ORIGINAL ARTICLE

Impact of histone deacetylase inhibitor valproic acid on the anticancer effect of etoposide on neuroblastoma cells

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Submitted: 2012-09-01 Accepted: 2012-11-15 Published online: 2012-12-26

Key words: **etoposide; valproic acid; neuroblastoma; cytotoxicity; cell cycle**

Neuroendocrinol Lett 2012; **33**(Suppl.3):16–24 **PMID:** 23353839 NEL330912A02

Abstract

OBJECTIVES: Etoposide (Vepesid, VP-16), an inhibitor of topoisomerase II, is a chemotherapeutic drug commonly used for treatment of different types of malignant diseases. By inhibiting the topoisomerase II enzyme activity in cancer cells, this drug leads to DNA damage and subsequently to cell death. In this study, we investigated the effect of this anticancer drug alone and in combination with a histone deacetylase (HDAC) inhibitor, valproic acid (VPA), on a human UKF-NB-4 neuroblastoma cell line. **METHODS:** The effects of etoposide and VPA on UKF-NB-4 cells were tested under the normoxic and also the hypoxic (1% O_2) cultivation conditions. The cytotoxicity of etoposide and VPA to a UKF-NB-4 neuroblastoma cell line was evaluated with MTT assay. Apoptosis of the cells was analyzed by flow cytometry using an Annexin V and propidium iodide binding method. The effect of etoposide and VPA on the cell cycle distribution was determined by flow cytometric analysis using propidium iodide staining. **RESULTS:** The results of the study demonstrate that UKF-NB-4 neuroblastoma cells are sensitive both to etoposide and to VPA. They also indicate that the impact of VPA on cytotoxicity of etoposide in these tumor cells varies depending on the sequence of cultivation of the cells with the drugs. As a suitable sequence of cultivation, with a high rate of suppression of neuroblastoma cell growth was found the preincubation of the cells with etoposide, which was followed by their cultivation with VPA. In contrast, the reversed combination (preincubation of the cells with VPA before their treating with etoposide) did not give any increase in etoposide cytotoxicity. The effect of such combined treatment can be explained by measuring the cell cycle distribution, which shows that both etoposide and VPA change the cell cycle phase distribution. **CONCLUSION:** Etoposide and VPA were found as cycle phase specific drugs that are cytotoxic to human UKF-NB-4 neuroblastoma cells used either as single drugs or both together. However, whereas VPA might sensitize the cells to etoposide, inappropriate sequence of cultivation of the cells with VPA can decrease the etoposide cytotoxic efficacy. The results found here warrant further studies of combined treatment of neuroblastoma cells with etoposide with HDAC inhibitors and may help in the design of new protocols geared to the treatment of high risk neuroblastomas.

Abbreviations:

AP-1	- activating protein-1
ATM	- ataxia telangiectasia mutated
ATR	- ataxia telangiectasia and Rad3-related protein
CYP3A4	- cytochrome P450 3A4
ERK	- extracellular-regulated kinase
GSK-3beta	- glycogen synthase kinase-3beta
HDAC	- histone deacetylase
HR	- homologous recombination
CHK	- Csk homologous kinase
IC_{50}	- half maximal inhibitory concentration
IMDM	- Iscove´s modified Dulbecco´s medium
MDR	- multidrug resistance
MTT	- 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NHEJ	- non-homologous end-joining
PKC	- protein kinase C
PPAR	- peroxisome proliferator-activated receptor
VPA	- valproic acid

INTRODUCTION

Etoposide (Vepesid, VP-16) belongs to a component of basic anticancer regimens used in modern oncology. It is a standard part of treatment with highly significant clinical activity against a wide variety of neoplasms (e.g. Small cell lung cancer, Testicular cancer). Etoposide is a semisynthetic derivate of the natural product podophyllotoxin. Of the natural products, podophyllotoxins belong to an important class of compounds extracted from roots and rhizomes of the plants genus Podophyllum (Imbert 1998). Two important compounds were synthesized and selected for clinical trials – etoposide and teniposide (Li *et al.* 2012). Etoposide prevents cells from entering into mitosis, accumulates cells in a G2 phase and is a phase specific cytotoxic drug (Krishan *et al.* 1975). The first significant breakthrough in understanding of a mechanism of etoposide action was realized in 1976, when Loike and Horwitz suggested that etoposide induces single-strand breaks in DNA of HeLa cells and showed that these cells can repair such breaks within 150 minutes (Loike & Horwitz 1976). After this finding, it was also demonstrated that etoposide generates not only single-strand breaks, but the double-strand DNA breaks, too. Furthermore, it was found that DNA breakage can be detected in isolated nuclei, but such DNA damage was not induced in purified DNA (Wozniak & Ross 1983). Therefore, some nuclear enzymes should be involved in generation of breaks in DNA. These observations led to exploring a detailed mechanism of action of etoposide and identifying the main cell target of this drug, topoisomerase II (Chen *et al.* 1984).

