Cell Death and Differentiation (2006) 13, 446–453
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Valproic acid induces extracellular signal-regulated kinase 1/2 activation and inhibits apoptosis in endothelial cells

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Received 24.3.05; revised 10.6.05; accepted 20.7.05; published online 16.9.05
Edited by G Ciliberto

Abstract

The histone deacetylase (HDAC) inhibitor valproic acid (VPA) was recently shown to inhibit angiogenesis, but displays no toxicity in endothelial cells. Here, we demonstrate that VPA increases extracellular signal-regulated kinase 1/2 (ERK 1/2) phosphorylation in human umbilical vein endothelial cells (HUVEC). The investigation of structurally modified VPA derivatives revealed that the induction of ERK 1/2 phosphorylation is not correlated to HDAC inhibition. PD98059, a pharmacological inhibitor of the mitogen-activated protein kinase kinase 1/2, prevented the VPA-induced ERK 1/2 phosphorylation. In endothelial cells, ERK 1/2 phosphorylation is known to promote cell survival and angiogenesis. Our results showed that VPA-induced ERK 1/2 phosphorylation in turn causes phosphorylation of the antiapoptotic protein Bcl-2 and inhibits serum starvation-induced HUVEC apoptosis and cytochrome *c* release from the mitochondria. Moreover, the combination of VPA with PD98059 synergistically inhibited angiogenesis *in vitro* and *in vivo*.
Cell Death and Differentiation (2006) **13,** 446–453. doi:10.1038/sj.cdd.4401759; published online 16 September 2005

Keywords: valproic acid; endothelial cell; apoptosis; angiogenesis; ERK

Abbreviations: bFGF, basic fibroblast growth factor; CAM, chick chorioallantoic membrane; eNOS, endothelial nitric oxide synthase; ERK 1/2, extracellular signal-regulated kinases 1/2; FCS, foetal calf serum; HDAC, histone deacetylases; HDACI, histone deacetylase inhibitor; HUVEC, human umbilical vein endothelial cells; IMDM, Iscove's modified Dulbecco's medium; MEK 1/2, mitogen-activated protein kinase kinase 1/2; TSA, trichostatin A

Introduction

Valproic acid (VPA) is one of the most frequently prescribed antiepileptic drugs[1] and is employed clinically in the treatment of different pathologies including schizophrenia, bipolar disorders, and different forms of headache.[2–4] VPA is also currently under experimental and clinical investigation as an anticancer drug.[5] The most prominent adverse effects of VPA are teratogenicity and liver toxicity.[6] The teratogenic effects include neuronal tube defects such as exencephaly and spina bifida[7] and malformations of the heart.[8–10] Recently, VPA was shown to inhibit angiogenesis *in vitro* and *in vivo* by decreasing the expression of the endothelial nitric oxide synthase (eNOS) in endothelial cells.[11] Decreased eNOS expression and subsequent angiogenesis inhibition were preceded by the inhibition of histone deacetylases (HDAC). Indeed, a decrease in eNOS levels can be elicited by structurally unrelated HDAC inhibitors (HDACIs).[11,12] Although the mechanisms of VPA-induced teratogenicity are not yet fully understood, the ability of this substance to inhibit angiogenesis may play a role within this process.

The teratogenic and anticancer effects of VPA have been suggested to be at least in part attributable to HDAC inhibition.[13–15] HDACIs induce apoptosis in different cell types.[16] VPA induces apoptosis in a limited number of cell types while being nontoxic or even cytoprotective in other cells.[5,17–19] In different cells, VPA was shown to stimulate the phosphorylation/activation of the extracellular signal-regulated kinases 1/2 (ERK 1/2, mitogen-activated protein kinase (MAPK)),[15,20] which play an integral role in cellular survival signalling.[21]

Since VPA was shown to inhibit endothelial cell proliferation and tube formation but not to exert cytotoxicity in these cells,[11] we determined the influence of VPA on ERK 1/2 activation and on stress-induced cell death in response to serum starvation. Serum acts as trophic factor for endothelial cells and serum starvation induces endothelial cell apoptosis within 24 h.[22] Since ERK 1/2 activation is commonly involved in endothelial cell angiogenesis,[23] we examined the influence of VPA-induced ERK 1/2 activation on *in vitro* tube formation and on vessel formation in the chick chorioallantoic membrane (CAM) assay *in vivo*.

Results

VPA increases ERK 1/2 phosphorylation in endothelial cells

Confluent human umbilical vein endothelial cells (HUVEC) were kept in serum-free medium overnight and then incubated with VPA 1 mM for different times. An increase in the phosphorylation of ERK 1/2 was detected as soon as 5 min after addition of VPA (Figure 1a). After 120 min ERK 1/2 phosphorylation declined to basal levels (data not shown). In contrast, VPA did not influence phosphorylation of Akt (data not shown), another key player in endothelial cell angiogenesis and survival.[24]

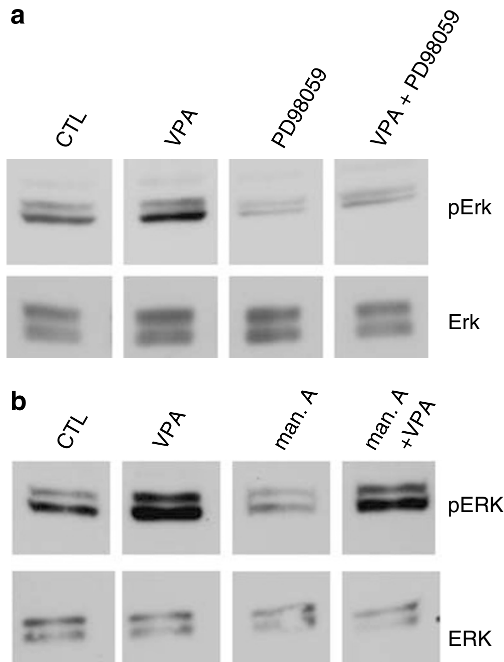

Figure 1 Influence of VPA on ERK 1/2 phosphorylation. (**a**) Representative Western blot showing ERK 1/2 phosphorylation in HUVEC after overnight cultivation in protein- and growth factor-free medium without (CTL = control) or with treatment with VPA (1 mM), the MEK inhibitor PD98059 (10 μM), or VPA (1 mM) + PD98059 (10 μM) for 5 min, (**b**) Representative Western blot showing ERK 1/2 phosphorylation in HUVEC after overnight cultivation in serum- and growth factor-free medium without (CTL = control) or with treatment with VPA (1 mM), the farnesyltransferase inhibitor manumycin A (man. A, 10 μM), or VPA (1 mM) + man. A (10 μM) for 5 min. Western blots are representative of three independent experiments

