ORIGINAL ARTICLE

Anticancer agent ellipticine combined with histone deacetylase inhibitors, valproic acid and trichostatin A, is an effective DNA damage strategy in human neuroblastoma

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Abstract

OBJECTIVES: Valproic acid (VPA) and trichostatin A (TSA) exert antitumor activity as histone deacetylase inhibitors, whereas ellipticine action is based mainly on DNA intercalation, inhibition of topoisomerase II and formation of cytochrome P450 (CYP)- and peroxidase-mediated covalent DNA adducts. This is the first report on the molecular mechanism of combined treatment of human neuroblastoma UKF-NB-3 and UKF-NB-4 cells with these compounds.
METHODS: HPLC with UV detection was employed for the separation and characterization of ellipticine metabolites formed by microsomes and peroxidases. Covalent DNA modifications by ellipticine in neuroblastoma cells and in incubations with microsomes and peroxidases were detected by ^{32}P-postlabeling. Expression of CYP enzymes, peroxidases and cytochrome b_5 was examined by Western blot.
RESULTS: The cytotoxicity of ellipticine to neuroblastomas was increased by pretreating these cells with VPA or TSA. A higher sensitivity of cells to ellipticine correlated with an increase in formation of covalent ellipticine-derived DNA adducts in these cells. To evaluate the mechanisms of this finding, we investigated the modulation by VPA and TSA of CYP- and peroxidase-mediated ellipticine-derived DNA adduct formation *in vitro*. The effects of ellipticine in the presence of VPA and TSA on expression of CYPs and peroxidases relevant for ellipticine activation and levels of cytochrome b_5 and P-glycoprotein in neuroblastoma cells were also investigated. Based on these studies, we attribute most of the enhancing effects of VPA and TSA on ellipticine cytotoxicity to enhanced ellipticine-DNA adduct formation caused by an increase in levels of cytochrome b_5, CYP3A4 and

CYP1A1 in neuroblastoma cells. A lower sensitivity of UKF-NB-4 cells to combined effects of ellipticine with VPA and TSA than of UKF-NB-3 cells is also attributable to high levels of P-glycoprotein expressed in this cell line.

CONCLUSION: The results found here warrant further studies and may help in the design of new protocols geared to the treatment of high risk neuroblastomas.

Abbreviations:

AhR	- aryl hydrocarbon receptor
COX	- cyclooxygenase
CYP	- cytochrome P450
DMSO	- dimethyl sulfoxide
GAPDH	- glyceraldehyde phosphate dehydrogenase
HDAC	- histone deacetylase
HPLC	- high-performance liquid chromatography
IMDM	- Iscove's modified Dulbecco's medium
LPO	- lactoperoxidase
MPO	- myeloperoxidase
MTT	- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide
NADP+	- nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	- nicotinamide adenine dinucleotide phosphate (reduced)
PBS	- phosphate buffered saline
PEI-cellulose	- polyethylenimine-cellulose
RAL	- relative adduct labeling
r.t.	- retention time
SDS	- sodium dodecyl sulphate
TLC	- thin layer chromatography
TSA	- trichostatin A
VPA	- valproic acid

INTRODUCTION

Neuroblastoma, a tumor of the peripheral sympathetic nervous system, is the most frequent solid extra cranial neurological tumor in children and is a major cause of death from neoplasia in infancy (Maris & Mathay 1999; Schwab 1999). These tumors are biologically heterogeneous, with cell populations differing in their genetic programs, maturation stage and malignant potential (Brodeur 2003). Neuroblastoma consists of three principal neoplastic cells (Voigt *et al.* 2000; Hopkins-Donaldson *et al.* 2002): i) neuroblastic or N-type: undifferentiated, round and small cells with scant cytoplasm and neuritic processes; ii) stromal or S-type: large, flattened and adherent differentiated cells; and iii) intermediate or I-type with morphological features of both above mentioned types, i.e. cells with short neurite-like processes such as adherent growth. As neuroblastoma cells seem to have the capacity to differentiate spontaneously *in vivo* and *in vitro* (Morgenstern *et al.* 2004), their heterogeneity could affect treatment outcome, in particular the response to apoptosis induced by chemotherapy. Neuroblastoma may regress spontaneously in infants, mature to benign ganglioneuromas, or grow relentlessly and be rapidly fatal (Brodeur 2003). Prognosis of patients with high risk tumors is poor, in spite of intensive therapy including megatherapy with subsequent hematopoietic progenitor cell transplantation, biotherapy and immunotherapy because drug resistance arises in the majority of those patients who initially responded to chemotherapy (Brodeur 2003). Little improvement in therapeutic options has been made in the last decade, requiring a need for the development of new therapies.

Recently, we have suggested novel treatment of neuroblastomas, utilizing a drug targeting DNA, the plant alkaloid ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*] carbazole, Figure 1). We have found that exposure of human neuroblastoma IMR-32, UKF-NB-3 and UKF-NB-4 cell lines to this agent resulted in strong inhibition of cell growth, followed by induction of apoptosis (Poljakova *et al.* 2009). These effects were associated with formation of two covalent ellipticine-derived DNA adducts, identical to those formed by the cytochrome P450 (CYP)- and peroxidase-mediated ellipticine metabolites, 13-hydroxy- and 12-hydroxyellipticine (Figure 1). In addition, other mechanisms, such as intercalation into DNA (Singh *et al.* 1994; Auclair 1987), and inhibition of DNA topoisomerase II activity (Auclair 1987; Monnot *et al.* 1991; Fossé *et al.* 1992; Froelich-Ammon *et al.* 1995) resulting in ellipticine toxicity to neuroblastoma cannot be excluded (for a summary see Stiborova *et al.* 2001; 2006c; 2011). The levels of covalent ellipticine-derived DNA adducts correlated with ellipticine toxicity in IMR-32 and UKF-NB-4 cell lines. In addition, hypoxic cell culture conditions resulted in a decrease in ellipticine toxicity to these cells and this correlated with lower levels of DNA adducts. Cells of both lines accumulated in S phase, suggesting that ellipticine-DNA adducts interfere with DNA replication. We therefore concluded that formation of ellipticine-DNA adducts was the predominant DNA-damaging mechanism of ellipticine action, resulting in its strong cytotoxicity to these neuroblastoma cells (Poljakova *et al.* 2009).

Because epigenetic modifications of chromatin play a role in the origin of human neuroblastomas, pharmaceutical manipulation of the epigenome may offer treatment options for this type of cancer (Furchert *et al.* 2007). Histone deacetylases (HDAC) and histone acetyl transferases modify histone proteins and contribute to an epigenetic code recognized by proteins involved in regulation of gene expression (Blaheta & Cinatl 2002; Blaheta *et al.* 2002; Marks *et al.* 2003; 2004; Hooven *et al.* 2005; Hrebackova *et al.* 2010). Indeed, former studies demonstrated the cytotoxicity of HDAC inhibitors to several neuroblastoma cells, resulting in growth inhibition of these tumor cells (Cinatl *et al.* 1996; Furchert *et al.* 2007; Michaelis *et al.* 2004; 2007). In neoplastic cells, where overexpression of different HDACs was frequently detected (for summary see Bolden *et al.* 2006; Hrebackova *et al.* 2010), the abundance of deacetylated histones is usually associated with DNA hypermethylation and gene silencing (Santini *et al.* 2007). Treatment with HDAC inhibitors induced the reactivation

