Valproic Acid, a Molecular Lead to Multiple Regulatory Pathways

(valproic acid / cancer / histone deacetylase)

M. KOSTROUCHOVÁ[1], Z. KOSTROUCH[1], M. KOSTROUCHOVÁ[2]

[1]Laboratory of Molecular Pathology, [2]Laboratory of Molecular Biology and Genetics, Institute of Inherited Metabolic Disorders of the 1st Faculty of Medicine, Charles University, Prague, Czech Republic

Abstract. Valproic acid (2-propyl pentanoic acid) is a drug used for the treatment of epilepsy and bipolar disorder. Although very rare, side effects such as spina bifida and other defects of neural tube closure indicate that valproic acid interferes with developmental regulatory pathways. Recently obtained data show that valproic acid affects cell growth, differentiation, apoptosis and immunogenicity of cultured cancer cells and tumours. Focused studies uncovered the potential of valproic acid to interfere with multiple regulatory mechanisms including histone deacetylases, GSK3 α and β, Akt, the ERK pathway, the phosphoinositol pathway, the tricarboxylic acid cycle, GABA, and the OXPHOS system. Valproic acid is emerging as a potential anticancer drug and may also serve as a molecular lead that can help design drugs with more specific and more potent effects on the one side and drugs with wide additive but weaker effects on the other. Valproic acid is thus a powerful molecular tool for better understanding and therapeutic targeting of pathways that regulate the behaviour of cancer cells.

Introduction

The trivial name valproic acid comes from an inversion of 2-**pro**pyl**val**e**ric** acid, its alternative chemical name. Valeric acid is a synonym for pentanoic acid, named after its original purification from the plant *Valeriana officinalis*. In the standardized chemical nomenclature, valproic acid should be denominated 2-propylpentanoic acid or dipropyl-acetic acid with the formula $(CH_3CH_2CH_2)_2CHCOOH$ (MW 144.21) (Fig. 1). Valproic acid was first synthesized by B.S. Burton, who searched for organic solvents (Burton, 1882). The real turn in the medical use of valproic acid came from experiments made by Pierre Eymard and his colleagues, who were using it as a solvent for compounds tested as potential anti-epileptic drugs on experimentally induced seizures. Eymard and colleagues realized that valproic acid was an efficient inhibitor of seizures itself (Meunier et al., 1963).

Although valproic acid is a small branched fatty acid, its chemical properties allow easy delivery to the organism and cells. It is slightly soluble in water and highly soluble in organic solvents and it is stable at room temperature. Since valproic acid exists in a dissociated form in water solutions containing alkali metals, it can be easily delivered to organisms in the form of sodium or magnesium salts which are soluble in water (Balbi et al., 1991). Branded products include Depakene (Abbott Laboratories in US & Canada), Convulex (Pfizer in UK and Byk Madaus in South Africa), Depakine and MicropakineLP (Sanofi-Aventis in Europe) and are based on sodium valproate.

Valproic acid is now an established drug for the treatment of epileptic seizures (absence, tonic-clonic (grand mal), and complex partial seizures) and mania in bipolar disorder (Bowden and Singh, 2005). In the human brain, valproic acid affects the function of the neurotransmitter GABA by potentiating the inhibitory activity of GABA through several ways including the inhibition of GABA degradation, increased synthesis of GABA, and decreased GABA turnover (Johannessen, 2000; Johannessen and Johannessen, 2003; Owens and Nemeroff, 2003). It was also found to attenuate NMDA-mediated excitation, block voltage-dependent Na^+ channels and modulate the firing frequency of neurons (Owens and Nemeroff, 2003; Gobbi and Janiri, 2006).

Although valproic acid proved to be a relatively safe drug, the side effects were soon recognized. Common

Received February 2, 2007. Accepted March 5, 2007.

Authors are supported by grants 304/07/0529 and 301/05/0859 from the Czech Science Foundation, and 0021620806 from the Ministry of Education, Youth and Sports of the Czech Republic.

Corresponding author: Marta Kostrouchová, Laboratory of Molecular Biology and Genetics, Institute of Inherited Metabolic Disorders, 1st Faculty of Medicine, Ke Karlovu 2, 128 01 Prague 2, Czech Republic. Phone: (+420) 224 967 088; Fax: (+420) 224 967 119; e-mail: marta.kostrouchova@lf1.cuni.cz

Abbreviations: Akt/PKB – Akt/protein kinase B, AML – acute myelocytic leukaemia, AP axis – anterio-posterial axis, APL – acute promyelocytic leukaemia, ATRA – all-trans retinoic acid, CREB – cAMP response element binding protein, ERK – extracellular signal-related kinase, GABA – γ-aminobutyric acid, GSK3 – glycogen synthase kinase 3, HDAC – histone deacetylase, HAT – histone acetyl transferase, MDS – myelodysplastic syndrome(s), NMDA – N-methyl-D-aspartic acid, RARα – retinoid receptor α, SAHA – suberoylanilide hydroxamic acid, TSA – trichostatin A.

clinical side effects of valproic acid are dyspepsia, weight gain, dysphoria, fatigue, dizziness, drowsiness, hair loss, headache, nausea, sedation and tremor. Valproic acid can impair liver function, cause thrombocytopoenia, and prolongate the blood coagulation times. When used in early pregnancy, valproic acid caused a 3-fold increased risk of congenital anomalies such as neural tube defects, mainly spina bifida (Lammer et al., 1987; Koren and Kennedy, 1999; Koren et al., 2006). Based on this, valproic acid is relatively contraindicated in pregnancy and in patients with pre-existing liver damage and haematological disorders.

A new view on valproic acid came from studies on cell cultures linking valproic acid to cell proliferation and differentiation (Slesinger and Singer, 1987; Searles et al., 1988; Cinatl et al., 1996, 1997; Skladchikova et al., 1998). The effect of valproic acid on cell cultures as well as its teratogenicity triggered a focused interest in its potential to affect cell behaviour and developmental pathways that may be targeted for therapeutic purposes of cancers (Reviewed in Blaheta and Cinatl (2002), Blaheta et al. (2002, 2005), Altucci et al. (2005),; Minucci and Pelicci (2006)).

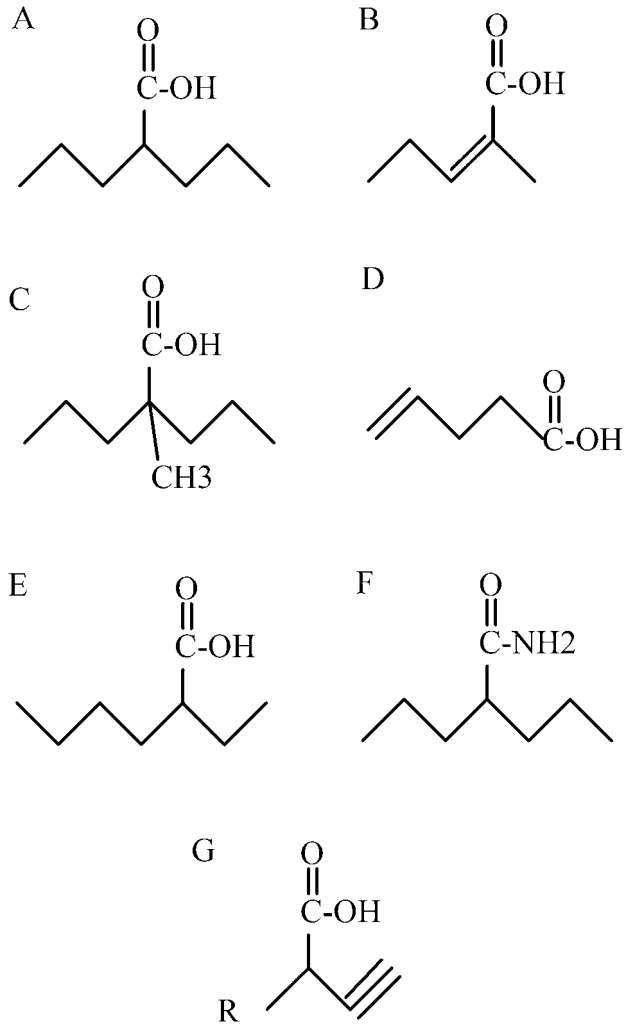

Fig. 1. Valproic acid and its selected analogues. A – Valproic acid, 2-propylpentanoic acid, B – 2-methylpent-2-enoic acid, C – 2-methyl-2-n-propylpentanoic acid, D – pent-4-enoic acid, E – 2-ethylhexanoic acid, F – valpromide, 2-propylpentanamide, G – 2-n-alkylbut-3-ynoic acid (R represents alkyl. Compounds containing propyl to octyl (for the alkyl residue) were tested.) (Bojic et al., 1998 ; Blaheta et al., 2005).

The molecular structure of valproic acid offers many possibilities of chemical modification (Fig. 1). Analogues of valproic acid including its metabolites show overlapping but distinct activities influencing convulsions, neural processes, cell proliferation, and differentiation and teratogenicity (Bojic et al., 1998; Gurvich et al., 2004; O'Loinsigh et al., 2004; Eickholt et al., 2005). Differential structure-activity relationships are key approaches in uncovering the molecular mechanisms responsible for biological effects of valproic acid.