ERK 1/2 is commonly phosphorylated by MEK 1/2[21] and the mitogen-activated protein kinase kinase 1/2 (MEK 1/2) inhibitor PD98059 (10 μM) completely blocked VPA-induced ERK 1/2 phosphorylation (Figure 1a). MEK 1/2 and ERK 1/2 are the two downstream kinases of the 'classical' MAPK pathway Ras/Raf/MEK/ERK.[21,24] The first step during Ras activation is Ras farnesylation by the farnesyltransferase followed by Ras localisation to the plasma membrane.[25] The farnesyltransferase inhibitor manumycin A inhibits Ras farnesylation and therefore activation.[26] Manumycin A reduced basal ERK 1/2 phosphorylation but did not influence VPA-induced ERK 1/2 phosphorylation (Figure 1b), indicating that VPA activates MEK 1/2 independently of Ras.

Ca^{2+} mobilisation is often involved as an upstream event in ERK 1/2 phosphorylation in endothelial cells.[24] However, VPA did not induce Ca^{2+} mobilisation at concentrations up to 1 mM whereas arachidonic acid (20 μM) and ionomycin (1 μM) evoked a clear elevation of the intracellular Ca^{2+} levels (Figure 2).

To determine whether HDAC inhibition may contribute to VPA-induced ERK 1/2 phosphorylation, two structurally modified VPA derivatives, the enantiomers *S*-2-pentyl-4-pentynoic acid and *R*-2-pentyl-4-pentynoic acid were investigated. As shown in Figure 3a, acetylated histone H4 levels in HUVEC increased after treatment with VPA or *S*-2-pentyl-4-pentynoic acid but not after treatment with *R*-2-pentyl-4-pentynoic acid. This indicates that VPA and *S*-2-pentyl-4-pentynoic acid but not *R*-2-pentyl-4-pentynoic acid inhibit HDAC subtypes that cause histone H4 deacetylation at the concentrations employed. Investigation of the influence of VPA, *S*-2-pentyl-4-pentynoic acid, or *R*-2-pentyl-4-pentynoic acid on ERK 1/2 phosphorylation revealed that all

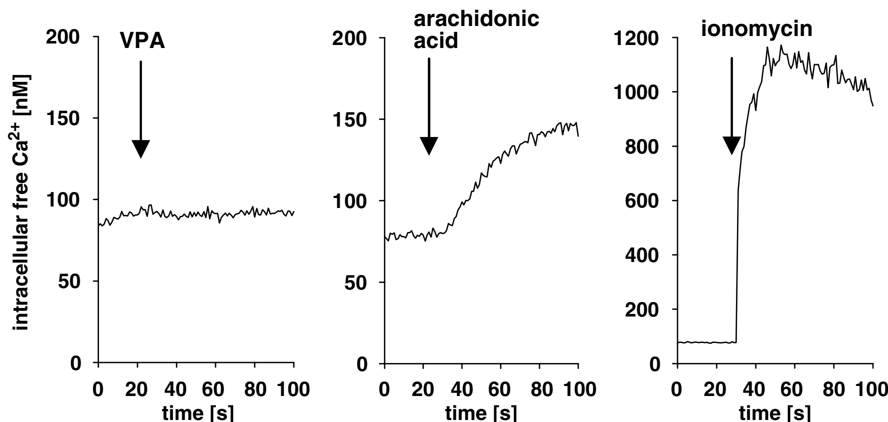

Figure 2 Influence of VPA on Ca^{2+} mobilisation in HUVEC. HUVEC, incubated in PBS containing 1 mg/ml glucose and 1 mM $CaCl_2$, were loaded with 2 μM Fura-2/AM for 30 min at 37°C, challenged with VPA (1 mM), arachidonic acid (20 μM), or ionomycin (1 μM), and the intracellular Ca^{2+} levels were determined. The results are representative of three independent experiments

three molecules induced ERK 1/2 phosphorylation independently of their influence on histone H4 acetylation (Figure 3b). The structurally nonrelated HDACI trichostatin A (TSA) did not stimulate ERK 1/2 phosphorylation (data not shown).

VPA inhibits endothelial cell cycle and endothelial cell death

VPA had been shown before to inhibit endothelial cell proliferation.[11] Therefore, we investigated the influence of VPA on the cell cycle of HUVEC. Subconfluent HUVEC were maintained for 24 h in serum- and growth factor-free medium prior to incubation of HUVEC in medium containing basic fibroblast growth factor (bFGF) (2.5 ng/ml), foetal calf serum (FCS) (15%), and pooled human serum (5%) with or without the addition of VPA 1 mM for 24 h. Cell cycle analysis of control cells revealed 52±3% of cells in G0/G1-phase, 27±2% in S-phase, and 14±4% in G2/M-phase. Analysis of VPA-treated cells resulted in 61±3% of cells in G0/G1-phase, 16±3% in S-phase, and 16±4% in G2/M-phase, indicating a G1/S-block induced by VPA-treatment.

ERK 1/2 activation is commonly involved in the survival signalling in endothelial cells.[27] Therefore, we investigated the effects of VPA on cell death induced by serum starvation. Confluent HUVEC were incubated for 48 h with Iscove's modified Dulbecco's medium (IMDM) in the absence of FCS, human serum, and bFGF. Determination of viable cells revealed that VPA protected HUVEC from cell death in a concentration-dependent manner (Figure 4a). The cytoprotective effect was not observed in cells cotreated with the MEK inhibitor PD98059 (Figure 4a).

The VPA derivatives *S*-2-pentyl-4-pentynoic acid and *R*-2-pentyl-4-pentynoic acid both protected HUVEC from cell death induced by serum starvation (Figure 4b). Prevention of ERK 1/2 activation by addition of the MEK inhibitor PD98059 abolished the *S*-2-pentyl-4-pentynoic acid and *R*-2-pentyl-4-pentynoic acid-induced cell death inhibition in a similar manner like the VPA-induced cell death (data not shown). In contrast, TSA did not protect HUVEC from cell death. Taken together, these data show that VPA protects HUVEC from cell death by ERK 1/2 phosphorylation independently of HDAC inhibition.

