with results obtained in the caspase activity assays. LEV had no effect on caspase protein expression.

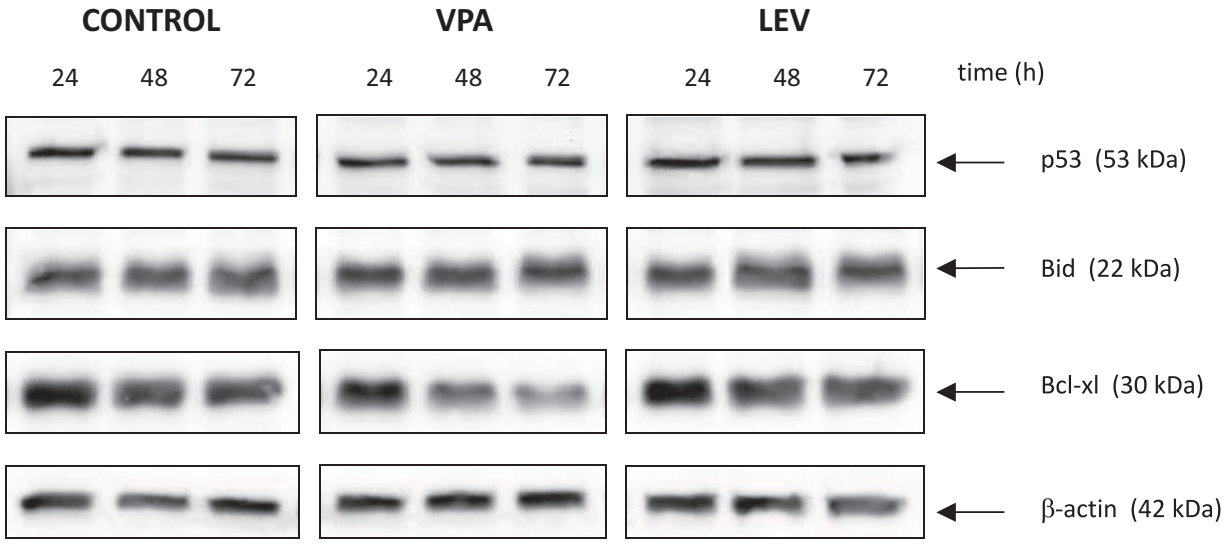

Fig. 2. The effect of VPA (5 mM) and LEV (10 mM) on p53, Bid and Bcl-xl protein expression after 24, 48 and 72 h of incubation. Blotting for β-actin indicated equal protein loadings