Valproic acid is a teratogenic agent in humans and in numerous animal model systems

The effect of valproic acid on neural tube development was studied on many animal models (Okada et al., 2004; Okada and Fujiwara, 2006). The defect of neural tube closure observed at highger incidence in children of mothers treated by valproic acid during pregnancy was also observed in mice (Nau et al., 1991), including spina bifida (Ehlers et al., 1992). Mice treated with valproic acid in early gestation time (GD8) developed exencephaly (Menegola et al., 1996), and skeletal malformations were also observed (Menegola et al., 1998). Mouse models indicated that high doses of valproic acid led to the formation of additional ribs or vertebra (or their fusions), suggesting that vertebral identity specification along the anterio-posterial axis (AP axis) was affected by valproic acid (Okada and Fujiwara, 2006). Other systems used to study valproic acid teratogenicity potential included *Xenopus laevis* and zebrafish (*Danio rerio*). Both models revealed the teratogenicity of valproic acid, although *X. laevis* with approximately 100-fold lower sensitivity. Valproic acid caused retardation of embryonic development, disrupted pigmentation and pericardial defects resembling the phenotype of the homeobox gene *Hop* inhibition (Gurvich et al., 2005).

The effect of valproic acid on cell lines and cancer cells in culture

Valproic acid and its analogues modulate the behaviour of various tumour cells by inducing differentiation and inhibiting cell proliferation, increasing apoptosis and immunogenicity, and decreasing angiogenic potency.

a/ Valproic acid induces differentiation and inhibits proliferation of cultured cancer cells

Valproic acid was shown to affect differentiation of many cell lines, mainly of neuroectodermal and leukaemic origin. The treatment of the cholinergic neuroblastoma × glioma hybrid cell line NG108-15 with valproic acid caused reduced growth of these cells. The activity of choline acetyltransferase, β-galactosidase and muscarinic cholinergic receptor binding were increased (Slesinger and Singer, 1987). Valproic acid reduced proliferation and induced differentiation of human neuroblastoma cells (SJ-N-KP, AF8) documented by neurite extension and by up-regulation of neuronal markers (Rocchi et al., 2005).

Human leukaemic cells HL-60 and MOLT-4 treated with 4 mmol/l valproic acid for three days showed induced differentiation, marked by the increase in CD11b and co-stimulatory/adhesion molecule CD86. Decrease in percentage of cells in the S phase of the cell cycle was also observed (Rezacova et al., 2006).

An antiproliferative effect of valproic acid has been first shown by Regan in 1985 (Regan, 1985), who observed a decreased mitotic index in mouse neuroblastoma (Neuro-2A) and rat glioma (C6) cells. This effect was reversible and was not caused by cytotoxicity. The influence of valproic acid on the cell cycle was determined to be in the G1 phase (Martin and Regan, 1991). The study on endometrial cancer cell lines, on myeloma cell lines and on poorly differentiated thyroid cancer cell lines (N-PA and BHT-101) confirmed the cell cycle arrest of valproate-treated cells in G0/1 phase. Valproic acid caused the accumulation of p21 and reduced levels of cyclin A and D1 (Takai et al., 2004; Catalano et al., 2005; Kaiser et al., 2006). Inhibition of proliferation by valproic acid in a dose-dependent manner was shown in myeloma cell lines OPM-2, NCI-H929 and LP-1 (Kaiser et al., 2006).

Valproic acid inhibited growth of the endometrial cancer cell lines (Ishikawa, Hec-1B, Hec59, RL95–2, KLE, and AN3CA) that each expressed a mutated, non-functional p53 protein and growth of human thyroid cancer cells (papillary and follicular thyroid cancer cell lines) by 26 to 59% when administered for 48 hours and up to 77% when used for 72 hours (Takai et al., 2004; Shen et al., 2005).

Long-term treatment with valproic acid inhibits prostate cancer cell growth *in vitro* and *in vivo*. Ten to 14 day administration of valproic acid decreased the net proliferation rate of prostate cancer cells – androgen receptor-positive (LNCaP and C4-2) as well as androgen receptor-negative (DU145 and PC3). This correlated with increased caspase-2 and caspase-3 activation and reduction of tumour xenograft growth *in vivo* (Xia et al., 2006).

The effect of valproic acid was investigated on hepatocellular carcinoma cell lines and on primary human hepatocytes. Despite the fact that hepatocellular carcinomas are generally very resistant to conventional chemotherapy, valproic acid inhibited proliferation in hepatocellular carcinoma cell lines but not in primary human hepatocytes (Armeanu et al., 2005b).

b/ Valproic acid induces apoptosis in cultured cancer cells

Valproic acid was shown to induce apoptosis in many human leukaemia cell lines of B-, T-, and myeloid lineage. Valproic acid triggered the release of cytochrome c from mitochondria as well as activation of caspases-3, -8, and -9 (Kawagoe et al., 2002). When MV4-11 cells were pre-treated with the caspase inhibitor, the pro-apoptotic effect of valproic acid was inhibited in the nucleus but not on the cell surface. These results led to suggestions that valproic acid can activate two different apoptotic signalling pathways: 1. caspase-dependent (in the nucleus) and 2. caspase-independent (on the cell membrane).

In myeloma cell lines, apoptosis was induced by valproic acid in OPM-2, NCI-H929, LP-1 cells as well as in sorted primary multiple myeloma cells (Kaiser et al., 2006). Valproic acid induced apoptosis in acute myeloic leukaemia patient cells expressing P-glycoprotein (P-gp) and/or MRP1 (multidrug resistance-associated protein 1) (Tang et al., 2004a), in human leukaemic cell lines HL-60 and MOLT-4, and in prostate carcinoma cell lines PC3, DU145, LNCaP (Angelucci et al., 2006).

Apoptosis was also induced in human thyroid cancer cells lines (papillary and follicular) treated with valproic acid. Nineteen to 30% of treated cells underwent apoptosis, compared with 4 to 8% of the control cells. The expression of pro-survival genes *bcl-2* and *bcl-x* was down-regulated, while the expression of the pro-apoptotic gene *bax* was up-regulated (Shen et al., 2005).

Induction of apoptosis was documented by chromatin condensation, DNA fragmentation and also phosphatidylserine externalization. Valproic acid at concentration greater than 300 μM induced a transient wave of apoptosis in the rat hepatoma cell line FaO. At the transcriptome level, the expression of Fas-ligand and caspase-11 were increased, and at the proteome level caspase-3 was activated (Phillips et al., 2003).

In hepatocellular carcinoma cells, down-regulation of anti-apoptotic factors and up-regulation of pro-apoptotic factors was found, indicating that the modulation of these factors is a key event in valproic acid-induced apoptosis (Armeanu et al., 2005b).

c/ Valproic acid increases immunogenicity of cultured cancer cells

Tumour-associated ligands engaged in the activation of NK cell receptors are largely unknown. The exceptions are the MHC class I chain-related molecules

MICA and MICB and the UL16-binding proteins (ULBP). They bind to the activating immuno-receptor NKG2D, which is expressed on cytotoxic lymphocytes. Valproic acid induced transcription of MICA and MICB in hepatocellular carcinoma cells, leading to increased cell surface and total MIC protein expression. The induction of MIC molecules increased lysis of hepatocellular carcinoma cells by NK cells (this was abolished by addition of a blocking NKG2D antibody). However, in primary human hepatocytes, valproic acid treatment did not induce MIC protein expression, suggesting that valproic acid mediates specific priming of malignant cells for innate immune effector mechanisms (Armeanu et al., 2005a).

d/ Valproic acid inhibits expression of angiogenic proteins in cultured cells

Valproic acid can inhibit angiogenesis directly by interfering with endothelial cells. It caused inhibition of proliferation, migration and tube formation of endothelial cells (Michaelis et al., 2004a). Valproic acid also induced a decrease of endothelial nitric-oxid synthase (eNOS), which was probably responsible for this activity. Hyperacetylation of histone H4 was also observed, indicating that HDAC inhibition preceded this event.

Valproic acid inhibits angiogenesis *in vivo*. This was documented using the chicken chorioallantoic membrane assay and the matrigel plug assay in mice. The treatment of human endothelial cells with 1 mM valproic acid for three days caused increased levels of the neutrophil chemo-attractants CXCL1, 3, 4, 5 and 6 (Engl et al., 2004). Morphological alterations as well as reduced cell growth were observed.

Valproic acid inhibits angiogenesis indirectly. Treatment of neuroblastoma cells with 1 mM valproic acid caused augmented expression of anti-angiogenic molecules thrombospondin-1 and activin A (Cinatl et al., 2002). Similarly the treatment of colon adenocarcinoma cell line Caco-2 with valproic acid caused significant reduction of vascular endothelial growth factor (VEGF) secretion, as well as down-regulation of mRNA and protein expression of VEGF. The highest inhibition was observed after treatment with 3 mM valproic acid for 48–72 hours. The same concentration of valproic acid caused a decreased basic fibroblast growth factor (bFGF) protein level and inhibition of the ubiquitin-proteasome proteolytic system activity (Zgouras et al., 2004).

Valproic acid has anti-neoplastic properties on human and animal malignancies

The effect of valproic acid on cultured malignant cells triggered interest in its potency to affect tumours *in vivo*. Valproic acid was shown, parallel to its antiproliferative effect on cultured neuroblastoma cells, to inhibit proliferation, increase expression of E-cadherin and induce apoptosis of tumours developed from injected UKF-NB-3 human neuroblastoma cells on athymic nude mice (Cinatl et al., 1997).

Acute myeloid leukaemias (AMLs) form a group of malignancies predominantly associated with chromosomal translocations, which lead to the formation of fusion genes that are transcribed and spliced to code for subsequent fusion proteins. The most studied AML subtype in this respect is acute promyelocytic leukaemia (APL; FAB M3). In this type of leukaemia the majority of patients carry the chromosomal translocation t(15; 17), which generates the PML-RARα (PML and retinoic acid receptor α) fusion proteins. The other translocations such as PLZF-RARα or AML-ETO are also known (Minucci and Pelicci, 1999; Minucci et al., 2000; Heibert et al., 2001; Kramer et al., 2001; Melnick and Licht, 2002).