DNA topoisomerases solve the topological problems associated with DNA replication, transcription, recombination and chromatin remodeling by introducing temporary single- or double-strand breaks in the DNA in an ATP-dependent reaction (Champoux 2001). The topoisomerase II enzyme changes its conformation particularly due to a binding of ATP, then allows the separation of the two strands of DNA and the passage of a second helix through the enzyme-bound DNA in the produced break. Finally, topoisomerase II religates the cleaved strands *via* the transesterification reaction, followed by a DNA release (Meresse *et al.* 2004). Topoisomerase II is a well established target in cancer therapy and its inhibition by etoposide leads to formation of DNA double-strand breaks and subsequently to cell death (Walker & Nitiss 2002). Besides etoposide, certain other antitumor agents acting as inhibitors of this enzyme are widely used for treatment of neoplasms (e.g. amsacrine, mitoxantrone, doxorubicin) (Nelson *et al.* 1984; Tewey *et al.* 1984; van der Graaf & de Vries 1990). They are targeting topoisomerase II, covalently attach it and increase double-strand breaks in DNA. The exact mechanism resulting from double-strand break formation to cell death is still a matter of debate. Cells react to DNA damage *via* cell cycle arrest, DNA repair or trigger apoptosis. Two processing pathways leading to double-strand break repair have, however, been described recently: non-homologous end-joining (NHEJ) and homologous recombination (HR) (Schonn *et al.* 2010). Etoposide can activate also two important initiators of cell cycle arrest (ataxia telangiectasia mutated – ATM and ataxia telangiectasia and Rad3-related protein – ATR) *via* phosphorylation of Csk homologous kinase (CHK) proteins that can inhibit DNA replication (Agner *et al.* 2005; Tanaka *et al.* 2007). As Schonn and co-workers (Schonn *et al.* 2010) showed in their study, 10 μM etoposide cause massive block in G2/M phase of cell cycle. It was also found that etoposide is approximately threefold more toxic when added to cells in late S and G2 phases than when added in G1 phase (Stacey *et al.* 2000). Another study demonstrated that cells with S-phase DNA content showed about 2–3 times less DNA damage caused by etoposide than cells with G1- or G2/M-phase DNA content (Olive & Banath 1993). Another study detected resistance to etoposide in G1 phase cells (Obata *et al.* 1990).

As it is the case for the most anticancer drug regimens used for treatment of tumor cells, the resistance to etoposide, a major obstacle to the clinical use of etoposide, may develop (Meresse *et al.* 2004). Two main mechanisms of resistance have been identified yet: multidrug resistance (MDR) and specific resistance to topoisomerase II inhibitors by alteration of this enzyme (Robert & Larsen 1998; Schroeder *et al.* 2003). In addition, several mechanisms are based on metabolism of etoposide, which can generate the inactive forms of this drug. One of its major metabolic pathways involves *O*-demethylation to etoposide catechol (etoposide-OH) that is catalyzed by cytochrome P450 3A4 (CYP3A4) (Zheng *et al.* 2006). The data found in *in-vitro* studies utilizing human liver microsomes suggest that CYP3A4 predominantly catalyzes the metabolism of etoposide and also another topoisomerase II inhibitor, ellipticine, thereby dictating their anticancer effects in humans (Zhao *et al.* 1998; Stiborova *et al.* 2010, 2011).

Etoposide is used in monotherapy, but mostly combined with other antitumor agents such as carboplatin, cisplatin or cyclophosphamide (Belani *et al.* 1994).

Recently, a promising chance to combine clinically used drugs with selected inhibitors of histone deacetylase (HDAC) has been found. HDAC inhibitors may contribute to recurrence of the tumor cells by affecting the chromatin structure, thereby increasing the expression of critical tumor suppressor genes [for a summary, see (Stiborova *et al.* 2012)]. Cancer cells have often hypoacetylated histones, due to overexpression of HDACs (Santini *et al.* 2007).