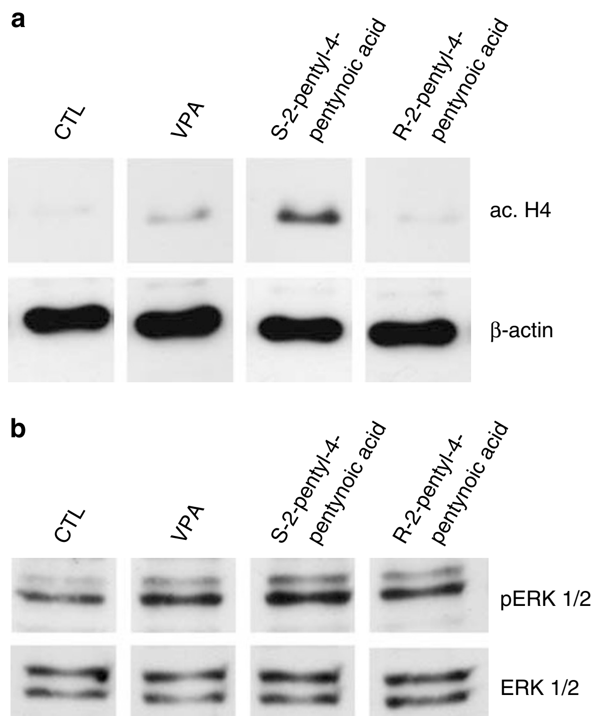

Figure 3 Influence of VPA derivatives on accumulation of acetylated histones and on ERK 1/2 phosphorylation in HUVEC, (**a**) Western blot showing levels of acetylated histone H4 (ac. H4) in HUVEC treated without (CTL = control) or with VPA (1 mM), *S*-pentyl-4-pentynoic acid (1 mM), or *R*-pentyl-4-pentynoic acid (1 mM) for 24 h, (**b**) Western blot showing ERK 1/2 phosphorylation in HUVEC cells after overnight cultivation in serum- and growth factor-free medium without (CTL = control) or with treatment with VPA (1 mM), *S*-pentyl-4-pentynoic acid (1 mM), or *R*-pentyl-4-pentynoic acid (1 mM) for 5 min. The results are representative of three independent experiments

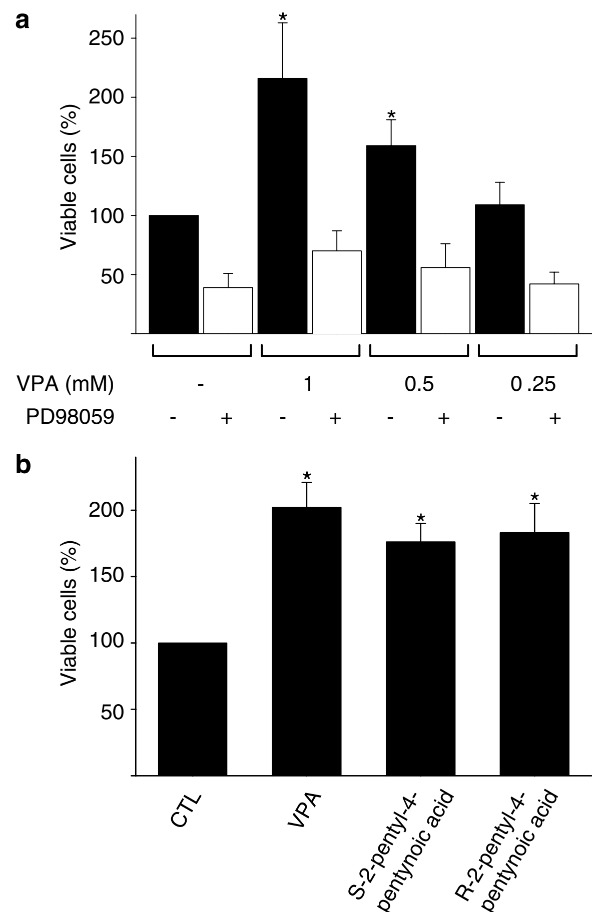

Figure 4 Influence of VPA on the viability of confluent HUVEC maintained for 48 h in serum- and growth factor-free medium, (**a**) HUVEC treated with VPA in different concentrations with and without the MEK inhibitor PD98059 (10 μM) relative to nontreated control, (**b**) HUVEC treated with VPA (1 mM), *S*-pentyl-4-pentynoic acid (1 mM), or *R*-pentyl-4-pentynoic acid (1 mM) relative to nontreated control. *$P \leq 0.05$ compared to control

Serum starvation of endothelial cells commonly results in the induction of apoptosis.[28] VPA decreased the number of apoptotic cells in HUVEC maintained for 48 h in protein-free medium as indicated by annexin V staining (Figure 5). PD98059 abolished the VPA-induced apoptosis inhibition. Detection of activated caspase 3 revealed similar results (data not shown). HDACIs are known to induce apoptosis in different cell types.[17] Therefore, we investigated if ERK activation is sufficient to inhibit apoptosis induced by serum starvation and the HDACI TSA, that does not induce ERK 1/2 phosphorylation. HUVEC were transfected with constitutively active ERK 2 (Figure 6a) and kept in serum- and growth factor-free medium for 48 h with and without the addition of TSA 200 ng/ml. Apoptotic cells were detected by annexin V staining. Transfection with constitutively active ERK 2 significantly reduced the number of apoptotic cells with and without TSA 200 ng/ml treatment (Figure 6b).

In HUVEC, ERK 1/2 phosphorylate the antiapoptotic protein Bcl-2, which in turn inhibits the ubiquitinylation of Bcl-2.[29] VPA 1 mM stimulated the phosphorylation of Bcl-2 as soon as 5 min after VPA addition, which was abolished by the MEK inhibitor PD98059 (Figure 7a). Since Bcl-2 inhibits cytochrome *c* release from the mitochondria, the influence of VPA on cytochrome *c* release was detected by flow cytometry. Cytochrome *c* release was significantly reduced by VPA 1 mM and PD98059 prevented VPA-induced inhibition of cytochrome *c* release (Figure 7b).