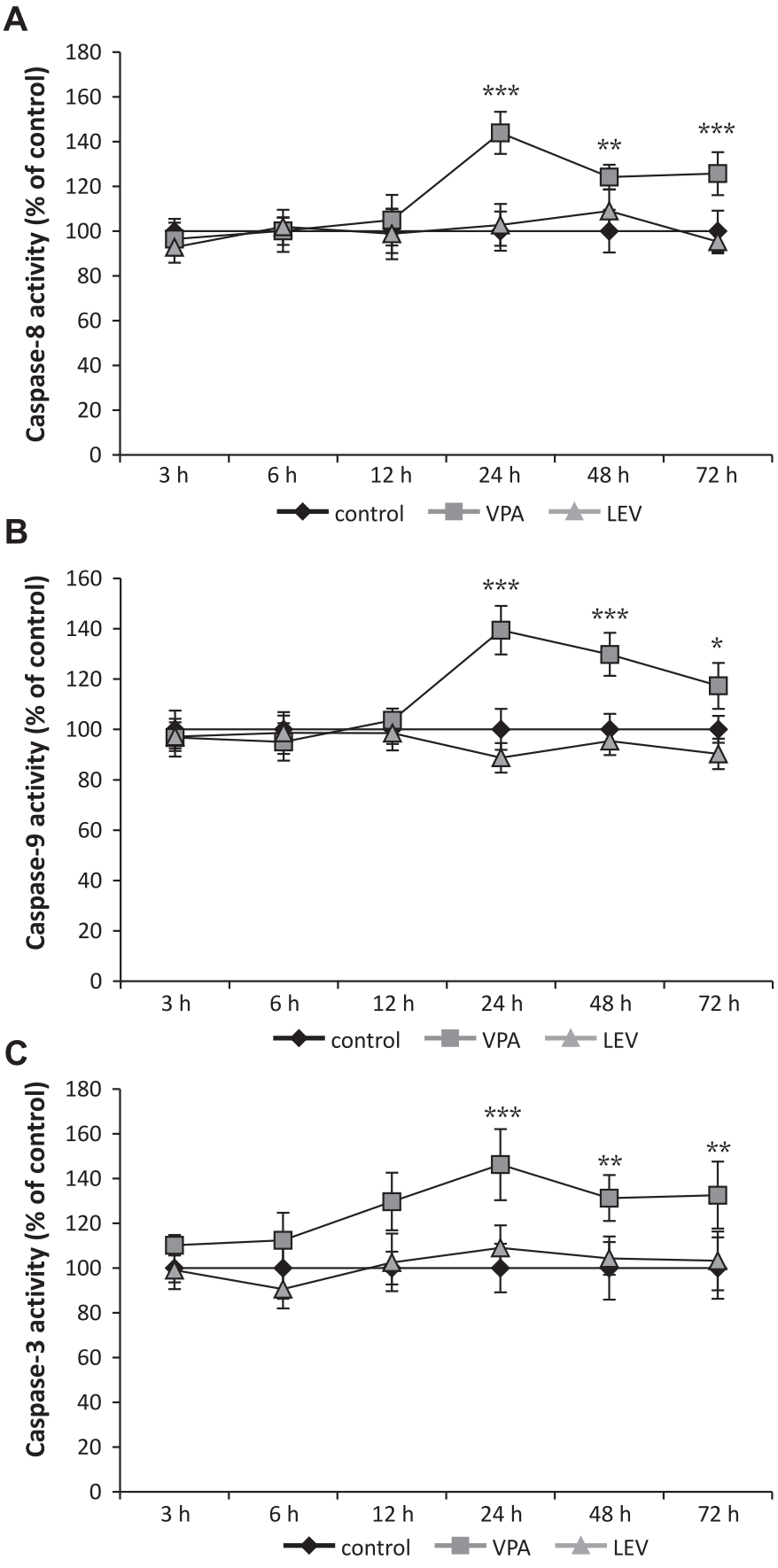

Fig. 3. The effects of VPA (5 mM) and LEV (10 mM) on caspase-8 (**A**), caspase-9 (**B**) and caspase-3 (**C**) activity. Each point represents the mean ± SEM of the results from three independent experiments with four replicates per treatment group. All means marked with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) are significantly different from control values