Valproic acid (VPA) has been extensively studied in the last decades because of its potential to inhibit HDACs [for a summary, see (Hrebackova *et al.* 2010)]. VPA can be taken orally and is commonly used for epilepsy treatment. This inhibitor of HDACs increases acetylation of histones H3 and H4, both under the normoxic and the hypoxic conditions (Hrebackova *et al.* 2010). An exact mechanism of anticancer effect of VPA is however still unclear. VPA not only suppresses tumor growth and metastatic processes, but it also induces tumor differentiation and apoptosis. Several mechanisms might be relevant for the biological activity of VPA: VPA increases the DNA binding of activating protein-1 (AP-1) transcription factor, and the expression of genes regulated by the extracellular-regulated kinase (ERK)-AP-1 pathway; VPA downregulates protein kinase C (PKC) activity; it inhibits glycogen synthase kinase-3beta (GSK-3beta), a negative regulator of the Wnt signaling pathway; and it also activates the peroxisome proliferator-activated receptors PPARgamma and delta (Blaheta & Cinatl 2002; Hrebackova *et al.* 2010; Stiborova *et al.* 2012).

Inhibitors of HDACs can also increase the cytotoxicity of topoisomerase II inhibitors (Marchion *et al.* 2004; Marchion *et al.* 2005; Stiborova *et al.* 2012). This phenomenon is not well understood and it seems to be dependent on many factors, being also dependent on a sequence of cultivation of cells with used drugs. The example of the different effects of combination of topoisomerase II inhibitor (etoposide) with an inhibitor of HDACs (VPA) was shown by Das and co-workers (Das *et al.* 2010). These authors used two different neuroblastoma cell lines and different times of their cultivation with the drugs. The influence of another HDAC inhibitor, karenitecin, to increase the effect of inhibitor of topoisomerase I was also found, in the work of Daud *et al.* (2009). The increased effect of treatment of cells with combined regimens containing inhibitors of topoisomerases could be connected with possible changes in levels of topoisomerase I and II mRNA by the HDAC inhibitors. The enhanced expression of genes of DNA topoisomerase II can be associated with increased etoposide sensitivity (Uesaka *et al.* 2007).

Neuroblastoma is the most common extracranial solid tumor in infancy. It originates from undifferentiated cells of the sympaticus nervous system and it is very biologically heterogeneous. Neuroblastomas are ranged to low risk neuroblastoma tumors, which can spontaneously regress, and to high risk neuroblastomas, which are typical with early metastasis and poor prognosis (Brodeur 2003). Despite of a great progress in modern oncology, some forms of neuroblastoma disease are still found very difficult to treat (Brodeur 2003; Poljakova *et al.* 2011). In this study, we investigated the effect of etoposide and VPA and their combination, on a human UKF-NB-4 neuroblastoma cell line.

MATERIAL AND METHODS

Cell cultures and chemicals

The UKF-NB-4 cell line, established from bone marrow metastases of high risk neuroblastoma, was a gift of Prof. J. Cinatl, Jr. (J. W. Goethe University, Frankfurt, Germany). This neuroblastoma cell line was derived from recurrent disease. VPA and etoposide were purchased from Sigma Chemical Co. (MO, USA). All other chemicals used in experiment were of analytical purity or better. Cells were grown at 37 °C and 5% CO_2, cultivated in Iscove´s modified Dulbecco´s medium (IMDM) with 10% fetal bovine serum. For hypoxia experiments, cells were maintained in modular incubator chamber (Billups-Rothenberg Inc., CA, USA) flushed with 1% O_2, 5% CO_2 and balance N_2 at 37 °C (hereafter referred to as hypoxia).

MTT assay

The cytotoxicity of etoposide and valproic acid to UKF-NB-4 cells in exponential growth was determined in a 96-well plate. For a dose-response curve, cells in exponential growth were seeded in 100 µl of medium with 10^4 cells per well. To investigate the effect of etoposide, VPA and their combination on UKF-NB-4 cells, the cells were treated with 0.125–64 µM or 0.125–32 µM for etoposide and 0.25–128 mM VPA. In experiments where both drugs were applied, the preincubation medium containing one of the drugs was removed and replaced with a fresh medium containing both drugs. Tumor cell viability was evaluated by MTT test as previously described (Cinatl *et al.* 1997; Poljakova *et al.* 2011). Briefly, after 72 hours incubation at 37 °C in 5% CO_2 the MTT solution (2 mg/ml PBS) was added, the plates were incubated for 3 hours and cells lysed in solution containing 20% of SDS and 50% *N,N*-dimethylformamide pH 4.5. The absorbance at 570 nm was measured for each well by multiwell ELISA reader Versamax (Molecular Devices, CA, USA). The IC_{50} values were calculated from at least 3 independent experiments using the linear regression of the dose-log response curves by SOFTmaxPro.

Annexin V/propidium iodide labeling

To determine apoptosis, 1.6×10^6 cells were plated in 100 mm dishes and treated with individual drugs or their combinations (using 1 mM VPA and 8 µM etoposide). For detection of apoptosis Annexin V-FITC Apoptosis Detection kit (Biovision, CA, USA) was used according to manufacturer´s instructions and samples

were analyzed using flow cytometry (FACSCalibur BD, San Jose, CA, USA).