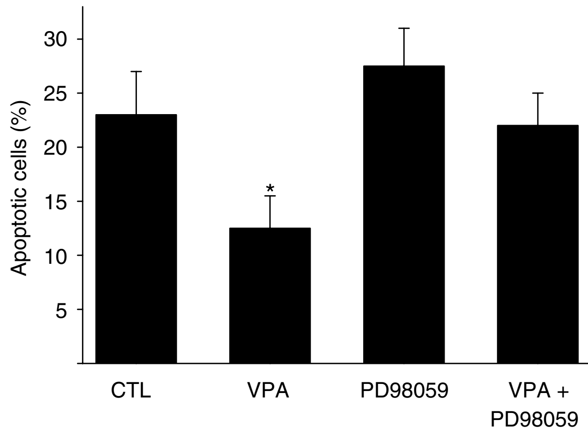

Figure 5 Influence of VPA on apoptosis of HUVEC after serum starvation. Confluent HUVEC were maintained for 48 h in serum- and growth factor-free medium without (CTL = control) or with treatment with VPA (1 mM), the MEK inhibitor PD98059 (10 μM), or VPA (1 mM) + PD98059 (10 μM). Apoptotic cells were detected by annexin V-staining

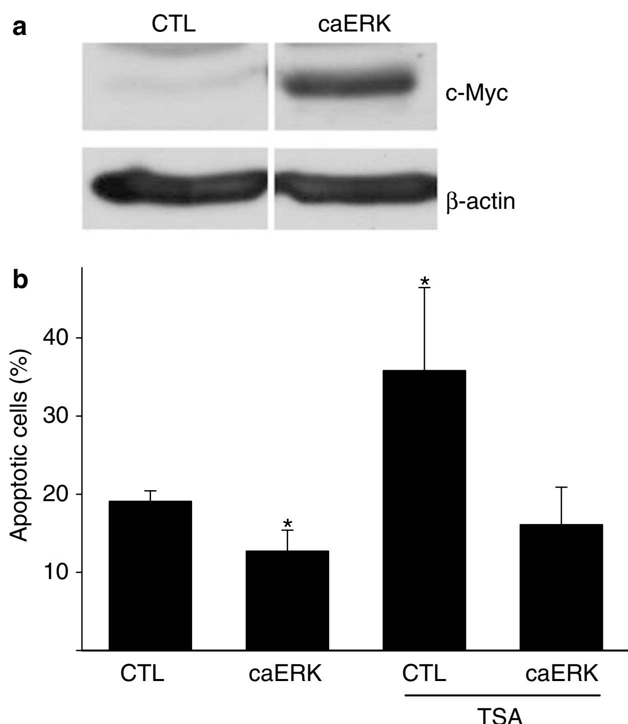

Figure 6 Influence of transfection of constitutively active ERK 2 on TSA and serum starvation-induced HUVEC apoptosis. HUVEC were transfected with a control plasmid (CTL) or c-Myc-tagged constitutively active ERK 2 (caERK) and maintained for 48 h in serum- and growth factor-free medium without (CTL = control) or with addition of TSA (200 ng/ml). (**a**) Representative Western blot showing expression of caERK indicated by the use of a c-Myc antibody. (**b**) Number of apoptotic cells detected by annexin V-staining. *$P \leq 0.05$ compared to control

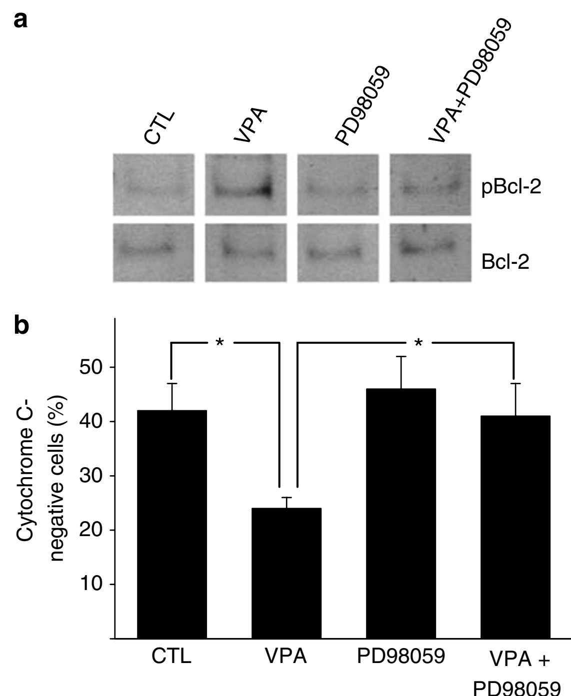

Figure 7 Influence of VPA on Bcl-2 phosphorylation and on cytochrome *c* release from the mitochondria. (**a**) Western blot showing Bcl-2 phosphorylation in HUVEC after overnight cultivation in serum- and growth factor-free medium without (CTL = control) or with treatment for 5 min with VPA (1 mM), the MEK inhibitor PD98059 (10 μM), or VPA (1 mM) + PD98059 (10 μM). The results are representative of three independent experiments. (**b**) Quantitative analysis of HUVEC that released cytochrome *c* from the mitochondria. Confluent HUVEC were maintained for 48 h in serum- and growth factor-free medium without (CTL = control) or with addition of VPA (1 mM), PD98059 (10 μM), or VPA (1 mM) + PD98059 (10 μM). *$P \leq 0.05$

VPA-induced angiogenesis inhibition is synergistically enhanced by MEK inhibition

VPA is known to inhibit angiogenesis.[11] However, the phosphorylation of ERK 1/2 in endothelial cells is generally thought to be a proangiogenic event.[27] Therefore, we determined whether or not inhibition of VPA-induced ERK 1/2 phosphorylation by PD98059 may influence the antiangiogenic effects of VPA. An *in vitro* tube formation assay showed a synergistic inhibition of HUVEC tube formation in the samples treated with a combination of VPA with PD98059 (Figure 8).

Additionally, the combination of VPA 1 mM and PD98059 10 μM inhibited vessel formation in the CAM assay *in vivo* also more effectively as compared to either single treatment. Representative photographs are shown in Figure 9.