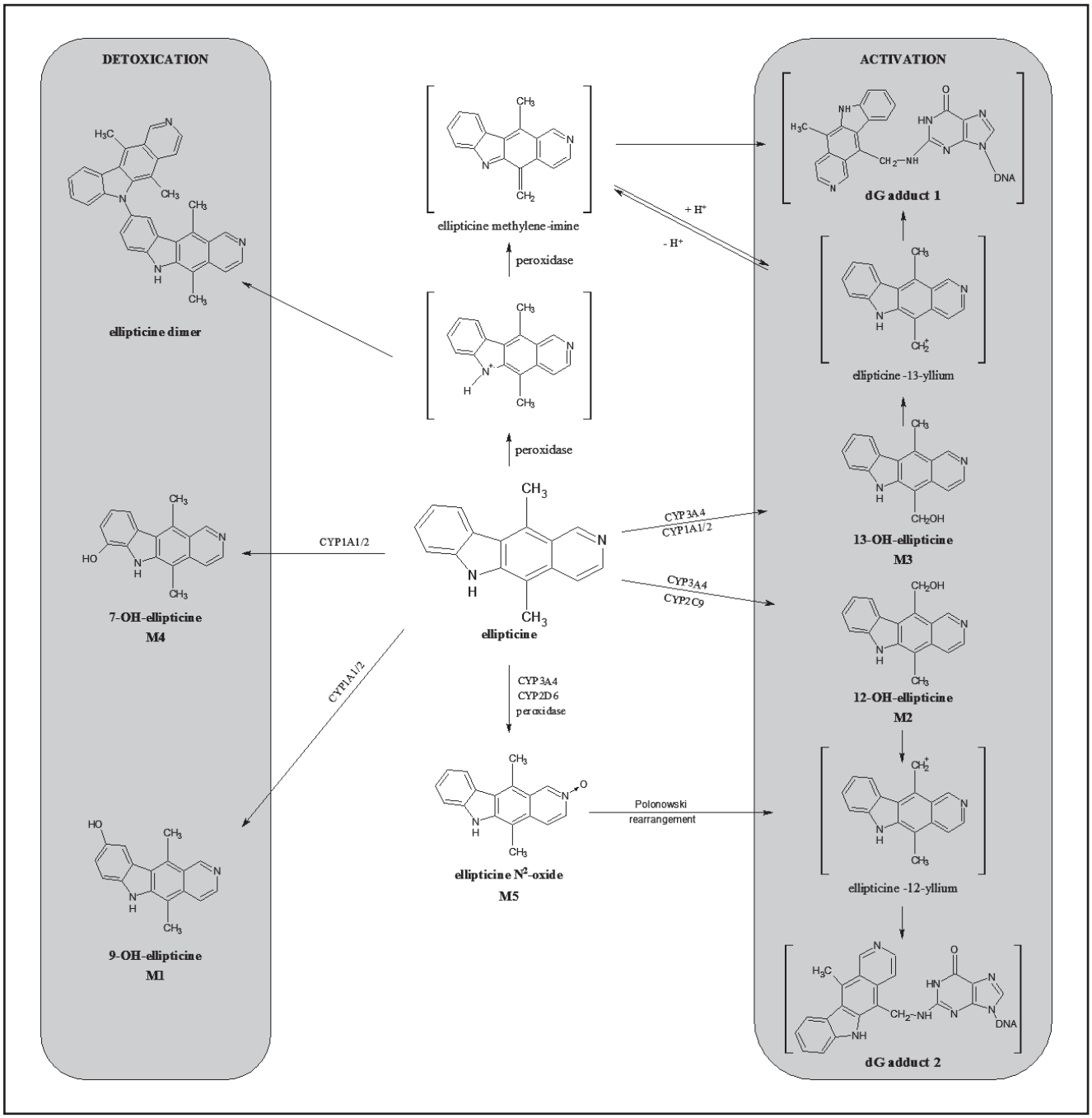

Fig. 1. Scheme of the metabolism of ellipticine by human CYPs and peroxidases showing the characterized detoxication metabolites and those proposed to form DNA adducts. The compounds shown in brackets are the hypothetical electrophilic metabolites postulated as ultimate arylating species or the postulated N^2-deoxyguanosine adducts.

of growth regulatory genes and consequently apoptosis in these cells. The HDAC inhibitor valproic acid (VPA) inhibits growth and induces differentiation of UKF-NB-2 and UKF-NB-3 human neuroblastoma cells *in vitro* at concentrations ranging from 0.5 to 2 mM, plasma concentrations that have been achieved in humans with no significant adverse effects. Nevertheless, it should be noted that common therapeutic concentrations of VPA are lower (Cinatl *et al.* 1996; Hrebackova *et al.* 2009).

Ultrastructural features of VPA-treated cells were consistent with the neuronal differentiation and are associated with decreased expression of N-myc oncoprotein and increased expression of neutral cell adhesion molecule in their membrane. In these cells VPA synergized interferon-alpha treatment leading to a massive accumulation of cells in G0/G1-phase. A synergism which was confirmed *in vivo*, where VPA plus interferon-alpha inhibited growth of UKF-NB-3 xenografts in nude mice and leading to complete cures in two out

of six animals, while single treatments merely inhibited tumor growth (Michaelis *et al.* 2004). Moreover, VPA combined with ABT-510, a peptide derivative of the natural angiogenic inhibitor thrombospondin-1, was even more efficient to inhibit neuroblastoma tumor growth and angiogenesis, suggesting that this combination may be an effective antiangiogenic treatment strategy for children with high-risk neuroblastoma (Yang *et al.* 2007). VPA also influences the resistance of neuroblastoma cells to several chemotherapeutic agents (Blaheta *et al.* 2007). This drug reverts the enhanced adhesion of drug-resistant UKF-NB-2, UKF-NB-6 and SK-N-SH neuroblastoma cells accompanied by diminished N-myc and enhanced p73 protein levels (Blaheta *et al.* 2007).

Besides VPA, another HDAC inhibitor, trichostatin A (TSA), was found to inhibit growth of neuroblastoma cells, at concentration as low as 50–250 nM (Hrebackova *et al.* 2009). This HDAC inhibitor in combination with anticancer drugs that act on DNA such as vepesid, ellipticine, doxorubicin, epirubicin, and cis-platin was recently found to enhance the efficacy of these in several tumor cells (Kim *et al.* 2003). The mechanisms are, however, unknown as well as the activity against neuroblastomas in combination with DNA damaging agents.

In this study we describe that pre-treatment of human neuroblastoma cells with the HDAC inhibitors, VPA or TSA, increases the toxicity of ellipticine to these human neuroblastoma cells and that this higher sensitivity to ellipticine correlates with an increase in formation of ellipticine-derived DNA adducts. In addition, we examined the molecular mechanisms of such effects in these neuroblastoma cells. Because neuroblastoma is heterogenous and this feature could affect its treatment, two types of neuroblastoma cell lines were tested for their response to combined treatment by ellipticine with VPA or TSA, UKF-NB-3 cells (the invasive N-type), and UKF-NB-4 cells (the less-aggressive S-type).

MATERIALS AND METHODS

Chemicals

Ellipticine, $NADP^+$ and NADPH were from Sigma Chemical Co. (St. Louis, MO, USA). Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained from sources described (Stiborova *et al.* 2001; 2003a; 2003b; 2004). 12-Hydroxy- and 13-hydroxyellipticine were isolated from multiple HPLC runs of ethyl acetate extracts of incubations containing ellipticine and human and/or rat hepatic microsomes as described (Stiborova *et al.* 2004). The human hepatic microsomal sample was a pooled sample obtained from Gentest corp. (Woburn, MA, USA) (catalog no. H161). Rat hepatic microsomes (0.6 nmol CYP/mg protein) were isolated as described (Stiborova *et al.* 2001; 2006a). The experiments were conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which complies with the Declaration of Helsinki. Bovine lactoperoxidase (LPO; 117 purporogallin units/mg protein, 13 guaiacol units/mg protein), human myeloperoxidase (MPO; 105 purporogallin units/mg protein, 11 guaiacol units/mg protein), and ovine cyclooxygenase 1 (prostaglandin H synthase 1, COX-1; 44371 oxygen consumption units/mg protein, one units consumes one nmole of O_2/min in the presence of 100 μM arachidonic acid, 2 mM phenol and 1 μM hematin) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All these and other chemicals used in the experiments were of analytical purity or better.

Cell cultures

The UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines, established from bone marrow metastases of high risk neuroblastoma, were a gift of Prof. J. Cinatl, Jr. (J. W. Goethe University, Frankfurt, Germany). UKF-NB-4 was derived from recurrent disease. Both two cell lines used were derived from high risk neuroblastoma with MYCN amplification, del1p and aneuploidy. Cells were grown at 37 °C and 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) (Lonza Inc, Allndale, NJ, USA), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml of penicilline and 100 μg/ml streptomycine (PAA Laboratories, Pasching, Austria).

MTT assay

The cytotoxicity of ellipticine to 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. Solution of ellipticine in dimethyl sulfoxide (DMSO) (1 μl) in final concentrations of 0.02–50 μM was added. To investigate the effects of VPA and TSA on ellipticine-cytotoxicity, neuroblastoma cells were pre-treated with 0.5–2.0 mM VPA (dissolved in IMDM) or 0.1–0.2 μM TSA (dissolved in DMSO) 24 h before adding ellipticine. Control cells and medium controls without cells received 1 μl of DMSO without drug. Tumor cell viability was evaluated by MTT test as previously described (Cinatl *et al.* 1997). Briefly, after incubation (3 days) at 37 °C in 5% CO_2 the MTT solution (2 mg/ml PBS) was added, the plates were incubated for 4 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 mean absorbance of medium controls was the background and was subtracted. The absorbance of control cells was taken as 100% viability and the values of treated cells were calculated as a percentage of control. Each value is the mean of 8 wells with standard deviations. The IC_{50} values were calculated from at least 3 independent experiments using the linear regression of the dose-log response curves by SOFTmaxPro.

Microsomal incubations

Incubation mixtures used to analyse DNA adduct formation by ellipticine *in vitro* consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, human or rat hepatic microsomes (0.5 mg protein), 0.1 mM ellipticine (dissolved in 7.5 µl methanol) and 0.5 mg of calf thymus DNA in a final volume of 750 µl. To investigate the effect of VPA and TSA on ellipticine-derived DNA adduct formation, 0.1 or 1.0 mM VPA (dissolved in distilled water) or 0.1 µM TSA (dissolved in 5 µl DMSO) were added into the incubation mixtures. Incubations were carried out at 37 °C for 30 minutes; ellipticine-DNA adduct formation was found to be linear up to 30 min of incubation (Stiborova *et al.* 2006a; 2010). Control incubations were carried out (*i*) without microsomes, (*ii*) without NADPH, (*iii*) without DNA, (*iv*) without ellipticine, and (*v*) without VPA and TSA. After the incubation, DNA was isolated by a standard phenol-chloroform extraction method and analyzed for DNA adduct formation by ^{32}P-postlabeling (see below).

Incubation mixtures used to form the ellipticine metabolites contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase (NADPH-generation system), 0.2 mg protein of rat hepatic microsomes and 10 µM ellipticine (dissolved in 5 µl methanol) in a final volume of 500 µl. The reaction was initiated by adding the substrate. To investigate the effect of VPA and TSA on ellipticine oxidation, 0.1, 0.5 or 1.0 mM VPA (dissolved in distilled water) or 50 and 100 nM TSA (dissolved in 5 µl DMSO) were added into the mixtures. In the control incubation, ellipticine was omitted from the incubation mixture. After incubation in open glass tubes (37 °C, 20 min) the reaction was stopped by adding 100 µl of 2 M NaOH. The oxidation of ellipticine is linear up to 30 min of incubation (Stiborova *et al.* 2006a). After incubation, 5 µl of 1 mM phenacetine in methanol was added as an internal standard and the ellipticine metabolites were extracted twice with ethyl acetate (2 × 1 ml). Analyses of ellipticine metabolites were performed by HPLC as described (Stiborova *et al.* 2004; 2006a). Recoveries of ellipticine metabolites were around 95%.