Cell cycle analysis

To determine cell cycle distribution, 7×10^5 cells were plated in 60 mm dishes and treated with individual drugs or their combinations for various incubation periods. In experiments where both drugs were applied, the preincubation medium containing one of the drugs was removed and replaced with a fresh medium containing both drugs. After treatment, the cells were collected by trypsinization, washed in PBS, stained using DNA PREP Reagents kit (Beckman Coulter Inc., CA, USA), incubated in the dark for 30 min at room temperature, measured by flow cytometry (FACSCalibur BD, San Jose, CA, USA) and the data were analyzed using ModFit LT software (Verity Software House, ME, USA).

Determination of doubling time

Defined amounts of neuroblastoma cells were cultivated with 1 mM VPA and after three days of cultivation they were collected and counted. Cells were counted using a Bürker chamber. Values of doubling time for control cells and cells cultivated with VPA were determined using a freeware program Doubling time (http://www.doubling-time.com/download.php).

RESULTS

The effect of etoposide on UKF-NB-4 human neuroblastoma cells

The effects of etoposide on UKF-NB-4 cells were evaluated by MTT test after treating these cells with different concentrations of etoposide for 72 hours under the normoxic (aerobic) and the hypoxic conditions. IC_{50} values for the used neuroblastoma cell line determined in three independent experiments are shown in Table 1. Hypoxic culture conditions (1% O_2) reduced etoposide toxicity to this tumor cell line, increasing the IC_{50} values of etoposide from 2.33±0.56 μM to 3.23±0.33 μM (Table 1).

To investigate the mechanism explaining the cytotoxicity of etoposide to neuroblastoma cells, we evaluated the effect of this drug on induction of apoptosis in the cells. Etoposide is capable of inducing apoptosis in UKF-NB-4 cells in time- and concentration-dependent manner. Etoposide-mediated apoptosis in UKF-NB-4 cells was measured using Annexin V and propidium iodid staining at 3, 6, 12, 24 and 48 hours, after addition of 2–8 μM etoposide. The increased levels of apoptotic cells were found after 24 hours of the cells growth in the medium containing 8 μM etoposide and after the 48 hours cell growth in the presence of all used concentrations of etoposide (data not shown).

Cultivation of UKF-NB-4 cells with 8 μM etoposide for 48 hours changed their morphology as compared with that of the control, untreated cells (Figure 1). Cells treated with etoposide, which did not undergo apoptosis, were flatter than normal (untreated) cells and contained an increased amount of vacuoles.

We investigated the effect of etoposide treatment on the cell cycle distribution of UKF-NB-4 neuroblastoma cells. Compared to controls, treatment of cells with various concentration of etoposide resulted in an appreciable arrest of the cells in G2/M and/or S phases of cell

Tab. 1. IC_{50} values for UKF-NB-4 neuroblastoma cells treated with etoposide or VPA.

	IC_{50} for etoposide (μM)		IC_{50} for VPA (mM)	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Exp. I	1.8	3.0	1.7	1.1
Exp. II	3.1	3.7	1.6	1.4
Exp. III	2.1	3.0	1.3	1.1
Mean value	2.33	3.23	1.53	1.20
Stand. dev.	0.56	0.33	0.17	0.14