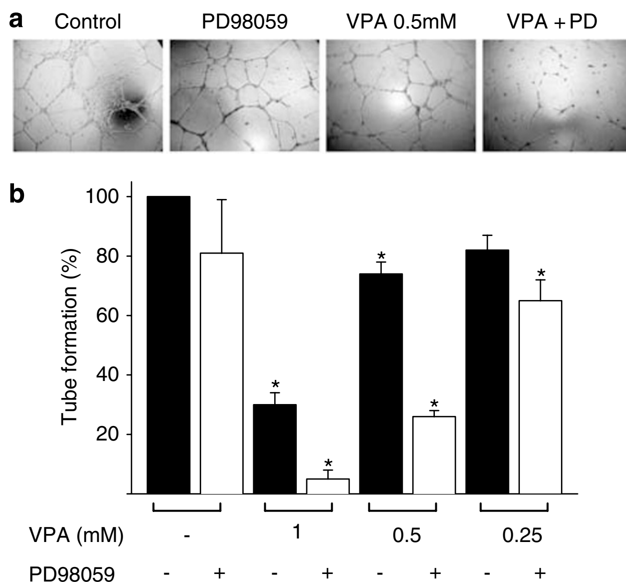

Figure 8 Influence of VPA with or without the MEK inhibitor PD98059 (PD) (10 μM) on formation of tube-like structures of HUVEC after 5-day pretreatment *in vitro*, (**a**) Representative photographs showing tube formation of HUVEC treated without (control) or with PD98059, VPA 0.5 mM, or PD98059 plus VPA 0.5 mM, (**b**) Quantitative analysis of HUVEC tube formation. Bar graph showing the results of three independent experiments. *$P \leq 0.05$ compared to control

Discussion

In this report, VPA was shown to induce ERK 1/2 phosphorylation in endothelial cells. VPA was used in concentrations $\leq$1 mM, which is in the range of therapeutical achievable plasma concentrations.[9] As such our observations are in accordance with previous reports showing VPA-induced ERK 1/2 phosphorylation in cells of animal origin including normal rat neuronal cells, Wistar rat thyroid cells, and the mouse neuroblastoma cell line Neuro2A as well as in human cancer cell lines including the human erythroleukaemia cell line K562 and the human neuroblastoma cell line SH-SY5Y.[15,20] Here, we show the induction of ERK 1/2 phosphorylation by VPA in a nonmalignant human cell. VPA-induced ERK 1/2 phosphorylation was inhibited by the MEK inhibitor PD98059, indicating that VPA-induced ERK 1/2 phosphorylation is mediated via MEK. MEK and ERK are commonly involved in the 'classical' MAPK cascade Ras/Raf/MEK/ERK.[21] However, inhibition of Ras activation by the farnesyltransferase inhibitor manumycin A did not inhibit VPA-induced ERK 1/2 phosphorylation demonstrating that VPA does not induce ERK 1/2 phosphorylation by this signalling pathway. Although an increase of intracellular Ca^{2+} levels is a common upstream event of MAP kinase phosphorylation,[24] VPA did not influence intracellular Ca^{2+} levels. Of interest, the long-chain fatty acid arachidonic acid, which activates ERK 1/2 in various cell types,[30] caused significant Ca^{2+} mobilisation. Hence, VPA-induced ERK 1/2 phosphorylation is independent of Ca^{2+}-signalling. A possible alternative route leading to ERK 1/2 phosphorylation without contribution of Ras or Ca^{2+} signalling may involve the small GTPases Rap1 and B-Raf, which in turn stimulate MEK.[31]

The contribution of HDAC inhibition to the VPA-induced ERK 1/2 phosphorylation was studied by the use of the enantiomers of the VPA derivative 2-pentyl-4-pentynoic acid. Our results showed that *S*-2-pentyl-4-pentynoic acid caused an increase in the amount of acetylated histone H4 in the tested concentrations of up to 1 mM in endothelial cells, whereas the *R*-enantiomer did not. Both enantiomers induced ERK 1/2 phosphorylation, while the HDACI TSA did not influence ERK 1/2 phosphorylation. To date, 17 HDACs are known.[17] Although it is not known which HDAC subtypes are inhibited by VPA, its derivatives, or TSA, and which HDAC subtypes are involved in histone H4 acetylation, the present data suggest that ERK 1/2 phosphorylation is likely to be induced by a mechanism independently of HDAC inhibition.

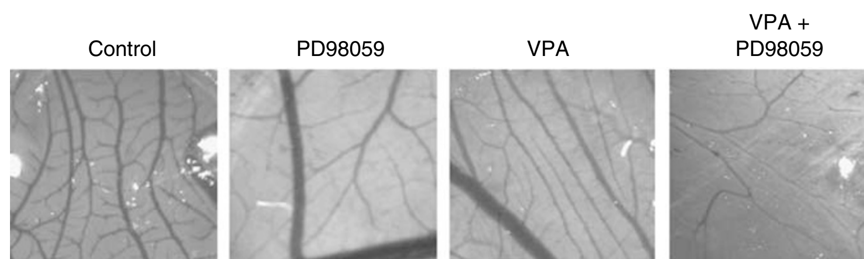

Figure 9 Representative photographs showing vessel formation in the CAM incubated from day 9 to 13 without (control) or with valproic acid (VPA) (1 mM), PD98059 (10 μM), or VPA (1 mM) plus PD98059 (10 μM) ($n = 10$)

The finding that ERK 1/2 phosphorylation and HDAC inhibition may be unrelated events in endothelial cells are in concordance with previous results, where VPA was found to induce ERK 1/2 phosphorylation in Wistar rat thyroid cells independently of HDAC inhibition.[15] In the same report, sodium butyrate, another HDAC inhibiting short-chain fatty acid, was found not to induce ERK 1/2 phosphorylation.[15]