PML-RARα functions like a retinoic acid receptor, but despite the fact that it contains most of the normal receptor, it acts as a transcriptional inhibitor at physiologic concentrations of the ligand, all-trans retinoic acid (ATRA). This partially reflects the function of normal RARα in the absence of the ligand. In the physiological situation, the RARα represses target gene transcription in the absence of ATRA by recruiting the histone deacetylase (HDAC) complex. This is done by direct binding of the HDAC-associated co-repressor N-CoR or SMRT. As a consequence, the transcription of numerous genes is silenced. Silenced genes include genes involved in cell maturation and death of numerous cell types. In the presence of high doses of retinoid ligands, the HDAC complex is dissociated from PML-RARα and replaced by histone acetyl transferase (HAT)/co-activator complexes (Warrell et al., 1998; Altucci et al., 2005). It was proposed that together with RA induction of the death ligand TRAIL, this allows the differentiation of leukaemic cells (Clarke et al., 2004; Nebbioso et al., 2005).

Although very promising, the treatment of AML by retinoids has only a partial therapeutic effect. Treatment of 26 patients with valproic acid/ATRA for three months resulted in transient disease control in a subset of patients with AML that had evolved from a myeloproliferative disorder, but not in patients with a primary myelodysplastic syndrome (MDS)-related AML (Bug et al., 2005).

As expected from the valproic acid effect on histone acetylation, valproic acid proved to be augmenting the anti-leukaemic effect of retinoids in AML. Administration of valproic acid to AML patients treated with retinoids significantly augmented the effects of retinoids and prolonged the period of leukaemic responsiveness to retinoid treatment (Bug et al., 2005).

Valproic acid, which could be used for the treatment of AML alone or in combination with ATRA, is usually used for the treatment of older people who cannot be treated with a more invasive type of treatment. There were numerous trials that addressed this question:

a pilot study of the valproic acid/ATRA combination was performed in 11 elderly patients with *de novo* AML. Complete bone marrow response was observed in three patients, including one complete remission. Two additional patients had haematological improvement (Raffoux et al., 2005).

Twenty older patients with recurrent or refractory AML or MDS were treated in a phase II protocol with sequential valproic acid and ATRA therapy. The patients were treated for two months. Haematological improvement was observed in six of the 20 patients. In five patients a major platelet response was observed, achieving platelet transfusion independence. Treatment with ATRA did not modify the response observed with valproic acid alone. This led to the conclusion that differentiation therapy with valproic acid was of clinical benefit in approximately 30% of elderly patients with AML or MDS (Pilatrino et al., 2005).

Valproic acid was used to treat patients with relapsed or primary resistant AML alone or in combination with ATRA. Complete haematological remission lasting for several months had been reported for a few patients only (< 5% of included patients), but increase of peripheral blood platelet counts was more common and has been observed in patients with AML and MDS (Bruserud et al., 2006).

In another trial, 31 patients were treated with valproic acid as monotherapy. ATRA was added later in 13 patients who did not respond to the initial therapy or who relapsed. Another 27 patients received a combination of valproic acid and ATRA. All patients in this trial were too old to receive intensive chemotherapy. The response rate was only 5% according to the International Working Group (IWG) criteria for AML and 16% when the IWG criteria were used for MDS. Based on this, the authors recommended to combine valproic acid with chemotherapy or demethylating agents in future trials (Kuendgen et al., 2006).

It is well established that multiple hits are necessary for the development of leukaemia beside the occurrence of the fusion proteins PML-RARα, PLZF-RARα and AML-ETO (Brown et al., 1997; Grisolano et al., 1997). Patients with acute promyelocytic leukaemia (APL) only rarely have mutations in p53, rather its function is affected. PML-RARα together with class I HDACs causes deacetylation of p53 and its degradation through the MDM2-proteasome pathway in haematopoietic precursors (Insinga et al., 2004, 2005). The effect of valproic acid on such additionally affected genes may be expected.

Other tumours are likely to be therapeutically influenced by valproic acid. Valproic acid affected the behaviour of prostate cancer (Annicotte et al., 2006). In a study where valproic acid and other anti-epileptic drugs were used for the treatment of seizures in patients suffering from glioblastomas, patients treated with valproic acid showed a tendency of longer survival time compared to the patients treated with carbamazepine, an anti-eptileptic drug known to augment expression of several p450 genes. It was concluded that such an effect may be a consequence of decreased efficiency of chemotherapy in patients treated by enzyme-inducing anti-epileptics (Oberndorfer et al., 2005). It may also be speculated that such an effect may be caused by the anti-neoplastic potential of valproic acid.

Valproic acid effect on gene expression

The recent development of DNA microarray techniques allowed wide or even genome-wide screening of the effect of valproic acid on gene transcription. Tang et al. studied gene expression in blood cells of paediatric patients treated chronically with valproic acid and identified 461 genes with altered expression compared to the control group. This group included 67 up-regulated and 394 down-regulated genes. Interestingly, down-regulated genes grouped similarly as the serine-threonine kinase signalling list generated by the Genespring computer program (Affymetrix) (Tang et al., 2004b).

In an experimental model, brain tissue was assayed for gene expression in mice treated with low (approximately 0.2 mM) or high (approximately 0.6 mM) concentrations of valproic acid in blood (Chetcuti et al., 2006). This work identified 11 genes that were differentially expressed in brain tissues treated with valproic acid, eight were down-regulated and three up-regulated. The low-level valproic acid had a more pronounced effect on gene expression than the high-level valproic acid when the results were normalized against the expression of GAPDH, a supposedly house-keeping gene. The list of genes that were labelled as "present" indicated that 5475 genes were labelled as present in control tissues, while in the group with the low-level valproic acid, only 5170 genes were labelled as present and 5838 genes were labelled as present in the group with the high-concentration valproic acid. This suggests that the high concentration of valproic acid may actually be connected with increased gene expression of multiple genes and the group of up-regulated genes may be substantially larger (Chetcuti et al., 2006).

Okada and Fujiwara, who studied the relation between valproic acid-induced teratogenicity and gene expression assayed by microarrays, identified immediate early genes as targets of valproic acid. This supports the effect of valproic acid on the establishment of the AP axis and tissue identification (Okada and Fujiwara, 2006). Similar studies were done on *Xenopus* and zebrafish embryos. Valproic acid and its analogues that had teratogenic potential also affected gene expression. Thus, similarly as studies that indicated a differential effect of valproic acid and its derivatives on cell proliferation compared to the anti-seizure potential, this points at multiple mechanisms as targets of valproic acid (Gurvich et al., 2005; Okada and Fujiwara, 2006).

Valproic acid affects cell behaviour by multiple mechanisms

a/ Valproic acid inhibits the activity of histone deacetylases

Recent studies showed that valproic acid effect on proliferation and differentiation of malignant tumour cells is linked with its inhibiting activity on histone deacetylases. This was found in teratocarcinoma and neuroblastoma cells (Gottlicher et al., 2001; Phiel et al., 2001; Cinatl et al., 2002). The question to ask is whether the effect of valproic acid could be based on a direct inhibition of histone deacetylases (HDACs) (by a direct influence on enzymes that are responsible for acetylation and deacetylation of nucleosomal histones – mainly histones H3 and H4) or by modulation of other targets. Gurvich and co-workers (Gurvich et al., 2004) showed that valproic acid anti-neoplastic effect acts through the inhibition of HDAC. This was confirmed by an *in vitro* deacetylation assay on K562 and U937 cell lines using a synthetic acetylated substrate, and inhibition of HDAC activity was detected by assaying acetylation of histones H3 and H4 (Hoffmann et al., 2000).

Valproic acid and its analogues were shown to inhibit class I HDACs (HDACs 1–3) and class II HDACs (HDACs 4, 5 and 7). When valproic acid and its analogues 2-methyl-2-n-propylpentanoic acid (2M2PP), 4-pentenoic acid (4PA), 2-methyl-pentenoic acid (2M2P), 2-ethylhexanoic acid (2EH), and valpromide (VPM) were tested for HDAC activity, it was valproic acid that was shown to be the strongest inhibitor (Gurvich et al., 2004). The mechanism by which valproic acid affects behaviour of cancer cells differs from its anti-epileptic effect since the efficacy of antineoplastic and anti-epileptic effects differs between particular analogues.

Valproic acid, in contrast to another HDAC inhibitor trichostatin A (TSA), does not inhibit the activity of class II HDACs 6 and 10. This implies that valproic acid might be a more selective HDAC inhibitor than TSA. Valproic acid affects both class I and class II HDACs, but the effect on these two classes differs. Kramer and co-workers showed that valproic acid selectively inhibits the catalytic activity of class I HDACs and induces the proteasomal degradation of class II HDACs (Kramer et al., 2003).

Valproic acid and its analogues induce expression of multiple exogenous reporter genes, i.e. SV40, p21 and gelsolin, which are associated with HDAC inhibition (Gottlicher et al., 2001; Phiel et al., 2001).

Histone deacetylase inhibitors can be divided into four groups: short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides, and benzamides. Short-chain fatty acids such as sodium butyrate, valproic acid and trybutyrin are not very strong inhibitors of histone deacetylases, but they have been already used clinically and can be administered orally. Hydroxamic acids are stronger inhibitors of histone deacetylases, and trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) belong to this group. Cyclic tetrapeptides such as trapoxin (a depsipeptide) cause irreversible inhibition of HDACs and have not been used clinically because of toxicity. Benzamides MS-275 and CI-994 are synthetically derived inhibitors of HDACs (Yoo and Jones, 2006).