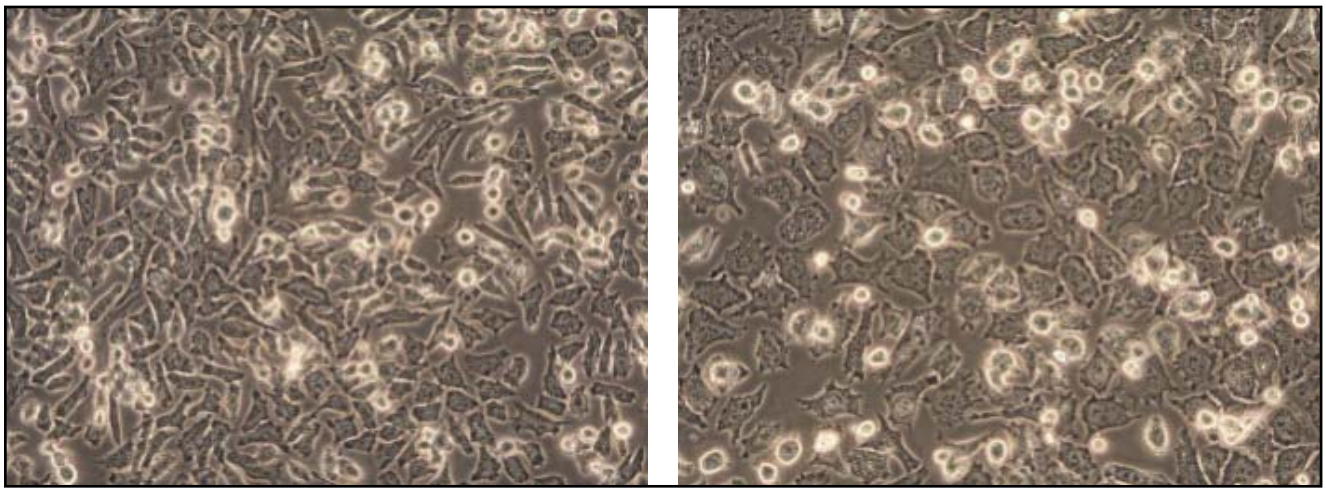

Fig. 1. Neuroblastoma cell line UKF-NB-4 (200 fold magnification) (A) and this cell line treated with 8 μM etoposide for 48 hours (B). Flatter and bigger cells with an increased amount of vacuoles can be seen in UKF-NB-4 cells treated with etoposide (200 fold magnification).

cycle with a concomitant decrease in G0/G1 phase (Figures 2 and 3). These changes in cell cycle were time-dependent, early after adding of etoposide increased percentage of S phase and later it was replaced by increasing of G2/M phase (Figure 3).

The effect of valproic acid on UKF-NB-4 human neuroblastoma cells

The effects of VPA on UKF-NB-4 human neuroblastoma cells were evaluated by MTT test after treating these cells with different concentrations of VPA for 72 hours under the normoxic (aerobic) and the hypoxic conditions. The analyzed neuroblastoma cells were sensitive to VPA. IC_{50} values for the UKF-NB-4 neuroblastoma cell line determined in three independent experiments are shown in Table 1. Hypoxic culture conditions (1% O_2) had essentially no effect on cytotoxicity of VPA to these cells (see the IC_{50} values shown in Table 1).

To determine whether 1 mM VPA induces apoptosis in UKF-NB-4 cells, flow cytometry using Annexin V and propidium iodide staining was used in further experiments. Exposure of the cells to 1 mM VPA for 3–48 hours had practically no effects on viability of the cells (data not shown). However, treatment of UKF-NB-4 neuroblastoma cells resulted in changes of the cell cycle distribution of these cells. Compared to controls, treatment of cells with 1 mM VPA for various time periods resulted in an appreciable arrest of the cells in G2/M phase of cell cycle, mainly after more than 12 hour incubations (Figure 4).

Exposure of UKF-NB-4 cells to 1 mM VPA also led to a ~1.4-fold increase in a doubling time of the cells (data not shown). These results correspond to changes in cell cycle of the cells treated with 1 mM VPA.

The combined effect of etoposide and valproic acid on UKF-NB-4 human neuroblastoma cells

Because VPA is known to modulate response of several tumor cells to chemotherapy using different anticancer drugs, we investigated the ability of this HDAC inhibitor to alter response of UKF-NB-4 neuroblastoma cells to etoposide. The UKF-NB-4 neuroblastoma cells were treated with various concentrations of etoposide and constant concentration of VPA for 72 hours after their 24 hour pretreatment with the drugs under their various combinations. For evaluation of the effect of etoposide combined with VPA on UKF-NB-4 cells, the MTT assay was again used. Several combinations of treating the UKF-NB-4 neuroblastoma cells with the drugs were used: i) the cells were preincubated with 1 mM VPA for 24 hours and then treated with various concentrations of etoposide for 72 hours (combination assigned as V/E), ii) the cells were preincubated with various concentrations of etoposide for 24 hours and then with 1 mM VPA for 72 hours (combination assigned as E/V), iii) the cells were preincubated with various concentrations of etoposide for 24 hours and after that with various concentrations of etoposide in the presence of 1 mM VPA for 72 hours (combination assigned as E/V,E), iv) the cells were preincubated for 24 hours with 1 mM VPA and then treated with various concentrations of etoposide in the presence of 1 mM VPA for 72 hours (combination assigned as V/V,E), v) the cells were treated with medium without any drug added for 24 hours and then treated with various concentrations of etoposide in the presence of 1 mM VPA for 72 hours (combination assigned as 0/V,E) and vi) the cells were treated with medium without any drug added for 24 hours and then treated with various concentrations of etoposide for 72 hours (control incubations with etoposide only) (assigned as 0/E). IC_{50} values for the cells in the presence of etoposide alone or etoposide and VPA under the various incubation combinations with the drugs are shown in Figure 5.