ERK 1/2 phosphorylation was shown to be an integral part of survival pathways in endothelial cells.[23] Therefore, we examined if VPA influences endothelial cell survival under stress conditions established by serum starvation. VPA prevented endothelial cells from serum starvation-induced cell death. Inhibition of ERK 1/2 phosphorylation by the MEK inhibitor PD98059 abrogated VPA-induced protective effects. Moreover, both VPA derivatives, *S*-2-pentyl-4-pentynoic acid, and *R*-2-pentyl-4-pentynoic acid inhibited cell death induced by serum starvation. In contrast, TSA did not protect endothelial cells. Together, these data indicate that VPA inhibits endothelial cell death by induction of ERK 1/2 phosphorylation probably independently of HDAC inhibition. Determination of apoptotic cells after serum starvation revealed that VPA inhibits endothelial cell death by inhibition of apoptosis. Transfection of endothelial cells with constitutively active ERK 2 inhibited apoptosis induced by TSA under serum starvation conditions. This indicates that ERK activation is sufficient to protect endothelial cells from apoptosis induced by serum starvation and HDAC inhibition. In endothelial cells, ERK 1/2 phosphorylates the antiapoptotic protein Bcl-2 and phosphorylation prevents Bcl-2 from ubiquitinylation.[29] Therefore, we investigated the influence of VPA on Bcl-2 phosphorylation. VPA 1 mM induced Bcl-2 phosphorylation, which was reversed by the MEK inhibitor PD98059. This shows that VPA increases Bcl-2 phosphorylation via ERK 1/2 phosphorylation. Bcl-2 is involved in the stabilisation of the mitochondrial membrane thereby preventing cytochrome *c* release from the mitochondria and in turn apoptosis.[32] In concordance, VPA inhibited cytochrome *c* release in endothelial cells in response to serum starvation, which again was reversed by PD98059. This shows that VPA decreases endothelial cell apoptosis by inhibition of mitochondrial cytochrome *c* release and that Bcl-2 phosphorylation may contribute to the stabilisation of the mitochondrial membranes.

A very recent report demonstrated that VPA increased the number of human haematopoietic stem cells in G0/G1-phase and inhibited apoptosis in human haematopoietic stem cells.[33] In concordance, VPA was previously found to inhibit endothelial cell proliferation[11] and we found here an increased number of endothelial cells in G0/G1-phase after VPA treatment. Taken these findings together with the ability of VPA to inhibit endothelial cell apoptosis, effects of VPA on cell cycle and apoptosis are similar to those observed in haematopoietic stem cells.

In addition to endothelial cell survival, ERK 1/2 phosphorylation is regarded to be a proangiogenic event.[23] Therefore, inhibition of ERK 1/2 phosphorylation should favour the antiangiogenic effects of VPA. In concordance, *in vitro* endothelial cell tube formation and vessel formation in the CAM assay were synergistically inhibited by the combination of VPA plus the MEK inhibitor PD98059. Therefore, the influence of VPA on angiogenesis appears to be mediated by two competing pathways. On the one hand, VPA like other HDACIs causes a decrease of cellular eNOS levels in endothelial cells.[11,12] On the other hand, VPA induces HDAC-independent ERK 1/2 phosphorylation, which in principle is a proangiogenic event. Inhibition of ERK 1/2 phosphorylation abrogates the proangiogenic signalling pathway thereby synergistically enhancing the antiangiogenic activity of VPA.

In conclusion, we show that VPA induces ERK 1/2 phosphorylation in endothelial cells thereby protecting endothelial cells from stress-induced apoptosis. In concordance with the fact that ERK 1/2 phosphorylation in endothelial cells represents a proangiogenic event, inhibition of VPA-induced ERK 1/2 phosphorylation synergistically enhances the antiangiogenic activity of VPA. Since small-molecule inhibitors of MEK have entered clinical trials as anticancer agents,[27] this finding may help to design more effective antiangiogenic treatment strategies. Moreover, these results show that VPA, a clinically very well-established drug whose therapeutic use is still increasing since it is becoming the first choice treatment for bipolar disorder worldwide,[34] substantially influences signal transduction and function of the endothelium.

Materials and Methods

Materials

VPA was obtained from Sigma (Taufkirchen, Germany). PD98059, manumycin A, and trichostatin A were purchased from Calbiochem (Darmstadt, Germany). The VPA derivatives *S*-2-pentyl-4-pentynoic acid and *R*-2-pentyl-4-pentynoic acid were synthesised according to the methods described before.[6,35,36] Standard GC-MS purity analysis procedures demonstrate a chemical purity of the derivatives $\geq$98%, and after suitable derivatisation an enantiomeric purity of $\geq$95% enantiomeric access of the chiral compounds. All VPA derivatives used in the *in vitro* experiments were dissolved in dimethylsulphoxide (DMSO) to give stock solutions of 1 M.

Cells

HUVEC were cultivated as described.[11] Cells were seeded onto Matrigel (BD Biosciences, Heidelberg, Germany; diluted 1 : 80 in culture medium)-coated culture flasks and grown in IMDM (Sigma, Taufkirchen, Germany) supplemented with 15% FCS, 5% pooled human serum (Blood Bank of The German Red Cross, Frankfurt am Main, Germany), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 ng/ml bFGF.

Apoptosis assay

Apoptotic cells were detected as described previously[37] using a commercial kit (R&D Systems, Wiesbaden, Germany) following the manufacturer's instructions by flow cytometry (FacsCalibur, BD Biosciences, Heidelberg, Germany). The assay is based on the detection of apoptotic cells using fluoresceinisothiocyanate-conjugated annexin V. Phosphatidylserine is exposed on the outer leaflet of the cytoplasm membrane early during apoptosis. Annexin V binds specifically to phosphatidylserine indicating apoptotic cells.

In addition, active caspase 3 was detected by flow cytometry using an antiactive caspase 3 antibody (BD Biosciences, Heidelberg, Germany) following the manufacturer's instructions.

Cell cycle analysis

Cell cycle was determined using a commercial kit (BD Biosciences, Heidelberg, Germany) following the manufacturer's instructions as described previously.[37] The assay is based on simultaneous detection of incorporated bromodeoxyuridine in the DNA of dividing cells and the determination of the amount of cellular DNA by staining with 7-amino-actinomycin. This combination allows to characterise cells that actively synthesise DNA depending on their position in the cell cycle (i.e. G0/G1, S, or G2/M phases) by flow cytometry.