Peroxidase incubations

Incubation mixtures used to quantify DNA adducts by ellipticine activated with peroxidases (LPO, MPO and COX-1) contained 50 mM potassium phosphate buffer (pH 7.4), 100 µM ellipticine dissolved in 7.5 µl methanol, 10 µg of peroxidase and 1 mg of calf thymus DNA in a final volume of 750 µl. In incubations with LPO and MPO, 100 M hydrogen peroxide was added as a cofactor. In incubations with COX-1, 100 µM arachidonic acid was added as a cofactor, in addition to hematin (1 µM) and $MgCl_2$ (5 mM). To investigate the effects of VPA and TSA on ellipticine-derived DNA adduct formation, 0.1 and 1.0 mM VPA (dissolved in distilled water) or 100 nM TSA (dissolved in 5 µl DMSO) were added into the incubation mixtures. Control incubations were without peroxidases, without hydrogen peroxide or without arachidonic acid, without DNA, without ellipticine or without VPA or TSA. Incubations with peroxidases were carried out at 37 °C for 30 min. All reactions were initiated by adding ellipticine. After the incubation and ethyl acetate extraction, DNA was isolated by a standard phenol-chloroform extraction method and analyzed for DNA adduct formation by ^{32}P-postlabeling (see below).

Incubation mixtures used to analyze ellipticine metabolites generated by peroxidase (LPO was used as a model) with a final volume of 500 µl consisted of 50 mM potassium phosphate buffer (pH 7.4), 10 µM ellipticine (dissolved in 5 µl methanol), 10 µg of LPO and hydrogen peroxide (20 µM). To investigate the effects of VPA and TSA on ellipticine oxidation by LPO, 0.1, 0.5 and 1.0 mM VPA (dissolved in distilled water) or 50 and 100 nM TSA (dissolved in 5 µl DMSO) were added into the incubation mixtures. Control incubations were without peroxidases, without hydrogen peroxide, without ellipticine, or without HDAC inhibitors. Incubations were carried out at 37 °C for 30 min. All reactions were initiated by adding ellipticine dissolved in methanol (final concentration of methanol was 1%). After incubations, 5 µl of 1 mM salicylamide in methanol was added as an internal standard, ellipticine and its metabolite extracted twice with ethyl acetate (2 × 1 ml) as described (Poljakova *et al.* 2005; Stiborova *et al.* 2007a). The extracts were evaporated under nitrogen and dissolved in 50 µl of methanol. Ellipticine and its metabolite were separated by HPLC. The column used was a 5 µm Ultrasphere ODS (Beckman, 4.6 × 250 mm) preceeded by a C-18 guard column. The eluent was 45–90% methanol in 10 mM ammonium acetate (pH 2.8), with flow rate of 0.8 ml/min, detection was at 296 nm (Poljakova *et al.* 2005; Stiborova *et al.* 2007a). Only one product peak with a retention time (r.t.) of 19.95 min corresponding to the ellipticine dimer (Stiborova *et al.* 2007a) and unconverted ellipticine with r.t. of 11.85 min were separated by HPLC. Recovery of the ellipticine dimer was around 95%.

Estimation of contents of CYPs, peroxidases and cytochrome b_5 in neuroblastoma cells

To determine the expression of cytochrome b_5, CYP1A1, 1B1 and 3A4, LPO and COX-1 proteins, cells were homogenized in 25 mM Tris-HCl buffer pH 7.6 containing 150 mM NaCl, 1% detergent NP-40 (Sigma, St. Louis, MO, USA), 1% sodium deoxycholate, 0.1% SDS and with solution of COMPLETE (protease inhibitor cocktail tablet, Roche, Basel, Swizerland) at concentration described by provider. The homogenates were centrifuged for 20 min at 14 000 g and supernatant was used for additional analysis. Protein concentrations were assessed using the DC protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard, and 10–45 µg of extracted proteins were sub-

jected to SDS-PAGE electrophoresis on a 11% gel for analysis of CYP1A1, 1B1 and 3A4, LPO and COX-1 protein expression, and a 17% gel for analysis of cytochrome b_5 protein expression (Stiborova *et al.* 2002; 2005). After migration, proteins were transferred to a nitrocellulose membrane and incubated with 5% non-fat milk to block non-specific binding. The membranes were then exposed to specific rabbit polyclonal anti-cytochrome b_5 (1:750, AbCam, MA, USA), anti-CYP1A1 (1:1000, Millipore, MA, USA), anti-CYP1B1 (1:500, AbCam, MA, USA), anti-CYP3A4 (1:5000, AbD Serotec, Oxford, UK), anti-LPO (2 μg/ml, AbCam, MA, USA) and anti-COX-1 (1:1000, AbCam, MA, USA) antibodies overnight at 4 °C. Membranes were washed and exposed to peroxidase-conjugated anti-IgG secondary antibody (1:3 000, Bio-Rad, Hercules, CA, USA), and the antigen-antibody complex was visualized by enhanced chemiluminiscence's detection system according to the manufacturer's instructions (Immun-Star HRP Substrate, Bio-Rad, Hercules, CA, USA). X-Rays films were from MEDIX XBU (Foma, Hradec Kralové, Czech Republic). Antibody against glyceraldehyde phosphate dehydrogenase (GAPDH) (1:750, Millipore, MA, USA) was used as loading control.

Treatment of neuroblastoma cells with ellipticine for DNA adduct analyses

Neuroblastoma cell lines were seeded 24 h prior to treatment at a density of 5×10^5 cells/ml in two 75 cm^2 culture flasks in a total volume of 20 ml of IMDM. Ellipticine was dissolved in 5 μl of DMSO, the final concentration was 0, 1 or 10 μM. After 48 h the cells were harvested after trypsinizing by centrifugation at 2 000 × g for 3 min and two washing steps with 5 ml of PBS yielded a cell pellet, which was stored at –80 °C until DNA isolation. DNA was isolated and labeled as described in the next section.

An analogous procedure was used to evaluate the effect of pre-treatment of neuroblastoma cells with VPA or TSA prior to adding ellipticine. In this case, cells were pre-treated with 0.5–2 mM VPA (VPA was dissolved in IMDM) or 0.1 μM TSA (dissolved in 5 μl DMSO) for 24 h before adding ellipticine. Further procedures were the same as described above.

DNA isolation and ^{32}P-postlabeling of DNA adducts

DNA from cells was isolated by the phenol-chloroform extraction as described (Frei *et al.* 2002; Borek-Dohalska *et al.* 2004; Poljakova *et al.* 2007; 2009). The ^{32}P-postlabeling of nucleotides using nuclease P1 enrichment procedure, found previously to be appropriate to detect and quantify ellipticine-derived DNA adducts formed *in vitro* (Stiborova *et al.* 2001; 2004; 2007a; Frei *et al.* 2002; Martinkova *et al.* 2009; 2010) and *in vivo* (Stiborova *et al.* 2003a; 2007b; 2008; 2011). From experiments performed earlier, calf thymus DNA incubated with 13-hydroxy- and 12-hydroxyellipticine (Stiborova *et al.* 2004; 2007a) and DNA of breast adenocarcinoma of rats treated i.p. with 4 mg ellipticine per kilogram body weight (Stiborova *et al.* 2011) were labeled with ^{32}P to compare adduct spot patterns.

Animal experiments

Female Wistar rats bearing the *N*-methyl-*N*-nitrosourea induced mammary adenocarcinoma (McCormick *et al.* 1981) were i.p. treated with 4 mg ellipticine per kilogram body weight. Ellipticine was administered dissolved in 1% acetic acid at a concentration of 2.5 mg/ml as described previously (Stiborova *et al.* 2008). One day after ellipticine treatment, the DNA from tumor tissues was isolated and analyzed for formation of DNA adducts using the nuclease P1 version of the ^{32}P-postlabeling assay as described (Stiborova *et al.* 2003a; 2007b; 2008; 2011). All experiments with animal models were conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki.

HPLC analysis of ^{32}P-labeled DNA adducts

HPLC analysis was performed essentially as described previously (Stiborova *et al.* 2003a). Individual spots detected by ^{32}P-postlabeling were excised from the thin layer plates and extracted with two 800 μl portions of 6 M ammonium hydroxide/isopropanol (1:1) for 40 min (Stiborova *et al.* 2003a). The eluent was evaporated in a Speed-Vac centrifuge, and the dried extracts dissolved in 100 μl of methanol/phosphate buffer (pH 3.5) 1:1 (v/v). Aliquots (50 μl) were analyzed on a phenyl-modified reversed-phase column (250 mm × 4.6 mm, 5 μm Zorbax Phenyl; Säulentechnik Knauer, Germany) with a linear gradient of methanol (from 40 to 80% in 45 min) in aqueous 0.5 M sodium phosphate and 0.5 M phosphoric acid (pH 3.5) at a flow rate of 0.9 ml/min. Radioactivity eluting from the column was measured by monitoring Cerenkov radiation on a Berthold LB 506 C-I flow-through radioactivity monitor (500 μl cell, dwell time 6 s) and integrated with the Borwin software (JMBS Developments, Grenoble, France).