The results found in these experiments indicate that in most cases VPA decreases the value of IC_{50}, thereby increasing the toxic effects of etoposide as compared with the effect of etoposide used for treating the cells

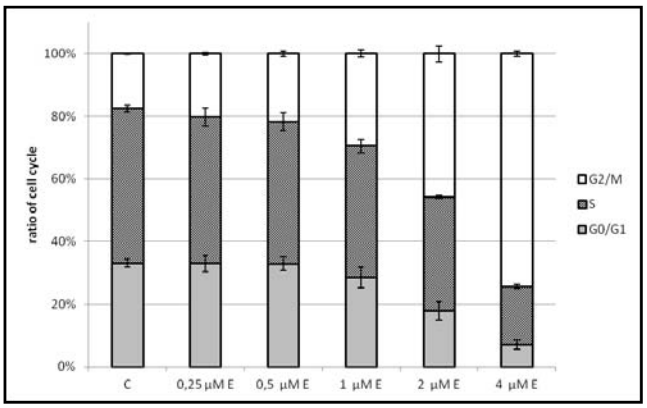

Fig. 2. Effect of various concentrations of etoposide (E) on cell cycle distribution in human UKF-NB-4 neuroblastoma cells after 48 hours etoposide treatment. C – control cells treated with medium without any drug added. The results shown in this figure are means and standard deviations of three experiments.

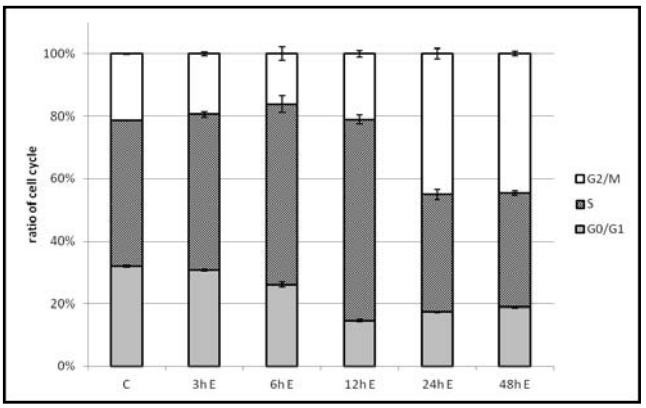

Fig. 3. Effect of 2 μM etoposide (E) on cell cycle distribution in human UKF-NB-4 neuroblastoma cells after 3 - 48 hours etoposide treatment. C – control cells treated with medium without any drug added. The results shown in this figure are means and standard deviations of three experiments.

for 72 hours alone (0/E). These results suggest that VPA sensitized cells to etoposide. However, in the case of pre-incubation of the cells with 1 mM VPA followed by their treating with etoposide (V/E), an increase in the IC_{50} value for etoposide was found (Figure 5). This finding suggests that VPA antagonizes the effect of etoposide on UKF-NB-4 cells at early time points of incubation. Even though we can only speculate how to explain this phenomenon, this sequence of cell incubations seems to slow cell cycle of the cells, thereby decreasing their sensitivity to etoposide.

The effect of combined treatment of UKF-NB-4 neuroblastoma cells with etoposide and VPA under the various combinations of treatment of these cells on their viability was also investigated using the Annexin V and propidium iodide staining. As shown in Figure 6, the most effective combination of the drug treatment to decrease viability of neuroblastoma cells was the combined treatment of cells with etoposide and VPA (cells pretreated with 8 μM etoposide for 24 hours and then treated with 1 mM VPA for 48 hours). These results indicate the synergistic effect of this combined treatment (see E/V in Figure 6).

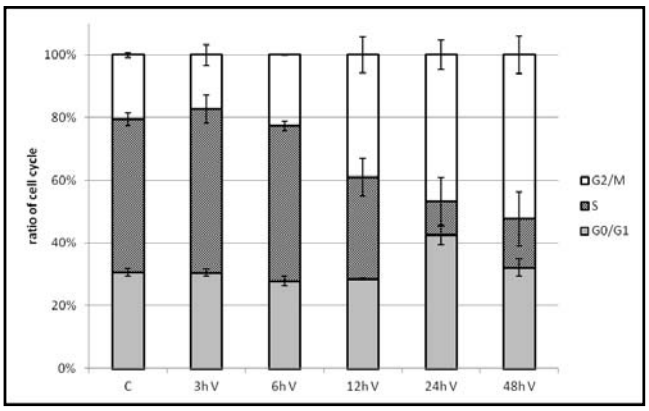

Fig. 4. Effect of 1 mM VPA (V) on cell cycle distribution in human UKF-NB-4 neuroblastoma cells after 3 - 48 hours VPA treatment. C – control cells treated with medium without any drug added. The results shown in this figure are means and standard deviations of three experiments.