Cytochrome *c* release

The cytochrome *c* release in apoptotic cells was detected as described by Waterhouse and Trapani.[38] Cells were harvested, washed with cold PBS, pelleted, and permeabilised (50 ng/ml digitonin in PBS) so that cytochrome *c* released from the mitochondria in apoptotic cells could be washed away. Upon fixation with 4% formaldehyde, cells were incubated for 1 h at room temperature with blocking buffer (0.05% saponin, 3% BSA in PBS), followed by incubation overnight at 4°C with primary anticytochrome *c* antibody (6H2.B4, BD Pharmingen, Heidelberg, Germany). After incubation with secondary FITC-labelled anti-mouse antibody for 40 min on ice, the percentage of nonapoptotic cells containing mitochondrial cytochrome *c* was quantified.

Immunoblotting

Cells were lysed in Triton X sample buffer and separated by SDS-PAGE, as described previously.[11] Proteins were detected using specific antibodies against β-actin (Sigma, Taufkirchen, Germany), acetylated histone H4 (Upstate Biotechnology, Lake Placid, NY, USA), ERK 1/2 or the phosphorylated forms of ERK 1/2 (each from New England Biolabs, Frankfurt am Main, Germany), or c-Myc (Santa Cruz, Heidelberg, Germany) and were visualised by enhanced chemiluminescence using a commercially available kit (Amersham, Germany).

Measurement of intracellular Ca^{2+} levels

The determination of intracellular Ca^{2+} levels was performed as described previously.[39] In brief, HUVEC were incubated with 2 μM Fura-2/AM for 30 min at 37°C in PGC buffer (PBS containing 1 mg/ml glucose and 1 mM $CaCl_2$). After washing, cells were transferred into a thermally controlled (37°C) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2, Thermo Spectronic, Rochester, NY, USA) with continuous stirring. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively. Intracellular Ca^{2+} levels were calculated according to the method of Grynkiewicz *et al.*,[40] whereas F_{max} (maximal fluorescence) was obtained by lysing the cells with 0.5% Triton-X 100 and F_{min} by chelating Ca^{2+} with 10 mM EDTA.

Plasmids and Transfection

The c-Myc-tagged constitutively active ERK 2 expression plasmid was kindly provided by Professor Stefanie Dimmeler (Frankfurt, Germany). Plasmids included LacZ (pcDNA3.1 Myc-LacZ) as a control and constitutively active ERK (pcDNA3.1 Myc-D321N ERK2sem). HUVEC (60–70% confluent) were transfected using the SuperFect reagent (Quiagen, Hilden, Germany). Cells were incubated with the DNA-SuperFect complexes at 37°C for 4 h followed by recovery in the presence of 4% FCS. Transfection efficiency was about 30% as determined using green fluorescent protein and maximal levels of protein expression were observed between 24 and 48 h.

In vitro tube formation assay

Endothelial cell tube formation was assessed as described.[11] Briefly, 96-well plates were coated with cold Matrigel (50 μl/well), which was allowed to polymerise at room temperature for about 30 min. Thereafter, 100 μl of a suspension of HUVEC (5×10^4 cells/ml) were seeded onto the Matrigel and cultured overnight in IMDM, supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5% (v/v) FCS. Tube formation was assessed after 12 h and quantified by determining the mean number of branching points in at least three different wells.

Chick chorioallantoic membrane assay

All experiments with chick embryos were carried out *in vivo* as described.[11] A window (7–10 mm in diameter) was cut into the egg shell of 3-day-old embryos, resealed with transparent film and incubated for further 5 days. For the CAM assay, solutions of the substances were mixed with an 1% methylcellulose solution. Aliquots (10 μl) of the resulting 0.5% methylcellulose solution were pipetted onto bacteriological-grade Petri dishes, air-dried for 1 h and the resulting discs were placed onto the 9-day-old CAMs. Two to three discs were placed on each CAM about 10 mm apart. Control discs contained the appropriate solvent. Evaluation of the CAMs was performed 4 days after the application of the disc. To better visualise the vascular system of the CAM, 20% Luconyl Black (BASF, Ludwigshafen, Germany) in PBS was injected into a vitelline vein using glass capillaries. Photographs were taken using a Nikon SMZ1000 stereomicroscope.

Statistics

Results are the mean $\pm$ S.D. of at least three experiments. Comparisons between two groups were performed using Student's *t*-test, three and more groups were compared by ANOVA followed by the Student–Newman–Keuls test. *P*-values lower than 0.05 were considered to be significant.

Acknowledgements

We gratefully acknowledge the support of the 'Hilfe für krebskranke Kinder Frankfurt eV' and its foundation 'Frankfurter Stiftung für krebskranke Kinder'. The work was further supported by the Arthur und Margarete Ebert-Stiftung, the Deutsche Forschungsgemeinschaft, the EU-Research Training Network 'Nutriceptors' Contract HPRN-CT-2002-00268, and by the Edith von Heyden-Vermächtnis.

References

1. Perucca E (2002) Pharmacological and therapeutic properties of valproate: a summary after 35 years of clinical experience. CNS Drugs 16: 695–714
2. Citrome L (2003) Schizophrenia and valproate. Psychopharmacol. Bull. 37: 74–88
3. Belmaker RH (2004) Bipolar disorder. N. Engl. J. Med. 351: 476–486