Studies that focused on similarities between gene expression profiles caused by compounds with related cellular mechanisms showed that valproic acid clearly belongs to the histone deacetylase inhibitors group. Interestingly, the similarities in gene expression induced by different members of the molecular group indicate that gene expression profiles affected by the genetically distinct mechanisms may have overlapping characteristics (Williams et al., 2002).

b/ Valproic acid interferes with MAPK signalling

Protein kinases are key regulators of cell proliferation and differentiation by transmitting extracellular stimuli to the cell nucleus (Toker, 2000). The mitogen-activated protein (MAP) kinase pathways lead to the activation of extracellular-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38 (Gudermann et al., 2000). It was documented that valproic acid increased DNA binding and transactivation activity of the AP-1 transcription factor (Manji et al., 1996; Chen et al., 1997, 1999b; Asghari et al., 1998) , which is also a target of the JNK and p38 signal cascades. *In vitro* studies showed that valproic acid specifically triggers phosphorylation of ERK, the upstream modulator of AP-1, but does not act via the JNK and p38 pathways (Yuan et al., 2001). Therefore, valproic acid-induced increase in AP-1 binding and function are likely due, at least in part, to the activation of ERK followed by phosphorylation and increase in the expression of c-Jun. The analogues of valproic acid (named above) have stronger effects on the activation of MAPK than valproic acid itself and this is in contrast to their effect on HDAC I inhibition. This seems to support the possibility that the effect on MAPK is not, at least entirely, mediated by HDAC inhibition. HDAC activity is, however, connected to the kinase transduction pathways through HDAC3, which is both cytoplasmic and nuclear, and its activity is linked to numerous signal transduction cascades (Chen et al., 2005).

c/ Valproic acid affects the β catenin-Wnt signalling pathway

The Wnt signalling pathway plays an important role in embryonic development as well as in cellular proliferation, cell migration, polarity, organo- and carcino-

genesis (Miller et al., 1999, 2001; Bienz and Clevers, 2000; Christiansen et al., 2000; Miller, 2002; Bienz, 2005). One of four known different pathways of Wnt signalling is the canonical Wnt/β-catenin cascade. In this pathway Wnt binds to Lrp5 or 6 and Frizzled co-receptor complex and this leads to the accumulation of β-catenin in cells. β-catenin then translocates to the nucleus, binds to TCF/Lef transcription factors and activates the transcription of Wnt-dependent genes. β-catenin is phosphorylated by glycogen synthase kinase 3β (GSK-3β), which leads to its rapid degradation (Koch et al., 2001).

Valproic acid has been reported to inhibit GSK-3β-mediated phosphorylation of a peptide derived from the CREB protein in human embryonic kidney 293T and murine Neuro2A neuroblastoma cells and increase levels of β-catenin in human neuroblastoma cells and colon cancer cells (Chen et al., 1999a; Vincan et al., 2000). Nevertheless, it was proposed that valproic acid activates Wnt-dependent gene expression through inhibition of histone deacetylase activity (Phiel et al., 2001).

The involvement of histone deacetylase inhibition by valproic acid in β-catenin dependent regulation is supported by its effect on the expression of E-cadherin (Takai et al., 2004). The expression of numerous tumour suppressor genes including E-cadherin is linked to hypermethylation of specific regions of DNA and may be partially reverted by increased histone acetylation (Mielnicki et al., 2001; Ohira et al., 2003; Digel and Lubbert, 2005).

d/ Valproic acid may influence additional pathways

Valproic acid was shown to be involved in other regulatory pathways. In many of them, it is likely that the effect is mediated primarily by the inhibition of histone deacetylase activity, but some may be distinct.

Valproic acid increases the levels of 5-lipoxygenase (5-LOX) protein in murine hippocampus. 5-LOX produces leukotriens from arachidonic acid and this is likely to be involved in the regulation of chromatin remodelling. Since increased levels of 5-LOX are linked to aging and neurodegeneration, it may be expected that an increase in 5-LOX expression may contribute to tumour cell aging and differentiation through chromatin remodelling (Manev et al., 2000; Qu et al., 2000).

Valproic acid is likely to inhibit invasiveness of cancer cells at concentrations lower than those necessary for its anti-proliferative effect. Increased expression of genes that inhibit invasiveness of cancer cells was observed in response to the treatment with relatively low doses of valproic acid (150 μmol/l). These genes included signal transducer and activator of transcription 6 (STAT6), Ring1 and YY1 binding protein (RYBP), and protocadherin γ subfamily C3 (PCDHGC3) (McGarry et al., 2004).

Valproic acid may affect regulation by nuclear receptors. The regulation of nuclear receptors is critically influenced by HDACs. Some members of this superfamily of genes may be affected by valproic acid more directly, such as the peroxisome proliferator-activated receptors (PPARs). PPARδ and PPARγ (but not PPARα) were activated by valproic acid in Chinese hamster ovary cells and F9 teratocarcinoma cells (Lampen et al., 1999; Werling et al., 2001). The effect, at least in the case of PPARδ, was not based on the binding and activation of the receptor by valproic acid as an agonistic ligand since it did not induce formation of heterodimers of PPARδ with retinoid X receptor on DNA response elements (Lampen et al., 2001). The expression of PPARγ-dependent genes may significantly contribute to the anticancer activity of valproic acid, because PPARγ regulates the expression of the tumour suppressor gene *PTEN* (phosphatase and tensin homologue deleted from chromosome 10) that is often mutated in cancers (Patel et al., 2001). PTEN, a lipid phosphatase, dephosphorylates phosphatidylinositol (3,4,5)-triphosphate (PIP-3) to phosphatidylinositol (4,5)-diphosphate (PIP-2) and antagonizes the regulation by phosphatidylinositol-3 kinase (PI-3K).

Studies aimed at the elucidation of the mechanism of the anti-epileptic effect of valproic acid provided strong evidence that valproic acid and its derivatives interfere with multiple enzymatic pathways. Valproic acid forms an ester with coenzyme A, and despite the fact that this was shown not to be connected with the anti-epileptic potential, it may also be a link to multiple different interactions (Deutsch et al., 2003). Other metabolites, such as the E isomer of 2-ene-valproic acid, possess antiepileptic potential with no or minimal teratogenic potency but may also interfere with other processes than GABA-dependent regulation.

Valproic acid is degraded by β oxidation and glucuronidation (Wong et al., 2000). Its effect on mitochondrial metabolism and β oxidation is well characterized (Bjorge and Baillie, 1991). An interesting connection between valproic acid-induced generation of reactive oxygen species and enhanced binding of SP1 to antioxidant response element-containing promoters was proposed by Kawai and Arinze (Kawai and Arinze, 2006).

e/ Multiple pathways may be affected by related mechanisms

Valproic acid affects multiple cell regulatory pathways (Fig. 2). The best substantiated molecular mechanism of action of valproic acid is clearly its inhibitory effect on HDACs. This effect will likely influence other pathways including kinases due to an upstream change of gene expression. This would certainly critically affect the regulation by kinases, including GSK3β and ERK.

Is there a connection between valproic acid's beneficial effects on such complex diseases as mood disorder and cancers? The common link may be an impaired and deregulated metabolism with a characteristic gene expression profile including specifically silenced genes. Keeping with this, valproic acid seems to have long-term and preventive effects in both neurological disorders and cancers.

Histone deacetylases recently emerged as regulators of histone acetylation-independent events including interactions with protein phosphatase PP1 (Fig. 3) (Brush et al., 2004; Chen et al., 2005). Trichostatin A, HDAC42 and SAHA disrupted HDAC1 and the HDAC/PP1 complex, resulting in dephosphorylation and deactivation of Akt. Thus, HDAC inhibitors are likely to critically affect expression of genes downstream of Akt, GSK3 and other transcription factors including CREB (Taylor et al., 2000).

Numerous non-histone proteins are substrates for histone deacetylases. Androgen receptor, SMAD7, Stat3, NF-κB (RelA), and SRY are substrates for class I HDACs (Lin et al., 2006) that are directly inhibited by valproic acid.

The direct effect of valproic acid on numerous enzymes is also well documented. It seems likely that the resulting effect of valproic acid on cells and tissues is based on specific interactions that depend on a particular cellular context.

Combination treatment with valproic acid and other drugs

Valproic acid effect on the regulation by PPARγ was studied in relation to the treatment with ciglitizone, a PPARγ ligand, in combination with phenylbutyrate on non-small cell lung cancer cells. The combinatory treatment led to more profound growth arrest than treatment with either drug alone (Chang and Szabo, 2002).

The therapy combining sodium butyrate and interpheron (IFN) α has been shown to suppress the growth of human lung cancer cells. This was caused by marked apoptosis, even when butyrate alone barely induced irreversible differentiation (Yamamoto-Yamaguchi et al., 2003). This combination therapy of sodium butyrate and type I IFN also reduced invasion and metastasis of human liver cancer cells (Kaneko et al., 2004). Similarly as in the case of butyrate, valproic acid anticancer activity was increased by IFN-α in neuroblastoma cell lines (Cinatl et al., 2002; Michaelis et al., 2004b). Keeping with this, the expression pattern in epileptic children treated with valproic acid included alteration of a number of IFN-regulating genes (Tang et al., 2004b).