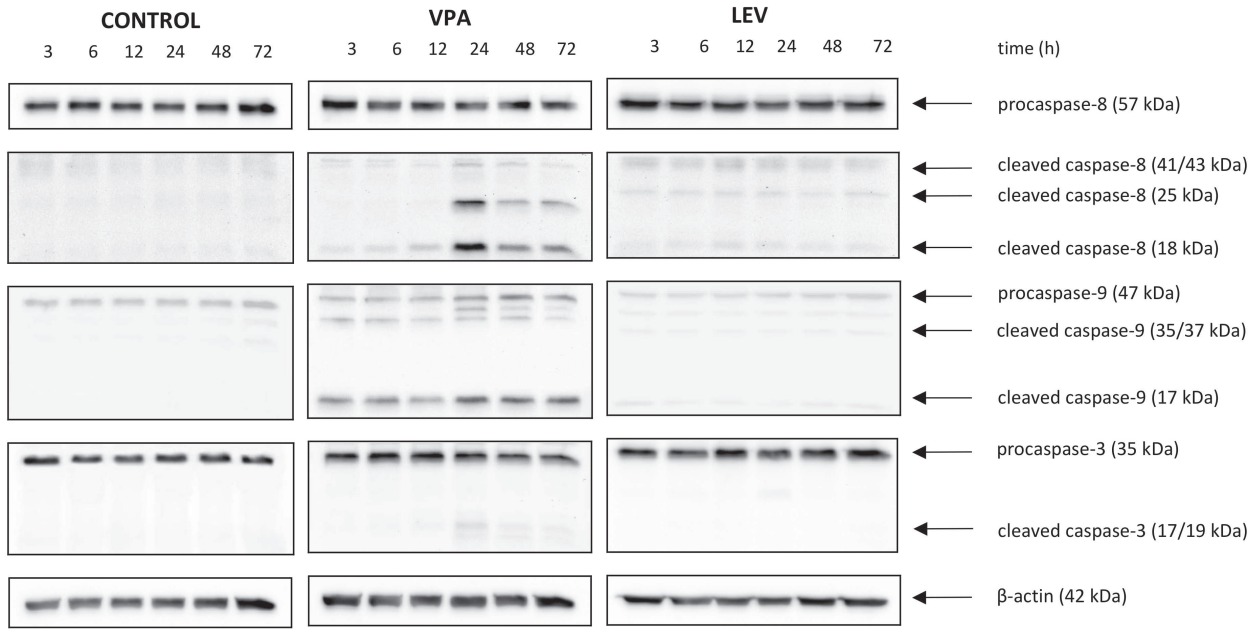

Fig. 4. The effect of VPA (5 mM) and LEV (10 mM) on full length and cleaved caspase-8, caspase-9 and caspase-3 protein expression after 3, 6, 12, 24, 48 and 72 h of incubation. Blotting for β-actin indicated equal protein loadings

Discussion

The results of the present study show that exposure of OVCAR-3 cells to VPA increases apoptosis, as demonstrated by DNA fragmentation assay, in a concentration-dependent manner. These results are in agreement with data published by Takai et al. [38], who showed that VPA treatment significantly increased the number of apoptotic OVCAR-3 cells, as determined by the TUNEL assay.

Following the demonstration that VPA induces OVCAR-3 cell death, it was pertinent to examine the underlying mechanisms that cause this apoptotic response. We showed that exposure of cells to VPA resulted in up-regulation of *TP53* (tumor protein p53) gene expression, with no effect on p53 protein expression. The tumor-suppressor protein, p53, has one of the most commonly altered genes, *TP53,* in human cancer and plays an important role in cellular response to genotoxic stress [15]. By enforcing cell cycle arrest and triggering apoptosis, p53 plays a key role in limiting the further expansion of cells containing damaged genomes. These results regarding altered *TP53* gene expression correspond with our previously published data [24].

The absence of effect of VPA exposure on the level of p53 protein noted here, has also been observed in myeloblastic leukemia cells [40] and in neuroblastoma cells [10]. Moreover, Condorelli et al. [10] showed that VPA was able to induce nuclear translocation and p53 hyperacetylation, despite its lack of effect on the total level of p53. Legube et al. [26] has suggested that VPA is able to activate the p53-pathway, not only by increasing p53 protein levels, but also by its hyperacetylation, which is thought to stabilize its nuclear active form. This supports the observation of Zhao et al. [45] that HDAC inhibitor-triggered activation of p53 pathways is a consequence of p53 hyperacetylation, rather than an induction of protein levels.

An impact of VPA on the expression of genes, *BCL2L1* and *BID*, of the anti- and pro-apoptotic Bcl-2 protein superfamily was also found. Modulation of the expression of these proteins is thought to play a role in sensitizing cancer cells to apoptosis. *BCL2L1* is a pro-survival, anti-apoptotic gene, encoding Bcl-xl protein, and, although in our experiment *BCL2L1* gene expression was up-regulated by VPA treatment, Bcl-xl protein expression was down-regulated. Significant down-regulation of *BCL2L1* gene expression after VPA treatment has been observed in human thyroid cancer cells [36] and also myeloma cells [31].

Results similar to ours concerning Bcl-xl protein level reduction in the presence of VPA have also been reported in hepatoma cells [1] and lung cancer cells [16]. In contrast, studies on thoracic cancer cells [46], neuroblastoma cells [10] and mesothelioma cells [41] have shown no effect of VPA treatment on Bcl-xl protein levels. Interestingly, we observed that expression of the pro-apoptotic *BID* gene was up-regulated in the course of VPA treatment, while Bid protein level was not affected. Our findings contrast with data from Neri et al. [31] who demonstrated significant down-regulation of the *BID* gene in myeloma cells. The lack of influence of VPA on Bid protein expression, as observed in our study, concurs with the results of Ziauddin et al. [46] and Condorelli et al. [10]. However, other data have shown that VPA increased the expression of Bid, as well as its active cleaved form in hepatoma cells [1], mesothelioma cells [41] and lung cancer cells [16].