Detection of P-glycoprotein by flow cytometry

P-glycoprotein expression was detected by flow cytometry using monoclonal antibody anti CD243 PE labeled (Beckmann Coulter, Nyon, Switzerland). Cultivation was performed in 12-well plates, three samples from every well were prepared and two wells measured. The fluorescence intensity of at least 10,000 cells was measured by FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with 488 nm laser and list mode data were analyzed with the CellQuest software (Lanza *et al.* 1997). Expression was evaluated as mean intensity of fluorescence. The fluorescence measurements were calibrated for each run by FITC-conjugated bead standards (DAKO cat. no. K0110). Results were expressed as mean of six determinations ± standard deviation.

Statistical analyses

The data were analyzed statistically by Student's *t*-test. All *p*-values are two-tailed and considered significant at the 0.05 level.

RESULTS

VPA and TSA increase cytotoxicity of and DNA adduct formation by ellipticine in human neuroblastoma cells

To determine the effect of VPA and TSA on cytotoxicity of ellipticine to human neuroblastoma cells, these were treated with increasing concentrations of ellipticine without or with pre-treatment with HDAC inhibitors. The VPA and TSA concentrations used for cell pre-treatment were close to their respective IC_{50} values to neuroblastoma cells (Hrebackova *et al.* 2009). The pre-treatment of UKF-NB-3 and UKF-NB-4 cells with either HDAC inhibitor made cells more sensitive to ellipticine. The decrease in IC_{50} values for ellipticine caused by both compounds in UKF-NB-3 and UKF-NB-4 cells was dose-dependent, being higher in UKF-NB-3 cells than in UKF-NB-4 cells (Table 1).

Because formation of covalent DNA adducts by ellipticine was found to be one of the most important DNA damaging mechanisms responsible for ellipticine cytotoxicity to neuroblastoma cells, the effect of VPA and TSA on ellipticine-DNA adduct formation was investigated with the nuclease P1 version of ^{32}P-postlabeling assay (Stiborova *et al.* 2001; 2003a; 2003b; 2004; 2007a; 2007b). Two major ellipticine-DNA adducts were formed in both neuroblastoma cells under all conditions tested in this study (spots 1 and 2 in Figure 2A,B). The two adducts are identical to those found previously after *in vitro* incubation of calf thymus DNA with ellipticine and isolated CYPs (Stiborova *et al.* 2001; 2003b), or peroxidases (Stiborova *et al.* 2007a) or after treatment of cells in culture with this anticancer drug (Frei *et al.* 2002; Borek-Dohalska *et al.* 2004; Martinkova *et al.* 2009) or *in vivo*, in several tissues, including mammary adenocarcinoma (Figure 2C) of rats (Stiborova *et al.* 2003a; 2007b; 2011) and mice (Stiborova *et al.* 2008) exposed to this agent. These adducts are generated from ellipticine-13-ylium and ellipticine-12-ylium (Figure 1), the reactive species formed from ellipticine metabolites, 13-hydroxy- and 12-hydroxyellipticine (Stiborova *et al.* 2004; 2007a; Poljakova *et al.* 2006; Moserova *et al.* 2008) (Figure 2D,E) as confirmed by co-chromatographic analysis using TLC and HPLC (data not shown). Besides these adducts, two additional minor adducts (spots 6 and 7 in Figure 2B) were detected in DNA of UKF-NB-4 cells treated with 10 μM ellipticine (Figure 2B and Table 2). Both minor adducts we have shown to be generated *in vitro* mainly by peroxidase-catalyzed oxidation of ellipticine (Poljakova *et al.* 2006; Stiborova *et al.* 2007a). The low level of these adducts prevented HPLC co-chromatographic analysis or their further characterization. No adducts were detected in DNA of control cells treated with solvent, VPA or TSA only.

Ellipticine-DNA adduct levels were ellipticine dose dependent in both neuroblastoma cell lines with an

Tab. 1. The effect of valproate and trichostatin A on toxicity of ellipticine to UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines.

	IC_{50} for ellipticine (μM)	
	UKF-NB-3 cells	**UKF-NB-4-cells**
None	0.440 ± 0.030	0.490 ± 0.035
+ 1 mM valproate	0.199 ± 0.011***	0.389 ± 0.029**
+ 2 mM valproate	0.047 ± 0.003***	0.252 ± 0.021***
+ 100 nM TSA	0.303 ± 0.030***	0.389 ± 0.030**

IC_{50} values were calculated from the linear regression of the dose-log response curves. Values are mean ± S.D. of at least 3 experiments. The data were analyzed statistically by Student's *t*-test. Values significantly different from individual cell lines cultivated without VPA or TSA: **p<0.01, ***p<0.001.

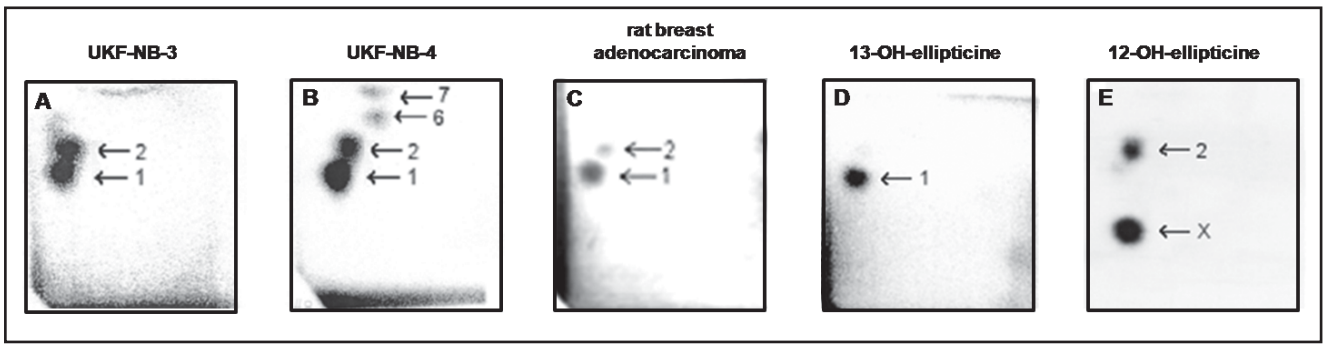

Fig. 2. Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labeled digests of DNA isolated from neuroblastoma UKF-NB-3 (**A**) and UKF-NB-4 (**B**) cells exposed to 10 μM ellipticine for 48 h, of DNA of breast adenocarcinoma of Wistar rats treated i.p. with 4 mg ellipticine per kilogram body weight (**C**), from calf thymus DNA reacted with 13-hydroxyellipticine (**D**) and 12-hydroxyellipticine (**E**). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay. (**A,B**) Scans of the plates from the imager for 6.5 min; (**C,D,E**) autoradiographs of films exposed for 1 h at –80 °C. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right).

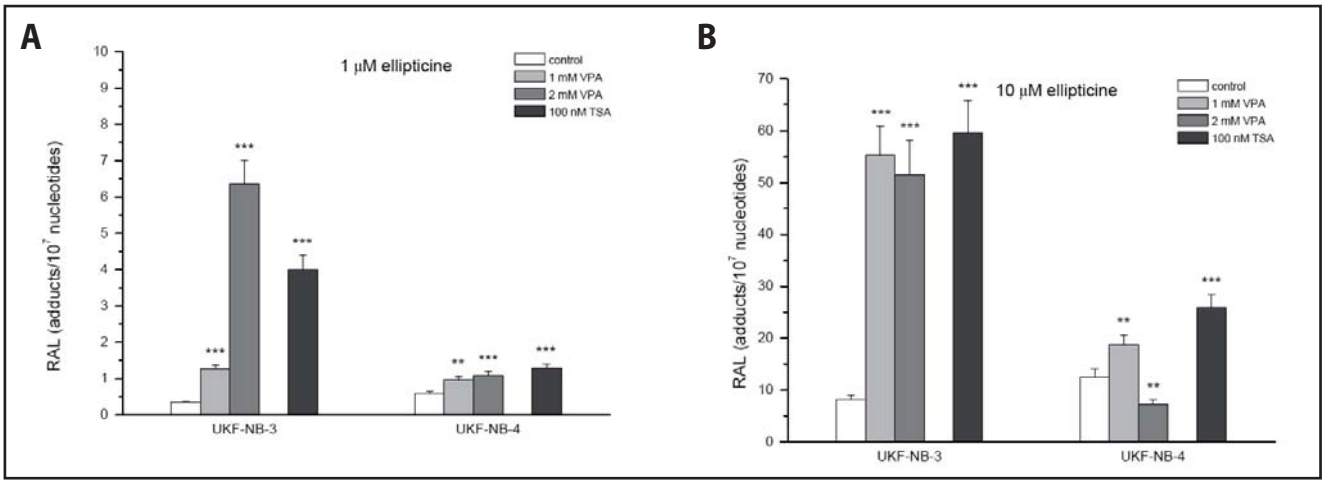

Fig. 3. The effect of valproate and trichostatin A on DNA adduct formation by 1 μM (A) and 10 μM ellipticine (B) in human UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines. *Columns*: Mean RAL (relative adduct labeling) ± standard deviations shown in the figure represent total levels of DNA adducts of three ^{32}P-postlabeling analyses. The data were analyzed statistically by Student's *t*-test. Values significantly different from cells exposed to ellipticine alone. ***p*<0.01, ****p*<0.001.

Tab. 2. The effect of valproate and trichostatin A on DNA adduct formation by ellipticine in human UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines.