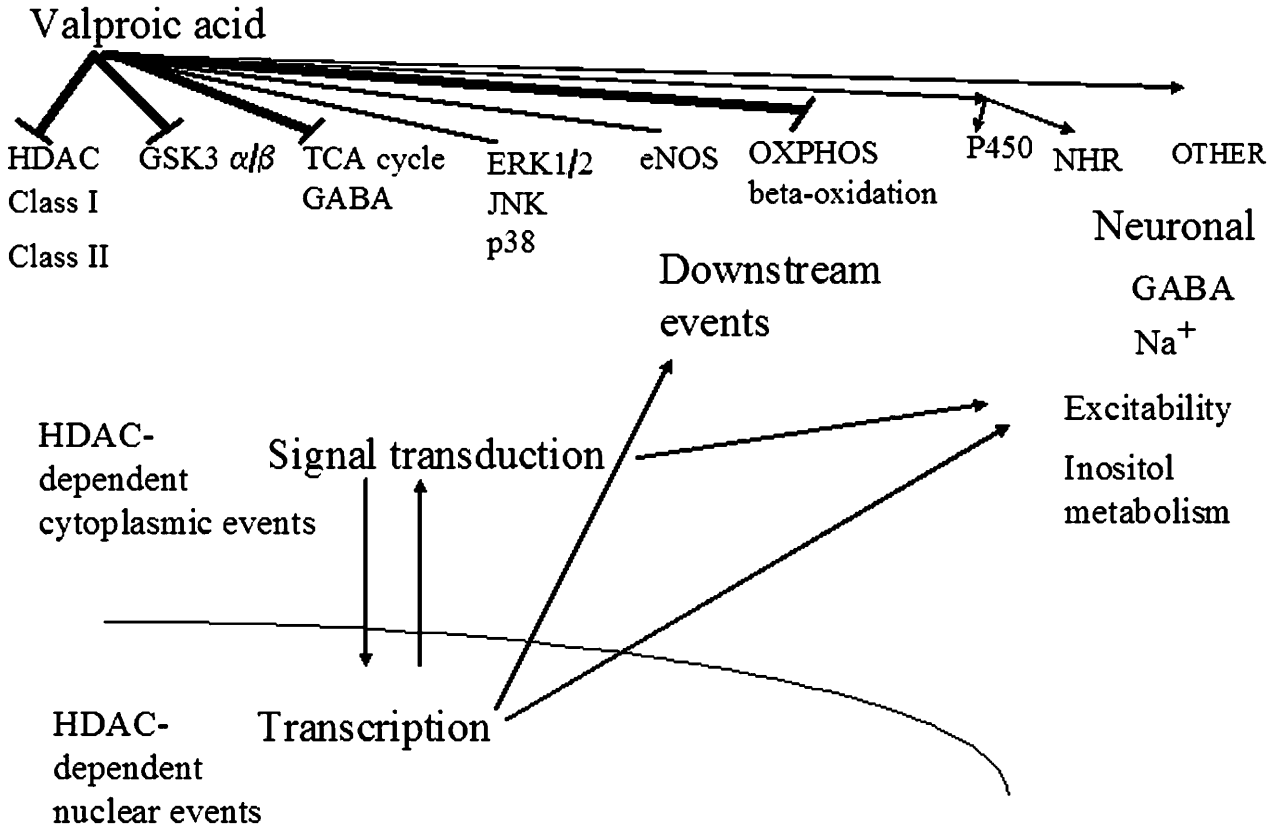

Fig. 2. Schematic representation of pathways affected by valproic acid and their interactions. Vaproic acid directly influences several pathways including HDACs, GSK3 β, TCA cycle, and GABA-dependent regulation. Many other interactions may be indirect through molecular cross-talk between pathways.

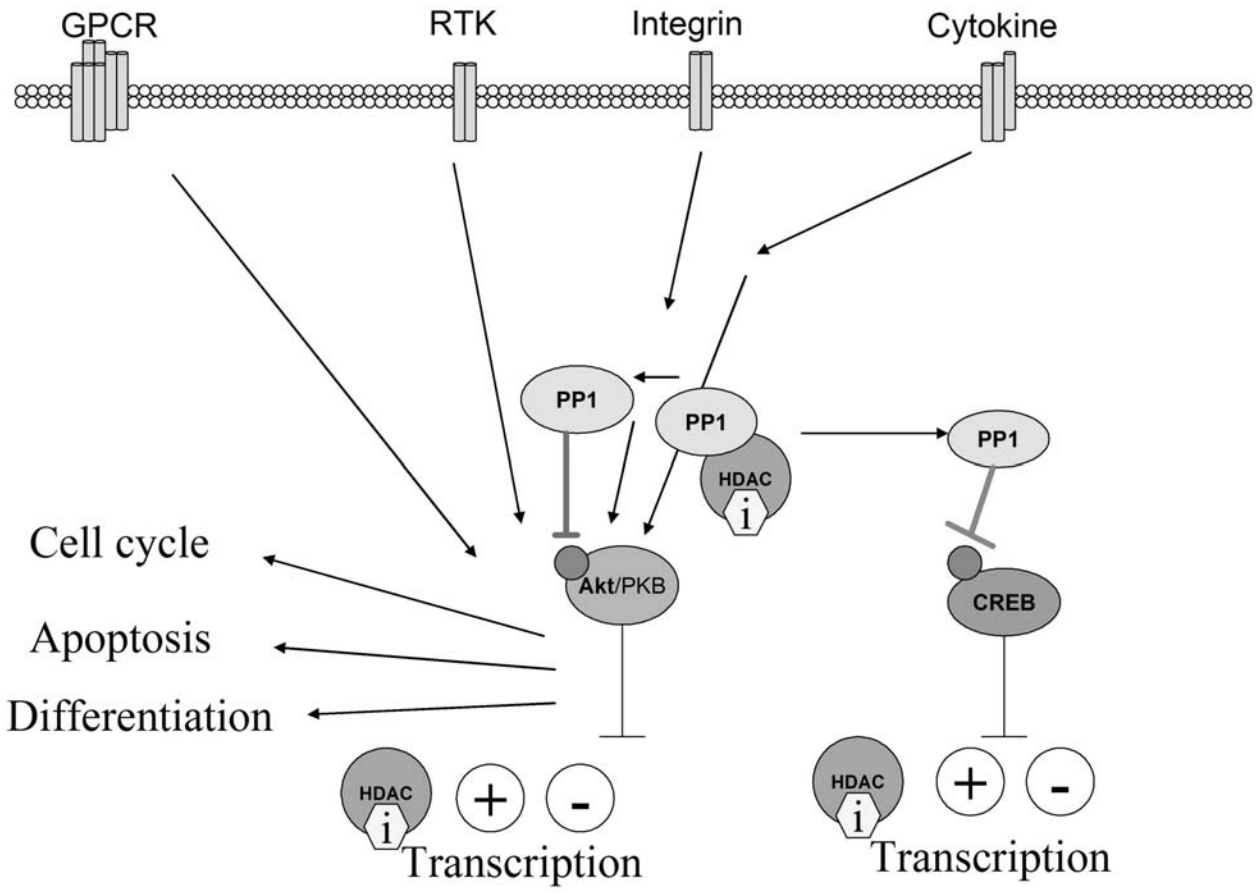

Fig. 3. Schematic representation of HDACs involvement in regulation of cytoplasmic and nuclear pathways including protein phosphatases (PP). An HDAC is associated with protein phosphatase. Inhibition of HDAC activity disrupts the PP/HDAC complex and relieves the PP for other interactions including Akt and CREB. As a result, inhibition of HDAC has both transcription activation and transcription inhibition consequences (Chen et al., 2005).

Valproic acid was found beneficial in the combination of differentiation and cytoreductive therapy of patients with advanced myeloid leukaemia. These patients with poor prognosis were treated with valproic acid and cytosine arabinoside (LD-AraC) or hydroxyurea (HU). The average treatment duration was 90 days. Valproic acid-related reduction of bone marrow blasts was observed in two out of eight patients (Guel et al., 2003).

Valproic acid as a molecular lead

The multiplicity of biological effects of valproic acid and its analogues offers molecular tools for mechanism-oriented studies. Functional studies and high throughput methods such as genome-wide microarrays and proteome-wide differential displays may uncover new potentials of valproic acid and its analogues in the regulation of multiple pathways affecting metabolism, proliferation and differentiation of cells. It may be anticipated that some compounds will prove to be more specific for particular enzymes and target molecules while others may posses a wider interaction spectrum. Irrespective of the outcome, valproic acid and its analogues are emerging as powerful molecular leads for such endeavours.

Acknowledgement

Authors thank Dr. Vladimír Saudek for critical reading of the manuscript and advice and Michal Pohludka for advice on chemical nomenclature.

References

Altucci, L., Clarke, N., Nebbioso, A., Scognamiglio, A., Gronemeyer, H. (2005) Acute myeloid leukemia: therapeutic impact of epigenetic drugs. *Int. J. Biochem. Cell. Biol.* **37**, 1752-1762.

Angelucci, A., Valentini, A., Millimaggi, D., Gravina, G. L., Miano, R., Dolo, V., Vicentini, C., Bologna, M., Federici, G., Bernardini, S. (2006) Valproic acid induces apoptosis in prostate carcinoma cell lines by activation of multiple death pathways. *Anticancer Drugs* **17**, 1141-1150.

Annicotte, J. S., Iankova, I., Miard, S., Fritz, V., Sarruf, D., Abella, A., Berthe, M. L., Noel, D., Pillon, A., Iborra, F., Dubus, P., Maudelonde, T., Culine, S., Fajas, L. (2006) Peroxisome proliferator-activated receptor γ regulates E-cadherin expression and inhibits growth and invasion of prostate cancer. *Mol. Cell. Biol.* **26**, 7561-7574.

Armeanu, S., Bitzer, M., Lauer, U. M., Venturelli, S., Pathil, A., Krusch, M., Kaiser, S., Jobst, J., Smirnow, I., Wagner, A., Steinle, A., Salih, H. R. (2005a) Natural killer cell-

mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate. *Cancer Res.* **65**, 6321-6329.