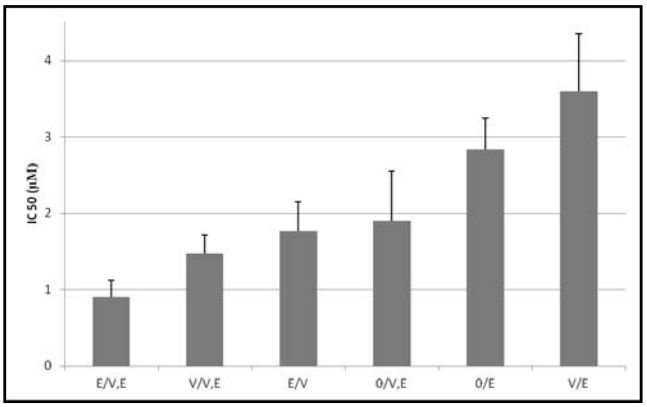

Fig. 5. Cytotoxicity of etoposide (E), VPA (V) and their combinations to human UKF-NB-4 cells expressed as IC_{50} values and standard deviations of three independent experiments determined by the MTT assay. The following combinations of treating the UKF-NB-4 neuroblastoma cells with the drugs were used: i) the cells were preincubated with 1 mM VPA for 24 hours and then treated with various concentrations of etoposide for 72 hours (V/E), ii) the cells were preincubated with various concentrations of etoposide for 24 hours and then with 1 mM VPA for 72 hours (E/V), iii) the cells were preincubated with various concentrations of etoposide for 24 hours and after that with various concentrations of etoposide in the presence of 1 mM VPA for 72 hours (E/V,E), iv) the cells were preincubated for 24 hours with 1 mM VPA and then treated with various concentrations of etoposide in the presence of 1 mM VPA for 72 hours (V/V,E), v) the cells were treated with medium without any drug added for 24 hours and then treated with various concentrations of etoposide in the presence of 1 mM VPA for 72 hours (0/V,E) and vi) the cells were treated with medium without any drug added for 24 hours and then treated with various concentrations of etoposide for 72 hours (control incubations with etoposide only) (0/E).

DISCUSSION

The poor response of high risk children neuroblastoma cancer to current treatment protocols indicates that novel therapeutical strategies are needed to be developed. Therefore, in this study, we investigated whether treatment of human UKF-NB-4 neuroblastoma cells with etoposide combined with the HDAC inhibitor, VPA, will increase the cytotoxicity of these drugs. Utilizing etoposide combined with VPA can, depending on the treatment regimens, both increased cytotoxicity of these drugs and decrease their effects on the treated cells (Das *et al.* 2010). Therefore, we also investigated various treatment regimens of these drugs in combination on UKF-NB-4 cells.

Etoposide, used as one of drugs in the study alone covalently binds to topoisomerase II enzyme stabilizing a cleavage complex, which subsequently leads to generation of double-strand breaks and cell death (Baldwin

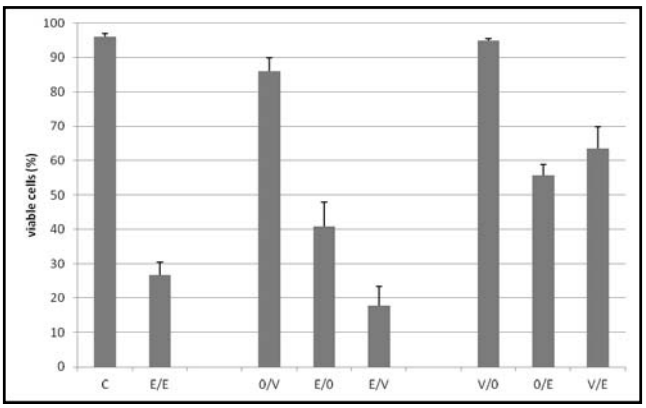

Fig. 6. Viability of neuroblastoma cells expressed as % of viability. C – cells treated with medium without any drug added, after combined treatment with 8 μM etoposide (E) and 1 mM VPA (V). Cells were pretreated for 24 hours either with medium without the drug (0) or with etoposide (E) or VPA (V) and then treated with the compounds for 48 hours. The results shown in this figure are means and standard deviations of three experiments.

& Osheroff 2005). Here, we demonstrate that this drug, when used at therapeutically relevant concentrations (~10 μM), modifies morphology of the UKF-NB-4 neuroblastoma cells. The cells also contain higher amounts of vacuoles. This finding is consistent with results found by Pérez *et al.* (1994), who found similar effects of etoposide on U-937 human promonocytic leukemia cells. This phenomenon can be caused by cell cycle arrest induced by etoposide in the cells, before they undergo apoptosis.