Cells	Levels of DNA adducts (RAL × 10^{-7})[a]				
	Adduct 1	Adduct 2	Adduct 6	Adduct 7	Total
UKF-NB-3					
1.0 μM ellipticine	0.12 ± 0.01	0.23 ± 0.02	n.d.	n.d.	0.35± 0.04
+ 1 mM valproate	0.35 ± 0.03***	0.92 ± 0.10***	n.d.	n.d.	1.27 ± 0.10***
+ 2 mM valproate	3.22 ± 0.30***	3.14 ± 0.30***	n.d.	n.d.	6.36 ± 0.65***
+ 100 nM TSA	2.46 ± 0.25***	1.53 ± 0.15***	n.d.	n.d.	3.99 ± 0.40***
10 μM ellipticine	3.27 ± 0.32	5.01 ± 0.50	n.d.	n.d.	8.28 ± 0.8
+ 1 mM valproate	21.71 ± 2.02***	33.60 ± 3.30***	n.d.	n.d.	55.31 ± 5.5***
+ 2 mM valproate	27.59 ± 2.76***	23.92 ± 2.90***	n.d.	n.d.	51.51 ± 6.7***
+ 100 nM TSA	30.86 ± 3.89***	27.60 ± 2.70***	0.59 ± 0.06	0.60 ± 0.06	59.65 ± 6.2***
UKF-NB-4					
1.0 μM ellipticine	0.20 ± 0.02	0.38 ± 0.04	n.d.	n.d.	0.58 ± 0.06
+ 1 mM valproate	0.33 ± 0.03**	0.63 ± 0.07***	n.d.	n.d.	0.96 ± 0.10**
+ 2 mM valproate	0.38 ± 0.04***	0.71 ± 0.07***	n.d.	n.d.	1.09 ± 0.10***
+ 100 nM TSA	0.48 ± 0.04***	0.80 ± 0.08***	n.d.	n.d.	1.28 ± 0.12***
10 μM ellipticine	5.40 ± 0.56	6.50 ± 0.81	0.27±0.03	0.37±0.05	12.54 ± 1.51
+ 0.5 mM valproate	5.49 ± 0.51	5.65 ± 0.62	0.38±0.04	0.57±0.06	12.09 ± 1.24
+ 1 mM valproate	9.04 ± 0.99***	8.73 ± 0.89*	0.60±0.07**	0.39±0.04	18.72 ± 1.95**
+ 1.5 mM valproate	8.66 ± 0.84**	7.97 ± 0.82	0.17±0.02	0.17±0.02	16.97 ± 1.78*
+ 2 mM valproate	3.85 ± 0.41**	3.23 ± 0.32***	0.10±0.02**	0.11±0.02**	7.29 ± 0.89**
+ 100 nM TSA	13.08 ± 1.38***	11.90 ± 1.90***	0.47 ± 0.03**	0.40 ± 0.04	25.85 ± 2.60***

Neuroblastoma cells were treated with VPA and TSA for 24 h before ellipticine was added for another 48 h. [a]RAL, relative adduct labeling; averages and standard deviations of three ^{32}P-postlabeling analysis. n.d. - not detected (the detection limit of RAL was 1/10^{11} nucleotides). The data were analyzed statistically by Student's *t*-test. Values significantly different from cells exposed to ellipticine alone **p*<0.05, ***p*<0.01, ****p*<0.001.

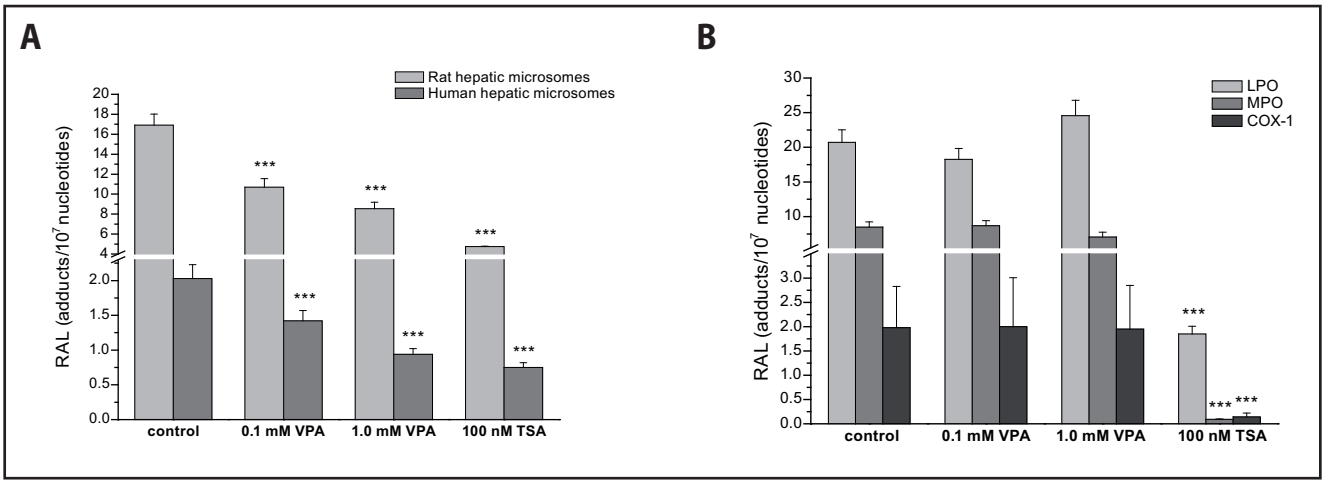

Fig. 4. The effect of VPA and TSA on DNA adduct formation by ellipticine activated with human or rat hepatic microsomes (A) or with peroxidases (B) as determined by TLC ^{32}P-postlabeling. *Columns*: Mean RAL (relative adduct labeling) ± standard deviations shown in the figure represent total levels of DNA adducts of four determinations (duplicate analyses of two independent *in vitro* incubations). The data were analyzed statistically by Student's *t*-test. Values significantly different from incubations without VPA or TSA: ***P<0.001. Control = without VPA or TSA; VPA = valproic acid; TSA = trichostatin A.

over-proportional increase between 1 μM and 10 μM ellipticine (Figure 3 and Table 2). Pre-treatment of UKF-NB-3 and UKF-NB-4 neuroblastoma cells with VPA and TSA had pronounced stimulatory effects on ellipticine-DNA adduct levels predominantly in UKF-NB-3 cells (Figure 3 and Table 2). TSA significantly increased DNA adducts at both ellipticine concentrations (1 and 10 μM) in both cell lines (up to 10-fold). However, VPA at concentrations of 1 and 2 mM increased only the levels of ellipticine-DNA adducts up to 20-fold at 1 and 10 μM ellipticine in UKF-NB-3 cells, and at 1 μM ellipticine in the UKF-NB-4 cell line (Figure 3 and Table 2). In the case of UKF-NB-4 cells treated with 10 μM ellipticine, even though VPA at 1 mM lead to a 1.5-fold increase in ellipticine-DNA adduct levels, this compound at 2 mM inhibited formation of all four ellipticine-DNA adducts, by 42% (Figure 3 and Table 2).

Because both VPA and TSA have been shown to be metabolized by CYPs and by peroxidases (Fisher *et al.* 1991; Rogiers *et al.* 1992; 1995; Isojärvi *et al.* 2001; Wen *et al.* 2001; Bort *et al.* 2004; Gupta *et al.* 2004; Hooven *et al.* 2005; Eyal *et al.* 2006; Perucca 2006; Cerveny *et al.* 2007) as is ellipticine (Stiborova *et al.* 2004; 2006c; 2007a), three experimental approaches were employed to elucidate their influence on ellipticine activation: (i) analysis of the effect of VPA and TSA on formation of ellipticine-DNA adducts in the CYP- and peroxidase-systems *in vitro*, (ii) analysis of the effect of these HDAC inhibitors on ellipticine oxidation to individual metabolites by both enzymatic systems *in vitro*, and (iii) analysis of the potential of VPA and TSA in the presence of ellipticine to influence expression levels of CYP and peroxidase enzymes responsible for ellipticine metabolism in neuroblastoma cells.

The effect of VPA and TSA on oxidative activation of ellipticine to DNA adducts by human and rat hepatic microsomes and peroxidases

Human and rat hepatic microsomes rich in CYP enzymes and isolated peroxidases (human MPO, bovine LPO and ovine COX-1 as models) that are capable of activating ellipticine to species forming DNA adducts (Stiborova *et al.* 2003b; 2004; 2007a) were used in the experiments. The DNA adduct pattern generated by ellipticine in the CYP-microsomal and peroxidase systems as determined by ^{32}P-postlabeling, again consisted of the two adducts derived from 13-hydroxy- and 12-hydroxyellipticine, spots 1 and 2, respectively, and of two additional adducts (adduct spots 6 and 7) (Stiborova *et al.* 2003b; 2004; 2007a) (Figure 2 and Table 3). In accordance to our former studies (Stiborova *et al.* 2001; 2003b), rat hepatic microsomes activated ellipticine more effectively than microsomes from human liver. In the case of peroxidases, the highest efficiency to activate ellipticine to DNA adducts exhibited LPO, followed by MPO and COX-1 (Figure 4). This finding corresponds to the results found previously, in our study investigating activation of ellipticine with peroxidases (Stiborova *et al.* 2007a).

As shown in Figure 4A and Table 3, VPA and TSA inhibited the activation of ellipticine to species forming DNA adducts mediated by human and rat hepatic microsomes. The peroxidase-catalyzed activation of ellipticine to DNA adducts, including adducts 6 and 7, was significantly inhibited by TSA, but VPA had no such effect (Figure 4B and Table 3).