Armeanu, S., Pathil, A., Venturelli, S., Mascagni, P., Weiss, T. S., Gottlicher, M., Gregor, M., Lauer, U. M., Bitzer, M. (2005b) Apoptosis on hepatoma cells but not on primary hepatocytes by histone deacetylase inhibitors valproate and ITF2357. *J. Hepatol.* **42**, 210-217.

Asghari, V., Wang, J. F., Reiach, J. S., Young, L. T. (1998) Differential effects of mood stabilizers on Fos/Jun proteins and AP-1 DNA binding activity in human neuroblastoma SH-SY5Y cells. *Brain. Res. Mol. Brain Res.* **58**, 95-102.

Balbi, A., Sottofattori, E., Mazzei, M., Sannita, W. G. (1991) Study of bioequivalence of magnesium and sodium valproates. *J. Pharm. Biomed. Anal.* **9**, 317-321.

Bienz, M. (2005) β-Catenin: a pivot between cell adhesion and Wnt signalling. *Curr. Biol*. **15**, R64-67.

Bienz, M., Clevers, H. (2000) Linking colorectal cancer to Wnt signaling. *Cell* **103**, 311-320.

Bjorge, S. M., Baillie, T. A. (1991) Studies on the β-oxidation of valproic acid in rat liver mitochondrial preparations. *Drug Metab. Dispos.* **19**, 823-829.

Blaheta, R. A., Cinatl, J., Jr. (2002) Anti-tumor mechanisms of valproate: a novel role for an old drug. *Med. Res. Rev.* **22**, 492-511.

Blaheta, R. A., Nau, H., Michaelis, M., Cinatl, J., Jr. (2002) Valproate and valproate-analogues: potent tools to fight against cancer. *Curr. Med. Chem.* **9**, 1417-1433.

Blaheta, R. A., Michaelis, M., Driever, P. H., Cinatl, J., Jr. (2005) Evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. *Med. Res. Rev.* **25**, 383-397.

Bojic, U., Ehlers, K., Ellerbeck, U., Bacon, C. L., O'Driscoll, E., O'Connell, C., Berezin, V., Kawa, A., Lepekhin, E., Bock, E., Regan, C. M., 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.

Bowden, C. L., Singh, V. (2005) Valproate in bipolar disorder: 2000 onwards. *Acta Psychiatr. Scand.* **111**, 13-20.

Brown, D., Kogan, S., Lagasse, E., Weissman, I., Alcalay, M., Pelicci, P. G., Atwater, S., Bishop, J. M. (1997) A PMLRARα transgene initiates murine acute promyelocytic leukemia. *Proc. Natl. Acad. Sci. USA* **94**, 2551-2556.

Bruserud, O., Stapnes, C., Tronstad, K. J., Ryningen, A., Anensen, N., Gjertsen, B. T. (2006) Protein lysine acetylation in normal and leukaemic haematopoiesis: HDACs as possible therapeutic targets in adult AML. *Expert. Opin. Ther. Targets* **10**, 51-68.

Brush, M. H., Guardiola, A., Connor, J. H., Yao, T. P., Shenolikar, S. (2004) Deactylase inhibitors disrupt cellular complexes containing protein phosphatases and deacetylases. *J. Biol. Chem.* **279**, 7685-7691.

Bug, G., Ritter, M., Wassmann, B., Schoch, C., Heinzel, T., Schwarz, K., Romanski, A., Kramer, O. H., Kampfmann, M., Hoelzer, D., Neubauer, A., Ruthardt, M., Ottmann, O. G. (2005) Clinical trial of valproic acid and all-trans retinoic acid in patients with poor-risk acute myeloid leukemia. *Cancer* **104**, 2717-2725.

Burton, B. S. (1882) On the propyl derivatives and decomposition products of ethylacetoacetate. *Am. Chem. J.* **3**, 385-395.

Catalano, M. G., Fortunati, N., Pugliese, M., Costantino, L., Poli, R., Bosco, O., Boccuzzi, G. (2005) Valproic acid induces apoptosis and cell cycle arrest in poorly differentiated thyroid cancer cells. *J. Clin. Endocrinol. Metab.* **90**, 1383-1389.

Chang, T. H., Szabo, E. (2002) Enhanced growth inhibition by combination differentiation therapy with ligands of peroxisome proliferator-activated receptor-γ and inhibitors of histone deacetylase in adenocarcinoma of the lung. *Clin. Cancer Res.* **8**, 1206-1212.

Chen, C. S., Weng, S. C., Tseng, P. H, Lin, H. P. (2005) Histone acetylation-independent effect of histone deacetylase inhibitors on Akt through the reshuffling of protein phosphatase 1 complexes. *J. Biol. Chem.* **280**, 38879-38887.

Chen, G., Yuan, P., Hawver, D. B., Potter, W. Z., Manji, H. K. (1997) Increase in AP-1 transcription factor DNA binding activity by valproic acid. *Neuropsychopharmacology* **16**, 238-245.

Chen, G., Huang, L. D., Jiang, Y. M., Manji, H. K. (1999a) The mood-stabilizing agent valproate inhibits the activity of glycogen synthase kinase-3. *J. Neurochem.* **72**, 1327-1330.

Chen, G., Yuan, P. X., Jiang, Y. M., Huang, L. D., Manji, H. K. (1999b) Valproate robustly enhances AP-1 mediated gene expression. *Brain Res. Mol. Brain Res*. **64**, 52-58.

Chetcuti, A., Adams, L. J., Mitchell, P. B., Schofield, P. R. (2006) Altered gene expression in mice treated with the mood stabilizer sodium valproate. *Int. J. Neuropsychopharmacol.* **9**, 267-276.

Christiansen, J. H., Coles, E. G., Wilkinson, D. G. (2000) Molecular control of neural crest formation, migration and differentiation. *Curr. Opin. Cell Biol.* **12**, 719-724.

Cinatl, J., Jr., Cinatl, J., Scholz, M., Driever, P. H., Henrich, D., Kabickova, H., Vogel, J. U., Doerr, H. W., Kornhuber, B. (1996) Antitumor activity of sodium valproate in cultures of human neuroblastoma cells. *Anticancer Drugs* **7**, 766-773.

Cinatl, J., Jr., Cinatl, J., Driever, P. H., Kotchetkov, R., Pouckova, P., Kornhuber, B., Schwabe, D. (1997) Sodium valproate inhibits in vivo growth of human neuroblastoma cells. *Anticancer Drugs* **8**, 958-963.

Cinatl, J., Jr., Kotchetkov, R., Blaheta, R., Driever, P. H., Vogel, J. U., Cinatl, J. (2002) Induction of differentiation and suppression of malignant phenotype of human neuroblastoma BE(2)-C cells by valproic acid: enhancement by combination with interferon-α. *Int. J. Oncol.* **20**, 97-106.

Clarke, N., Jimenez-Lara, A. M., Voltz, E., Gronemeyer, H. (2004) Tumor suppressor IRF-1 mediates retinoid and interferon anticancer signaling to death ligand TRAIL. *EMBO J.* **23**, 3051-3060.

Deutsch, J., Rapoport, S. I., Rosenberger, T. A. (2003) Valproyl-CoA and esterified valproic acid are not found in brains of rats treated with valproic acid, but the brain concentrations of CoA and acetyl-CoA are altered. *Neurochem. Res.* **28**, 861-866.

Digel, W., Lubbert, M. (2005) DNA methylation disturbances as novel therapeutic target in lung cancer: preclinical and clinical results. *Crit. Rev. Oncol. Hematol.* **55**, 1-11.

Ehlers, K., Sturje, H., Merker, H. J., Nau, H. (1992) The valproic acid metabolite E-2-n-propyl-2-pentenoic acid does not induce spina bifida in the mouse. *Dev. Pharmacol. Ther.* **19**, 196-204.

Eickholt, B. J., Towers, G. J., Ryves, W. J., Eikel, D., Adley, K., Ylinen, L. M., Chadborn, N. H., Harwood, A. J., Nau, H., Williams, R. S. (2005) Effects of valproic acid derivatives on inositol trisphosphate depletion, teratogenicity,

glycogen synthase kinase-3β inhibition, and viral replication: a screening approach for new bipolar disorder drugs derived from the valproic acid core structure. *Mol. Pharmacol.* **67**, 1426-1433.

Engl, T., Natsheh, I., Muller, I., Beecken, W. D., Jonas, D., Blaheta, R. A. (2004) Valproic acid induces expression of neutrophil chemoattractants of the CXC chemokine family in endothelial cells. *Int. J. Clin. Pharmacol. Ther.* **42**, 568-574.

Gobbi, G., Janiri, L. (2006) Sodium- and magnesium-valproate in vivo modulate glutamatergic and GABAergic synapses in the medial prefrontal cortex. *Psychopharmacology (Berl.)* **185**, 255-262.

Gottlicher, M., Minucci, S., Zhu, P., Kramer, O. H., Schimpf, A., Giavara, S., Sleeman, J. P., Lo Coco, F., Nervi, C., Pelicci, P. G., Heinzel, T. (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J.* **20**, 6969-6978.

Grisolano, J. L., Wesselschmidt, R. L., Pelicci, P. G., Ley, T. J. (1997) Altered myeloid development and acute leukemia in transgenic mice expressing PML-RAR α under control of cathepsin G regulatory sequences. *Blood* **89**, 376-387.

Gudermann, T., Grosse, R., Schultz, G. (2000) Contribution of receptor/G protein signaling to cell growth and transformation. *Naunyn Schmiedebergs Arch. Pharmacol.* **361**, 345-362.

Guel, H., Wassmann, B., Romanski, A., Hoelzer, D., Ruthardt, M., Ottmann, O. G., Bug, G. (2003) Effect of the histone deacetylase inhibitor valproic acid in combination with all-trans retinoic acid on malignant hematopoiesis. *Blood* **102**.