Etoposide kills UKF-NB-4 neuroblastoma cells in a time- and concentration-dependent manner. A high degree of apoptosis is produced by this drug after 48 hours of cultivation of these cells. This anticancer drug also causes an appreciable arrest of the cells in G2/M and/or S phases of cell cycle with a concomitant decrease in G0/G1 phase in UKF-NB-4 cells.

The second drug used in this study, the inhibitor of HDAC, VPA, is known to sensitize several cancer cells to chemotherapeutic agents (Hajji *et al.* 2010; Stiborova *et al.* 2012). This compound increases acetylation of specific lysine residues of chromatin-associated histones H3 and H4, and decreases chromatin compaction thus making DNA more accessible to DNA damaging agents (Stiborova *et al.* 2012). VPA can also influence the levels of DNA repair proteins, induce DNA damage or induce cell cycle arrest (Catalano *et al.* 2005; Kaiser *et al.* 2006; Lee *et al.* 2010; Hrebackova *et al.* 2010; Stiborova *et al.* 2012). As we found in this study, VPA used at the well clinically tolerated concentrations (1 mM) (Hrebackova *et al.* 2010), is cytotoxic to UKF-NB-4 neuroblastoma cells and causes an arrest of the cells in G2/M and later in G0/G1 phase.

In the case of the use of both drugs in combination examined in this study, we found that VPA can influence cytotoxicity of etoposide to a human UKF-NB-4 cell line, when VPA is used in a pre-incubation, co-incubation and post-incubation treatment manner. The preincubation of the cells with etoposide, which was followed by their cultivation with VPA, was found to be a suitable sequence of cultivation producing a high degree of suppression of neuroblastoma cell growth. In contrast, the reversed combination (preincubation of the cells with VPA before their treating with etoposide) did not give any increase in etoposide cytotoxicity. The effect of such combined treatment can be explained by changes of the cell cycle distribution because etoposide is more effective in proliferating S-phase cells. In addition both VPA and etoposide assessed in combination treatments are metabolized by biotransformation enzymes that might influence their cytotoxicity. Moreover, expression levels of transporters that mediate drug efflux can protect cancer cells against both damaging agents. Recently, it has been found that the activities of these enzymes and transporters and their expression can be changed by several HDAC inhibitors (Stiborova *et al.* 2012). Indeed, VPA, is an inducer of CYP3A4 (Cerveny *et al.* 2007; Poljakova *et al.* 2011), which metabolizes etoposide (Kawashiro *et al.* 1998; Zheng *et al.* 2006), and CYP24, the enzyme important in vitamin D homeostasis (Vrzal *et al.* 2011). Likewise, one transporter protein that mediates drug elimination from cells, P-glycoprotein (encoded by MDR gene), is induced by VPA (Eyal *et al.* 2006; Cerveny 2007; Poljakova *et al.* 2011).

The effects of VPA combined with etoposide on other types of human neuroblastoma cells, SK-N-AS and SK-N-SH cells, was investigated by Das *et al.* (2010). The effect of this combined treatment was also dependent on exposure regimens. Although the combination of drugs modestly enhanced cell-death in SK-N-SH cells at longer incubation periods (72 and 96 hours), it caused a decline in etoposide-mediated cell killing at shorter exposure times (48 hours). In contrast, VPA synergistically increased the cytotoxicity of etoposide in SK-N-AS cells. The modest increase in topoisomerase-II β expression upon VPA treatment was suggested by Das *et al.* (2010) to potentially explain the additive increase in cell-death in SK-N-SH cells in the presence of both agents.

In summary, the combination of etoposide and VPA increases their anti-tumor effects on human UKF-NB-4 neuroblastoma cells. The results found suggest that impact of VPA on anticancer effect of etoposide on these cells depends on a variety of factors, mainly on the sequence of cultivation of the cells with these drugs. Nevertheless, because the combination of both drugs at the clinically relevant doses and in suitable sequence regimens increases their toxic effects on human neuroblastoma UKF-NB-4 cells, such combination regimens might be utilized for treatment of patients suffering from high risk neuroblastomas.

ACKNOWLEDGEMENTS

This study was supported by GACR (grant P301/10/0356), Charles University (grants 635712/2012 and UNCE 204025/2012) and by the project for conceptual development of research organization 00064203. We thank to Prof. J. Cinatl, Jr. (J. W. Goethe University, Frankfurt, Germany) for providing cell lines.

Potential Conflicts of Interest: *None disclosed.*

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