In order to evaluate whether the lower ellipticine-DNA adduct levels in the presence of VPA and TSA is caused directly by their inhibition of CYPs and peroxidases, the effect of these compounds on formation

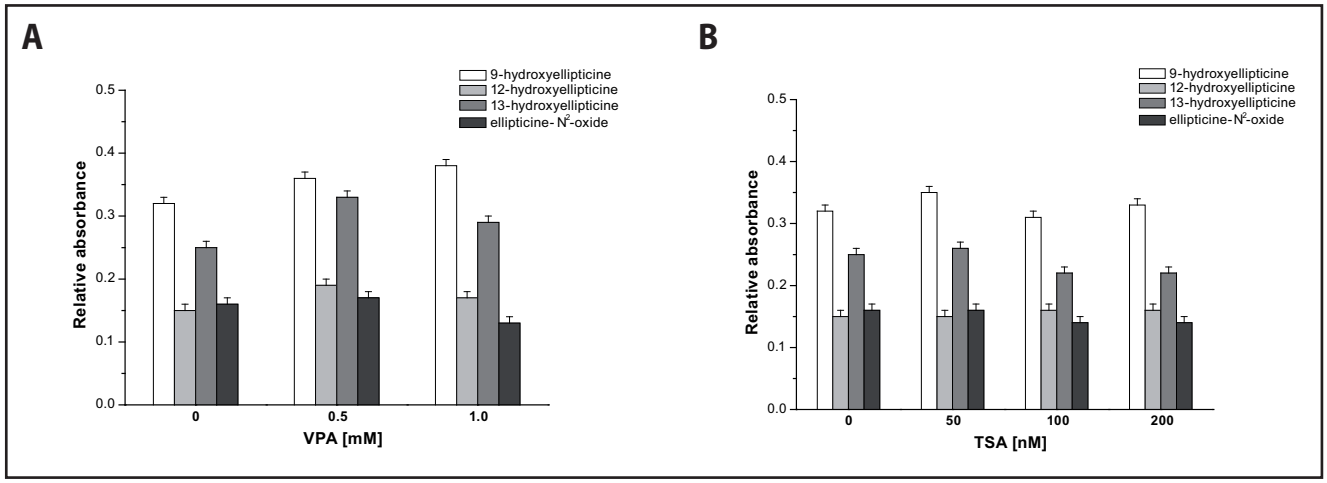

Fig. 5. The effect of VPA (**A**) and TSA (**B**) on ellipticine oxidation by rat hepatic microsomes. *Columns*: Mean values of individual ellipticine metabolites ± standard deviations of three experiments.

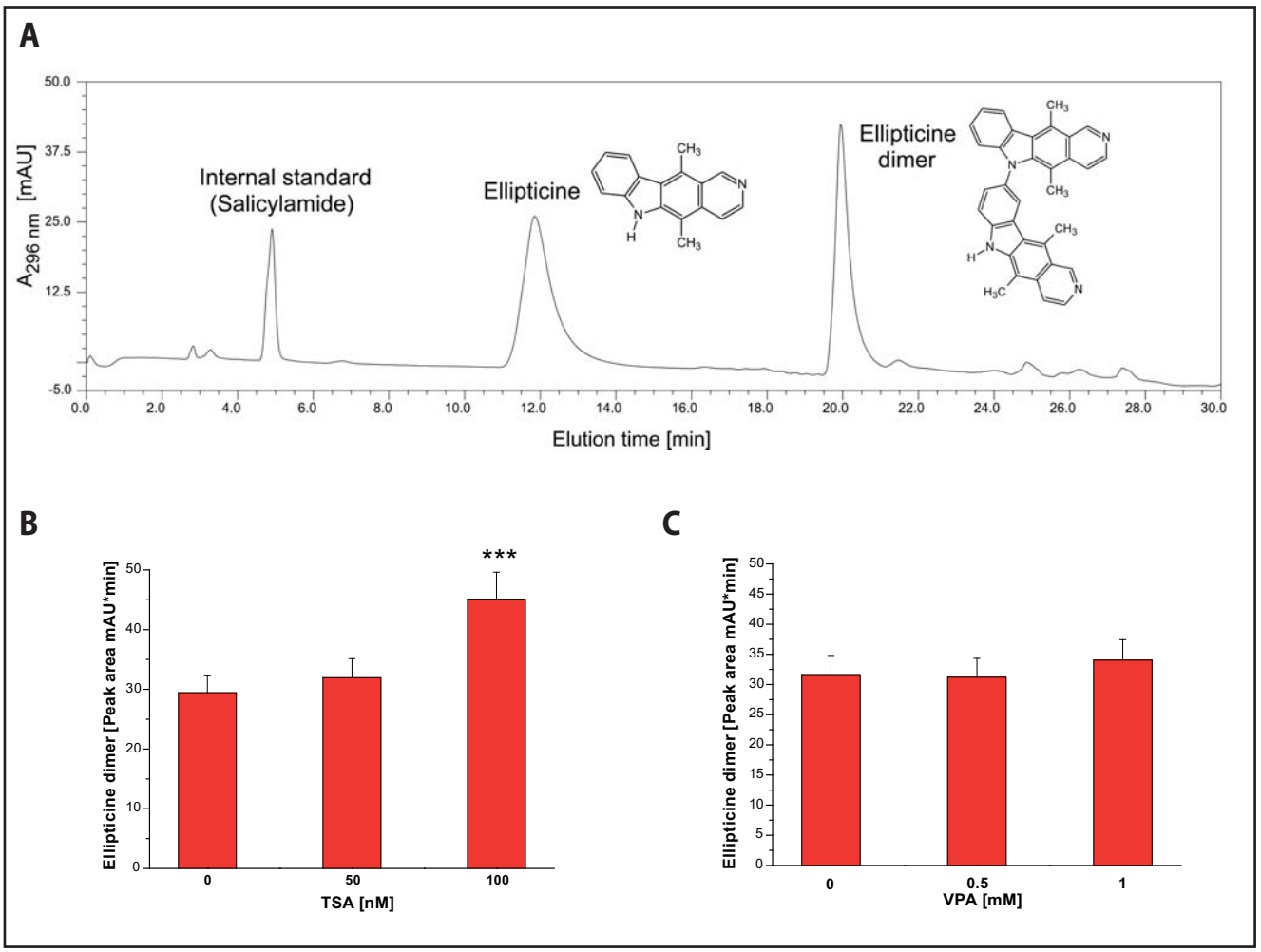

Fig. 6. HPLC separations of ellipticine from its dimer formed by oxidation of ellipticine with LPO (A) and the effect of TSA (B) and VPA (C) on ellipticine oxidation by LPO. *Columns*: Mean values of ellipticine dimer formed by LPO ± standard deviations of three experiments. ***$p<0.001$.

Tab. 3. The effect of valproate and trichostatin A on DNA adduct formation by ellipticine activated with either rat or human hepatic microsomes or peroxidases.

Enzymatic system	Levels of DNA adducts (RAL × 10^{-7})[a]				
	Adduct 1	Adduct 2	Adduct 6	Adduct 7	Total
Rat hepatic microsomes + ellipticine					
Without VPA and TSA	16.10 ± 0.80	0.26 ± 0.02	0.29±0.02	0.27±0.01	16.92 ± 1.10
+ 0.1 mM valproate	10.10 ± 0.80***	0.16 ± 0.01***	0.18±0.01***	0.26±0.01	10.70 ± 0.85***
+ 1.0 mM valproate	8.25 ± 0.54***	0.12 ± 0.05***	0.11±0.01***	0.05±0.01***	8.53 ± 0.65***
+ 100 nM TSA	4.20 ± 0.45***	0.12 ± 0.01***	0.21±0.01*	0.21±0.01*	4.74 ± 0.04***
Human hepatic microsomes + ellipticine					
Without VPA and TSA	1.60 ± 0.12	0.16 ± 0.02	0.18±0.02	0.07±0.01	2.03 ± 0.20
+ 0.1 mM valproate	1.09 ± 0.10**	0.13 ± 0.01	0.14±0.01**	0.06±0.01	1.42 ± 0.15***
+ 1.0 mM valproate	0.67 ± 0.06***	0.12 ± 0.09*	0.11±0.01***	0.04±0.01**	0.94 ± 0.08***
+ 100 nM TSA	0.50 ± 0.05***	0.09 ± 0.01***	0.11±0.01***	0.05±0.01*	0.75 ± 0.07***
Cyclooxygenase-1 + ellipticine					
Without VPA and TSA	1.50 ± 0.09	0.41 ± 0.02	0.02±0.001	0.05±0.002	1.98 ± 0.85
+ 0.1 mM valproate	1.51 ± 0.08	0.42 ± 0.03	0.03±0.	0.04±0.002	2.00 ± 1.01
+ 1.0 mM valproate	1.48 ± 0.08	0.41 ± 0.02	0.02±0.001	0.04±0.002	1.95 ± 0.90
+ 100 nM TSA	0.10 ± 0.01***	0.04 ± 0.01***	n.d.[b]	n.d.	0.14 ± 0.08***
Myeloperoxidase + ellipticine					
Without VPA and TSA	5.75 ± 0.48	1.61 ± 0.13	0.86±0.07	0.27±0.03	8.49 ± 0.74
+ 0.1 mM valproate	5.74 ± 0.42	1.81 ± 0.15	0.84±0.07	0.28±0.03	8.67 ± 0.75
+ 1.0 mM valproate	4.58 ± 0.35	1.16 ± 0.08	0.96±0.08	0.34±0.03	7.04 ± 0.71
+ 100 nM TSA	0.05 ± 0.01***	0.01 ± 0.01***	0.02±0.01***	0.01±0.01***	0.09 ± 0.01***
Lactoperoxidase + ellipticine					
Without VPA and TSA	13.36 ± 1.01	4.56 ± 0.32	2.27±0.18	0.51±0.04	20.70 ± 1.81
+ 0.1 mM valproate	12.12 ± 1.00	3.71 ± 0.29	1.89±0.12	0.54±0.05	18.26 ± 1.59
+ 1.0 mM valproate	16.29 ± 1.32	5.94 ± 0.46	1.78±0.17	0.55±0.05	24.56 ± 2.23
+ 100 nM TSA	1.42 ± 0.11***	0.18 ± 0.02***	0.14±0.01***	0.11±0.01***	1.85±0.16***

[a]Mean RAL (relative adduct labeling) of four determinations (duplicate analyses of two independent *in vitro* incubations).
[b]n.d. - not detected (the detection limit of RAL was $1/10^{11}$ nucleotides). The data were analyzed statistically by Student's *t*-test. Values significantly different from incubations without VPA or TSA: *p<0.05, **p<0.01, ***p<0.001.