4. Frediani F (2004) Anticonvulsant drugs in primary headaches prophylaxis. Neurol. Sci. 25: S161–S166
5. Blaheta RA, Michaelis M, Hernáiz Driever P and Cinatl Jr J (2005) The evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. Med. Res. Rev. 2005 [Epub ahead of print]
6. Nau H, Hauck RS and Ehlers K (1991) Valproic acid-induced neural tube defects in the mouse and human: aspects of chirality, alternative drug development, pharmacokinetics and possible mechanisms. Pharmacol. Toxicol. 69: 310–321
7. Nau H (1994) Valproic acid-induced neural tube defects. Ciba Found. Symp. 181: 144–152
8. Sonoda T, Ohdo S, Ohba K, Okishima T and Hayakawa K (1993) Sodium valproate-induced cardiovascular abnormalities in the Jcl:ICR mouse fetus: peak sensitivity of gestational day and dose-dependent effect. Teratology 48: 127–132
9. Thisted E and Ebbesen F (1993) Malformations, withdrawal manifestations, and hypoglycaemia after exposure to valproate *in utero*. Arch. Dis. Child. 69: 288–291
10. Andrews JE, Ebron-McCoy MT, Bojic U, Nau H and Kavlock RJ (1997) Stereoselective dysmorphogenicity of the enantiomers of the valproic acid analogue 2-*N*-propyl-4-pentynoic acid (4-yn-VPA): cross-species evaluation in whole embryo culture. Teratology 55: 314–318
11. Michaelis M, Michaelis UR, Fleming I, Suhan T, Cinatl J, Blaheta RA, Hoffmann K, Kotchetkov R, Busse R, Nau H and Cinatl Jr J (2004) Valproic acid inhibits angiogenesis *in vitro* and *in vivo*. Mol. Pharmacol. 65: 520–527
12. Rössig L, Li H, Fisslthaler B, Urbich C, Fleming I, Forstermann U, Zeiher AM and Dimmeler S (2002) Inhibitors of histone deacetylation downregulate the expression of endothelial nitric oxide synthase and compromise endothelial cell function in vasorelaxation and angiogenesis. Circ. Res. 91: 837–844
13. Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA and Klein PS (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J. Biol. Chem. 276: 36734–36741
14. Göttlicher M, Minucci S, Zhu P, Krämer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG and Heinzel T (2002) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J. 20: 6969–6978
15. Gurvich N, Tsygankova OM, Meinkoth JL and Klein PS (2004) Histone deacetylase is a target of valproic acid-mediated cellular differentiation. Cancer Res. 64: 1079–1086
16. Villar-Garea A and Esteller M (2004) Histone deacetylase inhibitors: understanding a new wave of anticancer agents. Int. J. Cancer 112: 171–178
17. Johnstone RW (2002) Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat. Rev. Drug Discov. 1: 287–299
18. Brunello N (2004) Mood stabilizers: protecting the mood…protecting the brain. J. Affect. Disord. 79: S15–S20
19. Facchetti F, Previdi S, Ballarini M, Minucci S, Perego P and La Porta CA (2004) Modulation of pro- and antiapoptotic factors in human melanoma cells exposed to histone deacetylase inhibitors. Apoptosis 9: 573–582
20. Yuan PX, Huang LD, Jiang YM, Gutkind JS, Manji HK and Chen G (2001) The mood stabilizer valproic acid activates mitogen-activated protein kinases and promotes neurite growth. J. Biol. Chem. 276: 31674–31683
21. Chang F, Steelman LS, Shelton JG, Lee JT, Navolanic PM, Blalock WL, Franklin R and McCubrey JA (2003) Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway. Int. J. Oncol. 22: 469–480
22. Karsan A, Yee E, Poirier GG, Zhou P, Craig R and Harlan JM (1997) Fibroblast growth factor-2 inhibits endothelial cell apoptosis by Bcl-2-dependent and independent mechanisms. Am. J. Pathol. 151: 1775–1784
23. Liu W, Ahmad SA, Reinmuth N, Shaheen RM, Jung YD, Fan F and Ellis LM (2000) Endothelial cell survival and apoptosis in the tumor vasculature. Apoptosis 5: 323–328
24. Zachary I (2003) VEGF signalling: integration and multi-tasking in endothelial cell biology. Biochem. Soc. Trans. 31: 1171–1177
25. Graaf MR, Richel DJ, van Noorden CJ and Guchelaar HJ (2004) Effects of statins and farnesyltransferase inhibitors on the development and progression of cancer. Cancer Treat. Rev. 30: 609–641
26. Hara M, Akasaka K, Akinaga S, Okabe M, Nakano H, Gomez R, Wood D, Uh M and Tamanoi F (1993) Identification of Ras farnesyltransferase inhibitors by microbial screening. Proc. Natl. Acad. Sci. USA 90: 2281–2285
27. Sebolt-Leopold JS and Herrera R (2004) Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat. Rev. Cancer 4: 937–947
28. Gerber HP, Dixit V and Ferrara N (1998) Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J. Biol. Chem. 273: 13313–13316
29. Yang Y and Yu X (2003) Regulation of apoptosis: the ubiquitous way. FASEB J. 17: 790–799
30. Bürkert E, Szellas D, Radmark O, Steinhilber D and Werz O (2003) Cell type-dependent activation of 5-lipoxygenase by arachidonic acid. J. Leukoc. Biol. 73: 191–200
31. Marinissen MJ and Gutkind JS (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol. Sci. 22: 368–376
32. Dias N and Bailly C (2005) Drugs targeting mitochondrial functions to control tumor cell growth. Biochem. Pharmacol. [Epub ahead of print]
33. De Felice L, Tatarelli C, Mascolo MG, Gregorj C, Agostini F, Fiorini R, Gelmetti V, Pascale S, Padula F, Petrucci MT, Arcese W and Nervi C (2005) Histone deacetylase inhibitor valproic acid enhances the cytokine-induced expansion of human hematopoietic stem cells. Cancer Res. 65: 1505–1513
34. Goodwin FK, Fireman B, Simon GE, Hunkeler EM, Lee J and Revicki D (2003) Suicide risk in bipolar disorder during treatment with lithium and divalproex. JAMA 290: 1467–1473
35. Bojic U, Ehlers K, Ellerbeck U, Bacon CL, O'Driscoll E, O'Connell C, Berezin V, Kawa A, Lepekhin E, Bock E, Regan CM and Nau H (1998) Studies on the teratogen pharmacophore of valproic acid analogues: evidence of interactions at a hydrophobic centre. Eur. J. Pharmacol. 354: 289–299
36. Gravemann U (2002) synthesis of achiral, racemic and enantiomerically pure valproic acid derivatives with anticonvulsant, neurotoxic and teratogenic potency. Thesis, University of Hannover
37. Michaelis M, Suhan T, Cinatl J, Hernáiz Driever P and Cinatl Jr J (2004) Valproic acid and interferon-alpha synergistically inhibit neuroblastoma cell growth *in vitro* and *in vivo*. Int. J. Oncol. 25: 1795–1799
38. Waterhouse NJ and Trapani JA (2003) A new quantitative assay for cytochrome *c* release in apoptotic cells. Cell Death Differ. 10: 853–855
39. Werz O, Bürkert E, Samuelsson B, Radmark O and Steinhilber D (2002) Activation of 5-lipoxygenase by cell stress is calcium independent in human polymorphonuclear leukocytes. Blood 99: 1044–1052
40. Grynkiewicz G, Poenie M and Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260: 3440–3450