Gurvich, N., Tsygankova, O. M., Meinkoth, J. L., Klein, P.S. (2004) Histone deacetylase is a target of valproic acid-mediated cellular differentiation. *Cancer Res.* **64**, 1079-1086.

Gurvich, N., Berman, M. G., Wittner, B. S., Gentleman, R. C., Klein, P. S., Green, J. B. (2005) Association of valproate-induced teratogenesis with histone deacetylase inhibition in vivo. *FASEB J.* **19**, 1166-1168.

Heibert, S. W., Lutterbach, B., Durst, K., Wang, L., Linggi, B., Wu, S., Wood, L., Amann, J., King, D., Hou, Y. (2001) Mechanisms of transcriptional repression by the t(8; 21)-, t(12; 21)-, and inv(16)-encoded fusion proteins. *Cancer Chemother. Pharmacol.* **48**, S31-34.

Hoffmann, K., Brosch, G., Loidl, P., Jung, M. (2000) First non-radioactive assay for in vitro screening of histone deacetylase inhibitors. *Pharmazie* **55**, 601-606.

Insinga, A., Monestiroli, S., Ronzoni, S., Carbone, R., Pearson, M., Pruneri, G., Viale, G., Appella, E., Pelicci, P., Minucci, S. (2004) Impairment of p53 acetylation, stability and function by an oncogenic transcription factor. *EMBO J.* **23**, 1144-1154.

Insinga, A., Pelicci, P. G., Inucci, S. (2005) Leukemia-associated fusion proteins. Multiple mechanisms of action to drive cell transformation. *Cell Cycle* **4**, 67-69.

Johannessen, C. U. (2000) Mechanisms of action of valproate: a commentatory. *Neurochem. Int.* **37**, 103-110.

Johannessen, C. U. Johannessen, S. I. (2003) Valproate: past, present, and future. *CNS Drug Rev.* **9**, 199-216.

Kaiser, M., Zavrski, I., Sterz, J., Jakob, C., Fleissner, C., Kloetzel, P. M., Sezer, O., Heider, U. (2006) The effects of the histone deacetylase inhibitor valproic acid on cell cycle, growth suppression and apoptosis in multiple myeloma. *Haematologica* **91**, 248-251.

Kaneko, F., Saito, H., Saito, Y., Wakabayashi, K., Nakamoto, N., Tada, S., Suzuki, H., Tsunematsu, S., Kumagai, N., Ishii, H. (2004) Down-regulation of matrix-invasive potential of human liver cancer cells by type I interferon and a histone deacetylase inhibitor sodium butyrate. *Int. J. Oncol.* **24**, 837-845.

Kawagoe, R., Kawagoe, H., Sano, K. (2002) Valproic acid induces apoptosis in human leukemia cells by stimulating both caspase-dependent and -independent apoptotic signaling pathways. *Leuk. Res.* **26**, 495-502.

Kawai, Y., Arinze, I. J. (2006) Valproic acid-induced gene expression through production of reactive oxygen species. *Cancer Res.* **66**, 6563-6569.

Koch, A., Waha, A., Tonn, J. C., Sorensen, N., Berthold, F., Wolter, M., Reifenberger, J., Hartmann, W., Friedl, W., Reifenberger, G., Wiestler, O. D., Pietsch, T. (2001) Somatic mutations of WNT/wingless signaling pathway components in primitive neuroectodermal tumors. *Int. J. Cancer* **93**, 445-449.

Koren, G., Kennedy, D. (1999) Safe use of valproic acid during pregnancy. *Can. Fam. Physician* **45**, 1451-1453.

Koren, G., Nava-Ocampo, A. A., Moretti, M. E., Sussman, R., Nulman, I. (2006) Major malformations with valproic acid. *Can. Fam. Physician* **52**, 441-442, 444, 447.

Kramer, O. H., Gottlicher, M., Heinzel, T. (2001) Histone deacetylase as a therapeutic target. *Trends Endocrinol. Metab.* **12**, 294-300.

Kramer, O. H., Zhu, P., Ostendorff, H. P., Golebiewski, M., Tiefenbach, J., Peters, M. A., Brill, B., Groner, B., Bach, I., Heinzel, T., Gottlicher, M. (2003) The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. *EMBO J.* **22**, 3411-3420.

Kuendgen, A., Schmid, M., Schlenk, R., Knipp, S., Hildebrandt, B., Steidl, C., Germing, U., Haas, R., Dohner, H., Gattermann, N. (2006) The histone deacetylase (HDAC) inhibitor valproic acid as monotherapy or in combination with all-trans retinoic acid in patients with acute myeloid leukemia. *Cancer* **106**, 112-119.

Lammer, E. J., Sever, L. E., Oakley, G. P., Jr. (1987) Teratogen update: valproic acid. *Teratology* **35**, 465-473.

Lampen, A., Carlberg, C., Nau, H. (2001) Peroxisome proliferator-activated receptor δ is a specific sensor for teratogenic valproic acid derivatives. *Eur. J. Pharmacol.* **431**, 25-33.

Lampen, A., Siehler, S., Ellerbeck, U., Gottlicher, M., Nau, H. (1999) New molecular bioassays for the estimation of the teratogenic potency of valproic acid derivatives in vitro: activation of the peroxisomal proliferator-activated receptor (PPARδ). *Toxicol. Appl. Pharmacol*. **160**, 238-249.

Lin, H. Y., Chen, C. S., Lin, S. P., Weng, J. R. (2006) Targeting histone deacetylase in cancer therapy. *Med. Res. Rev.* **26**, 397-413.

Manev, H., Uz, T., Sugaya, K., Qu, T. (2000) Putative role of neuronal 5-lipoxygenase in an aging brain. *FASEB J.* **14**, 1464-1469.

Manji, H. K., Chen, G., Hsiao, J. K., Risby, E. D., Masana, M. I., Potter, W. Z. (1996) Regulation of signal transduction pathways by mood-stabilizing agents: implications for the delayed onset of therapeutic efficacy. *J. Clin. Psychiatry* **57**, 34-47.

Martin, M. L., Regan, C. M. (1991) The anticonvulsant valproate teratogen restricts the glial cell cycle at a defined point in the mid-G1 phase. *Brain Res.* **554**, 223-228.

McGarry, L. C., Winnie, J. N., Ozanne, B. W. (2004) Invasion of v-Fos(FBR)-transformed cells is dependent upon histone deacetylase activity and suppression of histone deacetylase regulated genes. *Oncogene* **23**, 5284-5292.

Melnick, A., Licht, J. D. (2002) Histone deacetylases as therapeutic targets in hematologic malignancies. *Curr. Opin. Hematol.* **9**, 322-332.

Menegola, E., Broccia, M. L., Nau, H., Prati, M., Ricolfi, R., Giavini, E. (1996) Teratogenic effects of sodium valproate in mice and rats at midgestation and at term. *Teratog. Carcinog. Mutagen* **16**, 97-108.

Menegola, E., Broccia, M. L., Prati, M., Giavini, E. (1998) Stage-dependent skeletal malformations induced by valproic acid in rat. *Int. J. Dev. Biol*. **42**, 99-102.

Meunier, H., Carraz, G., Neunier, Y., Eymard, P., Aimard, M. (1963) Pharmacodynamic properties of dipropylacetic acid. 1st memory: anti-epileptic properties. *Therapie* **18**, 435-438. (in French)

Michaelis, M., Michaelis, U. R., Fleming, I., Suhan, T., Cinatl, J., Blaheta, R. A., Hoffmann, K., Kotchetkov, R., Busse, R., Nau, H., Cinatl, J. Jr. (2004a) Valproic acid inhibits angiogenesis in vitro and in vivo. *Mol. Pharmacol.* **65**, 520-527.

Michaelis, M., Suhan, T., Cinatl, J., Driever, P. H., Cinatl, J., Jr. (2004b) Valproic acid and interferon-α synergistically inhibit neuroblastoma cell growth in vitro and in vivo. *Int. J. Oncol.* **25**, 1795-1799.

Mielnicki, L. M., Asch, H. L., Asch, B. B. (2001) Genes, chromatin, and breast cancer: an epigenetic tale. *J. Mammary Gland Biol. Neoplasia* **6**, 169-182.

Miller, J. R. (2002) The Wnts. *Genome Biol.* **3**1-15.

Miller, J. R., Hocking, A. M., Brown, J. D., Moon, R. T. (1999) Mechanism and function of signal transduction by the Wnt/β-catenin and Wnt/Ca2+ pathways. *Oncogene* **18**, 7860-7872.

Miller, L. D., Park, K. S., Guo, Q. M., Alkharouf, N. W., Malek, R. L., Lee, N. H., Liu, E. T., Cheng, S. Y. (2001) Silencing of Wnt signaling and activation of multiple metabolic pathways in response to thyroid hormone-stimulated cell proliferation. *Mol. Cell. Biol*. **21**, 6626-6639.

Minucci, S., Pelicci, P. G. (1999) Retinoid receptors in health and disease: co-regulators and the chromatin connection. *Semin. Cell. Dev. Biol.* **10**, 215-225.

Minucci, S., Pelicci, P. G. (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat. Rev. Cancer* **6**, 38-51.

Minucci, S., Maccarana, M., Cioce, M., De Luca, P., Gelmetti, V., Segalla, S., Di Croce, L., Giavara, S., Matteucci, C., Gobbi, A., Bianchini, A., Colombo, E., Schiavoni, I., Badaracco, G., Hu, X., Lazar, M. A., Landsberger, N., Nervi, C., Pelicci, P. G. (2000) Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. *Mol. Cell* **5**, 811-820.