The precise molecular mechanisms involved in the potential cancer therapy effects of VPA may be tissue-specific and dependent on the genetic background of the cancer. It might be speculated that the observed discrepancies between mRNA and protein expression in ovarian cancer cells could be explained by post-transcriptional or post-translational modifications, caused by the action of VPA as a HDAC inhibitor. Further studies are necessary to investigate this hypothesis.

Although expression of caspase genes was not affected by either VPA or LEV, we decided to examine the caspase activation pathway. Evidence exists suggesting that transcriptional activation may not be essential for initiation of apoptosis, with protein localization and/or cleavage events being more important [11]. The caspase cascade system plays a crucial role in the induction, transduction and amplification of intracellular apoptotic signals. Caspase-3 is an executor caspase that can be cleaved (activated) by either caspase-8, the initiator caspase of the death receptor pathway (extrinsic pathway), or caspase-9, the initiator caspase of the mitochondrial pathway (intrinsic pathway) [5].

In order to investigate which pathway (the extrinsic or the intrinsic) could be involved in the action of the tested drugs, we assessed the activity of caspases (-8, -9, -3) and protein expression at different time points, from 3 h to 72 h, taking into consideration the fact that caspase activity varies with time in human ovarian cancer cells [22]. We observed that VPA elicited a significant stimulatory action on caspase-8, -9 and -3 activity and protein levels. Our findings agree with previously published data showing that VPA can activate both caspase pathways (extrinsic and intrinsic) and induce apoptosis *via* caspase-dependent mechanisms in ovarian cancer cells [38] and other types of cancer cells [4, 7–9, 13, 14, 19, 35, 44]. Our data suggest that VPA-mediated apoptosis of OVCAR-3 cells is caspase-dependent, but it should be noted that Kawagoe et al. [19] reported that VPA may induce apoptosis in human B-cells through both caspase-dependent and caspase-independent mechanisms.

In contrast with our findings regarding VPA, we found that LEV had no influence on the process of apoptosis in OVCAR-3 cells. Data concerning the effects of LEV on cell apoptosis are scarce. LEV has been shown to induce apoptosis in rat astrocyte primary cultures, but only at very high concentrations, as determined by COMET assay [33]. However, using TUNEL staining, a lack of pro-apoptotic effects in rats' brains due to LEV treatment has been shown by Manthey et al. [30] and Kim et al. [20, 21].

We also showed that the expression of apoptosis-related genes and proteins was unaffected by exposure to LEV, supporting our previously published data [24] which demonstrated that LEV did not affect the expression of genes involved in cell cycle regulation. LEV has also been shown to have no effect on the *TP53* gene and p53 protein expression in both normal human astrocytes and gliomas cells [3].

Additionally, we observed a lack of effect on the activity of all investigated caspases and on protein levels in LEV-exposed cells. However, in our previously published data [39], we demonstrated that LEV caused an increase in caspase-3 activity in human granulosa cells. Although these results are not directly comparable with the current research, it is possible that the difference in results may be due to epithelial ovarian cancers being susceptible to apoptosis [27], differentially from follicular cells that normally undergo apoptosis during the menstrual cycle [42]. It is also known that LEV acts in neuronal cells *via* SV2A protein [28], which is also widely distributed in endocrine tissue [6]. Granulosa cells represent the endocrine compartment of the ovary, while OVCAR-3 cells are of epithelial origin with no endocrine capacity. Although ovarian cancer in humans can arise from any of the cell types found in the ovary, almost 90% are derived from the ovarian surface epithelium [43].

In conclusion, based on both the results presented here and those from our previous paper [24], we suggest that VPA seems to have real potential as an anti-

cancer drug in ovarian cancer. Moreover, VPA has appropriate pharmacokinetic properties [12] and presents only moderate toxicity [7] which is acceptable in the context of an anticancer treatment. Additionally, we suggest that activation of caspases cascade is a major factor in the mechanisms of the apoptotic action of VPA. On the other hand, our results suggest that LEV has no antitumor activity in ovarian cancer.

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