Tab. 4. P-glycoprotein expression in neuroblastoma cells and its influencing by VPA and TSA.

Cells	P-glycoprotein levels (mean intensity of fluorescence)			
	Without pretreatment	1 mM VPA	2 mM VPA	100 nM TSA
UKF-NB-3	114.4 ± 5.4	165.5 ± 16.8**	135.2 ± 21.0**	136.1 ± 10.7**
UKF-NB-4	1417 ± 11	1710 ± 17***	2122 ± 20***	1542 ± 32*

Values are mean of six determinations ± standard deviation. The data were analyzed statistically by Student's *t*-test. Values significantly different from individual cell lines cultivated without VPA or TSA: *p<0.05, **p<0.01, ***p<0.001

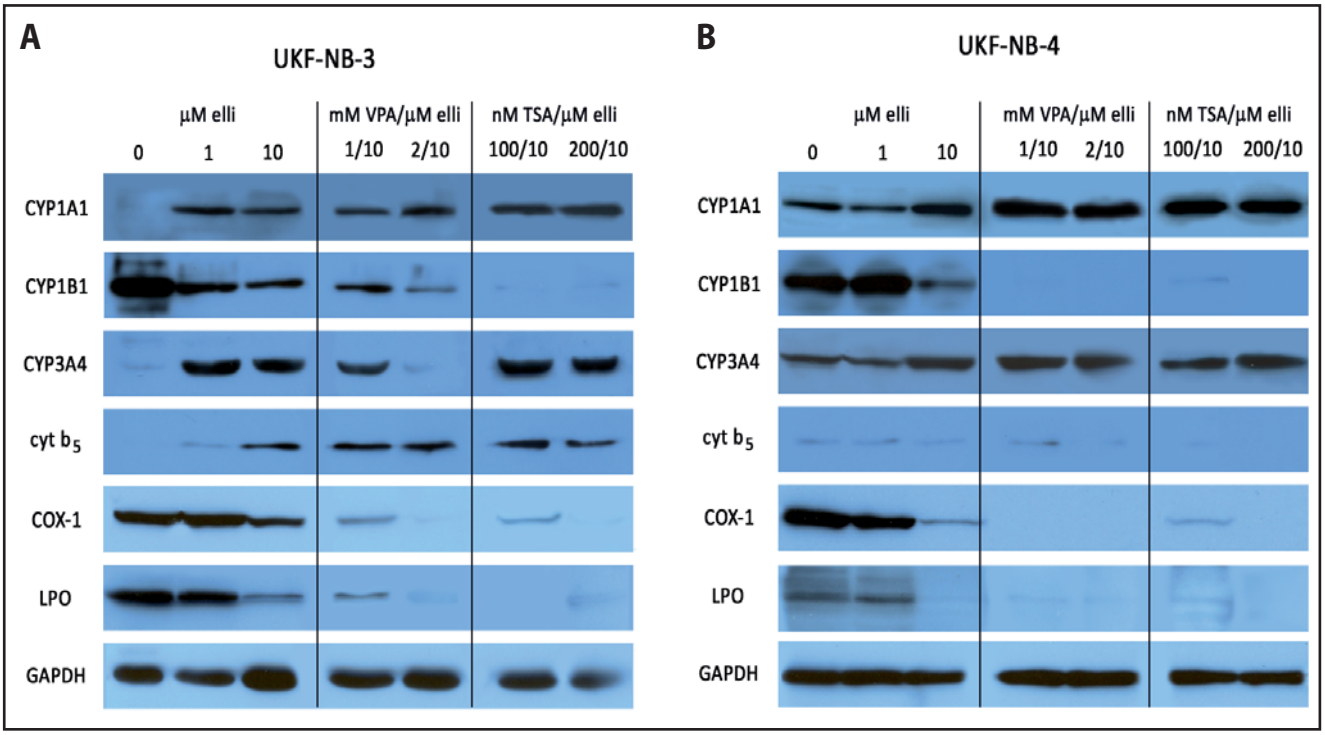

Fig. 7. Immunoblots showing the effects of exposing UKF-NB-3 cells (A) and UKF-NB-4 cells (B) to ellipticine with VPA or to ellipticine with TSA for 48 h on expression of CYP1A1, CYP1B1, CYP3A4, cytochrome b_5, COX-1 and LPO. Cell homogenates were subjected to SDS-PAGE, proteins transferred to nitrocellulose membranes and probed with antibodies as described in Material and Methods. GAPDH was used as loading control.

of individual ellipticine metabolites was investigated. Five ellipticine metabolites, 7-hydroxy-, 9-hydroxy-, 12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2-oxide, were produced by microsomal CYPs (see metabolites in Figure 1) (Stiborova *et al.* 2004; 2006a). But because of the low levels of 7-hydroxyellipticine formed by the rat microsomes (Stiborova *et al.* 2006a) that were used as a model in these experiments, they were not quantified in this study. Neither VPA nor TSA inhibited the oxidation of ellipticine by rat hepatic microsomes to any of the ellipticine metabolites (Figure 5A,B). These findings suggest that a decrease in ellipticine-derived DNA adduct levels by VPA and TSA (Figure 4A) was not the result of inhibition of CYP activity but mediated by other mechanisms.

VPA also had no effect on the LPO-catalyzed oxidation of ellipticine (Figure 6C) to the ellipticine dimer (see the peak of the ellipticine dimer in Figure 6A), the compound which is the major detoxification metabolite of ellipticine formed by peroxidases (compare Figure 1) (Stiborova *et al.* 2007a). TSA at 100 nM induced an increase in ellipticine dimer levels catalyzed by LPO (Figure 6B). This finding and the results above showing the inhibition of ellipticine-DNA adduct formation by TSA suggest that TSA alters the peroxidase-mediated activation of ellipticine from the reaction with DNA to the reaction with a second ellipticine molecule to form the dimer.

The effects of ellipticine and VPA or TSA on levels of proteins CYP1A1, 1B1, 3A4, COX-1, LPO and cytochrome b_5

Using Western blot analysis with antibodies raised against CYP1A1, 1B1, 3A4, LPO, COX-1 and cytochrome b_5, the effects of exposure of the cells to ellipticine with and without VPA or TSA on protein levels of these enzymes were analyzed.

Levels of CYP1A1 protein in neuroblastoma UKF-NB-3 and UKF-NB-4 cells were elevated by exposure of the cells to ellipticine; a dose dependent increase was seen in neuroblastoma cells by this compound (Figure 7). The two HDAC inhibitors had stimulation effects on CYP1A1 expression levels induced by ellipticine, predominantly in UKF-NB-4 cells (Figure 7).

Levels of CYP3A4 protein were increased only by ellipticine in both neuroblastoma cells (Figure 7). Treatment of cells with ellipticine and both two HDAC inhibitors had essentially no effects on CYP3A4 protein levels induced by ellipticine, but 2 mM VPA decreased the amounts of CYP3A4, mainly in a UKF-NB-3 neuroblastoma cell line (Figure 7).

In contrast to the increase in CYP1A1 and CYP3A4 protein levels by ellipticine, amounts of CYP1B1 protein were decreased in neuroblastoma cells treated with this drug (Figure 7). The combined treatment of cells with ellipticine and HDAC inhibitors leads to an additional decrease in CYP1B1 protein levels, resulting even in a complete lack of its expression caused by TSA in

UKF-NB-3 cells (Figure 7A) and by both two HDAC inhibitors in UKF-NB-4 cells (Figure 7B).

Amounts of cytochrome b_5, the protein that modulates the enzymatic activities of several CYPs including CYP3A4 (for a summary see Schenkman & Jansson 2003) and CYP1A1 (Stiborova *et al.* 2006b), were elevated in UKF-NB-3 by ellipticine, but VPA or TSA had practically no effect on this ellipticine-mediated induction (Figure 7A). On the contrary, no increase in cytochrome b_5 protein levels by ellipticine was found in UKF-NB-4 cells. In addition, its amounts were even decreased by VPA and TSA in combination with 10 μM ellipticine in these cells (Figure 7B).

The peroxidases metabolizing ellipticine, COX-1 and LPO, are expressed in UKF-NB-3 and UKF-NB-4 cells (Figure 7). Their amounts decreased with 10 μM ellipticine, increasing concentrations of VPA and TSA further decreased COX-1 and LPO levels in both two neuroblastoma cell lines (Figure 7).