Nau, H., Hauck, R. S., Ehlers, K. (1991) Valproic acid-induced neural tube defects in mouse and human: aspects of chirality, alternative drug development, pharmacokinetics and possible mechanisms. *Pharmacol. Toxicol.* **69**, 310-321.

Nebbioso, A., Clarke, N., Voltz, E., Germain, E., Ambrosino, C., Bontempo, P., Alvarez, R., Schiavone, E. M., Ferrara, F., Bresciani, F., Weisz, A., de Lera, A. R., Gronemeyer, H., Altucci, L. (2005) Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells. *Nat. Med.* **11**, 77-84.

Oberndorfer, S., Piribauer, M., Marosi, C., Lahrmann, H., Hitzenberger, P., Grisold, W. (2005) P450 enzyme inducing and non-enzyme inducing antiepileptics in glioblastoma patients treated with standard chemotherapy. *J. Neurooncol.* **72**, 255-260.

Ohira, T., Gemmill, R. M., Ferguson, K., Kusy, S., Roche, J., Brambilla, E., Zeng, C., Baron, A., Bemis, L., Erickson, P., Wilder, E., Rustgi, A., Kitajewski, J., Gabrielson, E., Bremnes, R., Franklin, W., Drabkin, H. A. (2003) WNT7a induces E-cadherin in lung cancer cells. *Proc. Natl. Acad. Sci. USA* **100**, 10429-10434.

Okada, A., Fujiwara, M. (2006) Molecular approaches to developmental malformations using analogous forms of valproic acid. *Congenit. Anom. (Kyoto)*, **46**, 68-75.

Okada, A., Aoki, Y., Kushima, K., Kurihara, H., Bialer, M., Fujiwara, M. (2004) Polycomb homologs are involved in teratogenicity of valproic acid in mice. *Birth Defects Res. A. Clin. Mol. Teratol.* **70**, 870-879.

O'Loinsigh, E. D., Gherardini, L. M., Gallagher, H. C., Foley, A. G., Murphy, K. J., Regan, C. M. (2004) Differential enantioselective effects of pentyl-4-yn-valproate on spatial learning in the rat, and neurite outgrowth and cyclin D3 expression in vitro. *J. Neurochem*. **88**, 370-379.

Owens, M. J., Nemeroff, C. B. (2003) Pharmacology of valproate. *Psychopharmacol. Bull.* **37**, 17-24.

Patel, L., Pass, I., Coxon, P., Downes, C. P., Smith, S. A., Macphee, C. (2001) Tumor suppressor and anti-inflammatory actions of PPARγ agonists are mediated via upregulation of PTEN. *Curr. Biol.* **11**, 764-768.

Phiel, C. J., Zhang, F., Huang, E. Y., Guenther, M. G., Lazar, M. A., Klein, P. S. (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J. Biol. Chem.* **276**, 36734-36741.

Phillips, A., Bullock, T., Plant, N. (2003) Sodium valproate induces apoptosis in the rat hepatoma cell line, FaO. *Toxicology* **192**, 219-227.

Pilatrino, C., Cilloni, D., Messa, E., Morotti, A., Giugliano, E., Pautasso, M., Familiari, U., Cappia, S., Pelicci, P. G., Lo Coco, F., Saglio, G., Guerrasio, A. (2005) Increase in platelet count in older, poor-risk patients with acute myeloid leukemia or myelodysplastic syndrome treated with valproic acid and all-trans retinoic acid. *Cancer* **104**, 101-109.

Qu, T., Uz, T., Manev, H. (2000) Inflammatory 5-LOX mRNA and protein are increased in brain of aging rats. *Neurobiol. Aging* **21**, 647-652.

Raffoux, E., Chaibi, P., Dombret, H., Degos, L. (2005) Valproic acid and all-trans retinoic acid for the treatment of elderly patients with acute myeloid leukemia. *Haematologica* **90**, 986-988.

Regan, C. M. (1985) Therapeutic levels of sodium valproate inhibit mitotic indices in cells of neural origin. *Brain Res.* **347**, 394-398.

Rezacova, M., Vavrova, J., Vokurkova, D., Zaskodova, D. (2006) Effect of valproic acid and antiapoptotic cytokines on differentiation and apoptosis induction of human leukemia cells. *Gen. Physiol. Biophys.* **25**, 65-79.

Rocchi, P., Tonelli, R., Camerin, C., Purgato, S., Fronza, R., Bianucci, F., Guerra, F., Pession, A., Ferreri, A. M. (2005) p21Waf1/Cip1 is a common target induced by short-chain fatty acid HDAC inhibitors (valproic acid, tributyrin and sodium butyrate) in neuroblastoma cells. *Oncol. Rep.* **13**, 1139-1144.

Searles, C. D., Slesinger, P. A., Singer, H. S. (1988) Effects of anticonvulsants on cholinergic and GABAergic properties in the neuronal cell clone NG108-15. *Neurochem. Res.* **13**, 1007-1013.

Shen, W. T., Wong, T. S., Chung, W. Y., Wong, M. G., Kebebew, E., Duh, Q. Y., Clark, O. H. (2005) Valproic acid inhibits growth, induces apoptosis, and modulates apoptosis-regulatory and differentiation gene expression in human thyroid cancer cells. *Surgery* **138**, 979-984.

Skladchikova, G., Berezin, V., Bock, E. (1998) Valproic acid, but not its non-teratogenic analogue 2-isopropylpentanoic acid, affects proliferation, viability and neuronal differentiation of the human teratocarcinoma cell line NTera-2. *Neurotoxicology* **19**, 357-370.

Slesinger, P. A., Singer, H. S. (1987) Effects of anticonvulsants on cell growth and enzymatic and receptor binding activity in a neuroblastoma x glioma hybrid cell culture. *Epilepsia* **28**, 214-221.

Takai, N., Desmond, J. C., Kumagai, T., Gui, D., Said, J. W., Whittaker, S., Miyakawa, I., Koeffler, H. P. (2004) Histone deacetylase inhibitors have a profound antigrowth activity in endometrial cancer cells. *Clin. Cancer Res.* **10**, 1141-1149.

Tang, R., Faussat, A. M., Majdak, P., Perrot, J. Y., Chaoui, D., Legrand, O., Marie, J. P. (2004a) Valproic acid inhibits proliferation and induces apoptosis in acute myeloid leukemia cells expressing P-gp and MRP1. *Leukemia* **18**, 1246-1251.

Tang, Y., Glauser, T. A., Gilbert, D. L., Hershey, A. D., Privitera, M. D., Ficker, D. M., Szaflarski, J. P., Sharp, F. R. (2004b) Valproic acid blood genomic expression patterns in children with epilepsy – a pilot study. *Acta Neurol. Scand.* **109**, 159-168.

Taylor, C. T., Furuta, G. T., Synnestvedt, K., Colgan, S. P. (2000) Phosphorylation-dependent targeting of cAMP response element binding protein to the ubiquitin/proteasome pathway in hypoxia. *Proc. Natl. Acad. Sci. USA*, **97**, 12091-12096.

Toker, A. (2000) Protein kinases as mediators of phosphoinositide 3-kinase signaling. *Mol Pharmacol.* **57**, 652-658.

Vincan, E., Leet, C. S., Reyes, N. I., Dilley, R. J., Thomas, R. J., Phillips, W. A. (2000) Sodium butyrate-induced differentiation of human LIM2537 colon cancer cells decreases GSK-3β activity and increases levels of both membrane-bound and Apc/axin/GSK-3β complex-associated pools of β-catenin. *Oncol. Res.* **12**, 193-201.

Warrell, R. P., Jr., He, L. Z., Richon, V., Calleja, E., Pandolfi, P. P. (1998) Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. *J. Natl. Cancer Inst.* **90**, 1621-1625.

Werling, U., Siehler, S., Litfin, M., Nau, H., Gottlicher, M. (2001) Induction of differentiation in F9 cells and activation of peroxisome proliferator-activated receptor δ by valproic acid and its teratogenic derivatives. *Mol. Pharmacol.* **59**, 1269-1276.

Williams, R. S., Cheng, L., Mudge, A. W., Harwood, A. J. (2002) A common mechanism of action for three mood-stabilizing drugs. *Nature* **417**, 292-295.

Wong, H., Kumar, S., Rurak, D. W., Kwan, E., Abbott, F. S., Riggs, K. W. (2000) Ontogeny of valproic acid disposition and metabolism: a developmental study in postnatal lambs and adult sheep. *Drug Metab. Dispos.* **28**, 912-919.

Xia, Q., Sung, J., Chowdhury, W., Chen, C. L., Hoti, N., Shabbeer, S., Carducci, M., Rodriguez, R. (2006) Chronic administration of valproic acid inhibits prostate cancer cell growth in vitro and in vivo. *Cancer Res.* **66**, 7237-7244.

Yamamoto-Yamaguchi, Y., Okabe-Kado, J., Kasukabe, T., Honma, Y. (2003) Induction of apoptosis by combined treatment with differentiation-inducing agents and interferon-α in human lung cancer cells. *Anticancer Res.* **23**, 2537-2547.

Yoo, C. B., Jones, P. A. (2006) Epigenetic therapy of cancer: past, present and future. *Nat. Rev. Drug Discov.* **5**, 37-50.

Yuan, P. X., Huang, L. D., Jiang, Y. M., Gutkind, J. S., Manji, H. K., Chen, G. (2001) The mood stabilizer valproic acid activates mitogen-activated protein kinases and promotes neurite growth. *J. Biol. Chem.* **276**, 31674-31683.

Zgouras, D., Becker, U., Loitsch, S., Stein, J. (2004) Modulation of angiogenesis-related protein synthesis by valproic acid. *Biochem. Biophys. Res. Commun.* **316**, 693-697.