P-glycoprotein expression in neuroblastoma cells

Expression levels of P-glycoprotein (encoded by multidrug resistance gene 1) were analyzed in UKF-NB-3 and UKF-NB-4 cell lines. As shown in Table 4, more than 12-fold higher expression levels of P-glycoprotein were found in the UKF-NB-4 line than in the UKF-NB-3 cells. In addition, a 1.5-fold increase in expression of this protein was produced by treating UKF-NB-4 cells with VPA, while only a 1.2-fold increase caused by VPA was found in UKF-NB-3 cell line (Table 4). In the case of pre-treatment of both neuroblastoma cell lines with TSA, only ~1.1-fold increase in P-glycoprotein expression levels were detected (Table 4).

DISCUSSION

The results of this study demonstrate that a combination of ellipticine with the HDAC inhibitors, VPA and TSA, leads to an increase in ellipticine toxicity to UKF-NB-3 and UKF-NB-4 neuroblastoma cells. The sensitivity of the cells to this combination seems to be related to the phenotype, with the invasive N-type (UKF-NB-3) being higher sensitive than the less-aggressive S-type cells (UKF-NB-4). The increase in toxic effects of ellipticine to neuroblastoma cells and the higher sensitivity of the N-type neuroblastoma UKF-NB-3 cells to ellipticine caused by VPA and TSA were associated with an increase in formation of ellipticine-derived DNA adducts, which we have shown to be one of the predominant DNA-damaging mechanisms of ellipticine action in several cancer cells including neuroblastomas (Borek-Dohalska *et al.* 2004; Poljakova *et al.* 2007; 2009; Martinkova *et al.* 2009). Hence, the increase in ellipticine–mediated DNA damage by both HDAC inhibitors should be an important consideration in their use as chemotherapeutic agents of the invasive N-type neuroblastomas.

At least two phenomena might determine the levels of ellipticine-derived DNA adducts in neuroblastoma cells: (i) the expression and activities of CYPs and peroxidases metabolizing (activating and detoxicating) ellipticine and levels of protein(s) influencing their activities, and/or (ii) the expression levels of P-glycoprotein dictating concentrations of drugs in the cells.

In this study, we have found that the CYP-mediated formation of ellipticine-derived DNA adducts in tested neuroblastoma cells depends on expression levels of CYP1A1 and 3A4 enzymes and also on expression of another protein present in the membrane of endoplasmic reticulum, cytochrome b_5. The finding that CYP3A4 is expressed in UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines is interesting, because this CYP is highly expressed mainly in the liver and in the intestine (Rendic & Di Carlo 1997), but not in the brain. The ellipticine–mediated modulations of levels of cytochrome b_5 seem to play a key role in higher formation of ellipticine-derived DNA adducts in UKF-NB-3 cells. This heme protein was found to influence oxidation of ellipticine catalyzed by CYP3A4, the enzyme oxidizing this compound mainly to 12-hydroxy- and 13-hydroxylated metabolites that form DNA adducts (see Figure 1), and by CYP1A1, which predominantly detoxicate ellipticine (Stiborova *et al.* 2006a; 2006c; 2004; 2011). In the case of CYP3A4, stimulating effects of cytochrome b_5 on oxidation of ellipticine to 12-hydroxy- and 13-hydroxyellipticine lead to an increase in ellipticine-DNA adduct formation (Stiborova *et al.* 2004). In the case of CYP1A1, cytochrome b_5 has been recently found to alter the ratio of ellipticine metabolites formed by this CYP, "switching" CYP1A1-mediated oxidation of this anticancer drug from detoxication (9-hydroxy- and 7-hydroxyellipticine) to DNA-forming metabolites (12-hydroxy- and 13-hydroxyellipticine). These changes resulted in an increase in formation of covalent DNA adducts by ellipticine (Kotrbova *et al.* 2011).

In the present study, we have found that ellipticine increases amounts of cytochrome b_5 in UKF-NB-3 cells, but not in UKF-NB-4 cells. The higher levels of cytochrome b_5 and the increase in CYP1A1 levels induced by ellipticine with VPA or TSA in UKF-NB-3 cells, therefore, promote the activation of ellipticine catalyzed by CYP1A1 in these cells. On the contrary, because of low levels of cytochrome b_5 in UKF-NB-4 cells, the elevated expression levels of CYP1A1 caused by exposure to ellipticine in the presence of VPA and TSA results in higher ellipticine detoxication in these cells. Hence, both these induction effects produce concerted regulatory effects of ellipticine, VPA and TSA on ellipticine pharmacological action.

The finding that CYP1A1 is induced by ellipticine is in accordance with data found in the former studies (Fernandez *et al.* 1988; Gasiewicz *et al.* 1996; Aimova *et al.* 2007; Martinkova *et al.* 2009). Expression of CYP1A1 protein as well as its enzymatic activity was

significantly induced by ellipticine in rats treated with this compound (Aimova *et al.* 2007). This induction was explained by several authors (Fernandez *et al.* 1988; Gasiewicz *et al.* 1996) to be predominantly a consequence of the ellipticine binding to aryl hydrocarbon receptor (AhR). Surprisingly, CYP1B1, another CYP enzyme expressed in both two neuroblastoma cell lines metabolizing ellipticine mainly to detoxication metabolites (9-hydroxy- and 7-hydroxyellipticine) (Stiborova *et al.* 2004), whose transcriptional activation (*CYP1B1* gene) is believed to also involve the AhR (Kerzee & Ramos 2001), was, however, not induced by ellipticine in these cells. Its expression was even decreased by treating the cells with ellipticine plus VPA and TSA. The mechanisms of this phenomenon as well as mechanisms of the potential of ellipticine to increase levels of CYP3A4 and cytochrome b_5 in neuroblastoma cells were, however, not evaluated in this work and await further investigation.

In contrast to CYP1A1 and 3A4 enzymes, the peroxidases COX-1 and LPO that are expressed in UKF-NB-3 and UKF-NB-4 cells do not participate in the increased sensitivity of these neuroblastoma cells to ellipticine in the presence of VPA and TSA. Their expression levels are even decreased in neuroblastoma cells exposed to ellipticine plus either HDAC inhibitor.

Another reason for the differences between UKF-NB-3 and UKF-NB-4 neuroblastoma cell's sensitivities to the combined effect of ellipticine with HDAC inhibitors might be their different genetic programs (Cinatl *et al.* 1999; Bedrnicek *et al.* 2005). The UKF-NB-4 line was established from a recurrent disease and, in contrast to UKF-NB-3, the P-glycoprotein causing multidrug resistance, was found previously to be expressed at high levels in this cell line (Cinatl *et al.* 1999). Indeed, here we found that more than 12-fold higher levels of P-glycoprotein are expressed in the UKF-NB-4 line than in UKF-NB-3 cells. This transport protein might efficiently eliminate VPA, TSA and ellipticine (and their metabolites) from cells, thereby decreasing the toxic effects of these compounds. Moreover, because VPA acts as an inducer of P-glycoprotein expression in several human tumor cell lines (Eyal *et al.* 2006; Cerveny *et al.* 2007), including neuroblastoma cells (up to 1.5-fold) (present study), elimination of both these HDAC inhibitors and ellipticine from the UKF-NB-4 cell line might be even higher. In contrast to VPA, ellipticine has not been identified to be a substrate of P-glycoprotein (Huang *et al.* 2005), indicating its suitability to be used in combined therapy with other drugs.

Collectively, the results presented in this paper are the first report demonstrating that VPA and TSA increase sensitivity of neuroblastomas to ellipticine. The higher sensitivity of neuroblastomas to ellipticine caused by VPA and TSA corresponds to the increase in DNA-damage responsible for ellipticine cytotoxicity in these cells (Poljakova *et al.* 2009). This increase is not caused by the direct stimulation effects of these HDAC inhibitors on the enzymes catalyzing reactions leading to formation of ellipticine-DNA adducts; these enzymes were either not influenced by VPA and TSA or even inhibited by these HDAC inhibitors. Therefore, the increase in DNA adduct formation is mediated by elevated levels of CYP3A4 and 1A1 enzymes and/or cytochrome b_5 caused by their induction with ellipticine and/or VPA and TSA.

Concerning formation of ellipticine-derived DNA adducts, the risk of treating children with this compound might be considered. Nevertheless, our *in vivo* studies using the rat experimental model mimicking the fate of ellipticine in human (Stiborova *et al.* 2003b; 2006a) demonstrated that ellipticine-DNA adducts did not persist in healthy tissues of rats treated with ellipticine (Stiborova *et al.* 2007b). Therefore, these results suggest a relatively low risk of the genotoxic side effects of ellipticine during the cancer treatment in human.

It should be also emphasized that besides the above mechanism explaining elevated levels of ellipticine-derived DNA adducts in neuroblastoma cells another mechanism, based on epigenetic modifications of chromatin by VPA and TSA, which was not examined in this work, might modulate ellipticine-induced cytotoxicity in these cells, too. Namely, it was found that loosening-up the chromatin structure by histone acetylation is caused by HDAC inhibitors (Kim *et al.* 2003; Marchion *et al.* 2005a; 2005b; Catalano *et al.* 2006). Such changes in the chromatin structure, leading to chromatin decondensation, might make cell DNA more accessible to the ellipticine–mediated damage such as formation of ellipticine-DNA adducts or intercalation of ellipticine into DNA and inhibition of topoisomerase II activity. Therefore, stimulation effects of VPA and TSA on ellipticine toxic effects to neuroblastoma cells following from these phenomena should also be taken into account. Such effects of VPA and TSA in neuroblastoma cells are, hence, planned to be studied in our further work.

The results of the present study strongly suggest that combined therapy of ellipticine with VPA or TSA might be an effective treatment strategy for children with high risk neuroblastomas.

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