J. Cell. Mol. Med. Vol 13, No 8B 2009 pp. 2376-2385

The histone deacetylase inhibitor valproic acid alters growth properties of renal cell carcinoma *in vitro* and *in vivo*

Jon Jones, Eva Juengel, Ausra Mickuckyte, Lukasz Hudak, Steffen Wedel, Dietger Jonas, Roman A. Blaheta*

Department of Urology and Pediatric Urology, Goethe-University, Frankfurt am Main, Germany

Received: March 11, 2008; Accepted: July 21, 2008

Abstract

Histone deacetylase (HDAC) inhibitors represent a promising class of antineoplastic agents which affect tumour growth, differentiation and invasion. The effects of the HDAC inhibitor valproic acid (VPA) were tested *in vitro* and *in vivo* on pre-clinical renal cell carcinoma (RCC) models. Caki-1, KTC-26 or A498 cells were treated with various concentrations of VPA during *in vitro* cell proliferation 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays and to evaluate cell cycle manipulation. *In vivo* tumour growth was conducted in subcutaneous xenograft mouse models. The anti-tumoural potential of VPA combined with low-dosed interferon-α (IFN-α) was also investigated. VPA significantly and dose-dependently up-regulated histones H3 and H4 acetylation and caused growth arrest in RCC cells. VPA altered cell cycle regulating proteins, in particular CDK2, cyclin B, cyclin D3, p21 and Rb. *In vivo*, VPA significantly inhibited the growth of Caki-1 in subcutaneous xenografts, accompanied by a strong accumulation of p21 and bax in tissue specimens of VPA-treated animals. VPA–IFN-α combination markedly enhanced the effects of VPA monotherapy on RCC proliferation *in vitro*, but did not further enhance the anti-tumoural potential of VPA *in vivo*. VPA was found to have profound effects on RCC cell growth, lending support to the initiation of clinical testing of VPA for treating advanced RCC.

Keywords: HDAC • valproic acid • renal cell carcinoma • proliferation • tumour growth

Introduction

Renal cell carcinoma (RCC) accounts for 2–3% of adult cancers with over 30,000 new cases per year in the United States and an associated 13,000 deaths [1]. Age-adjusted incidence rates of 16.2/100,000 in men and 8.0/100,000 in women have been recorded during 1998–2002. In Europe, the highest incidence of kidney cancer was observed in the Czech Republic, with rates of approximately 22/100,000 in men and 11/100,000 in women. High rates were also observed in the other eastern Europe countries and in Germany, in contrast to most of the Asian and African populations and some South American populations where rates up to 10–15 times lower are reported [2]. Thirty per cent of RCC patients have metastatic disease at the time of diagnosis. An additional 20–30% with clinically localized disease at the time of nephrectomy will subsequently develop metastatic disease. [1].

Metastatic RCC carries a dismal prognosis. Almost half of the patients with RCC die within 5 years of diagnosis and 5-year survival rate for those with metastatic disease is less than 10% [3]. RCC is generally unresponsive to cytotoxic, hormonal and radiation adjuvant therapies. Immunotherapies such as interleukin-2 and interferon-α (IFN-α) have been shown to be effective, albeit in a minority of patients and often with severe drug-associated toxicity. Identification of tumour-specific target proteins and development of potent anticancer compounds is, therefore, a high priority in the treatment of advanced RCC.

Histone deacetylases (HDACs) represent one of the most important intracellular molecules, as they modulate a wide variety of cellular functions. Abnormal histone acetylation status can result in undesirable phenotypic changes, including developmental disorders and cancer. Hence, HDAC inhibitors may be

*Correspondence to: Prof. Dr. phil. nat. Roman BLAHETA, Goethe-University-Hospital, Department of Urology and Pediatric Urology, Science Center, Bldg. 25, R204, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany.
Tel.: +49-69-6301-7109
Fax: +49-69-6301-7108
E-mail: Blaheta@em.uni-frankfurt.de

doi:10.1111/j.1582-4934.2008.00436.x

useful in cancer prevention, due to their ability to 'reactivate' the expression of epigenetically silenced genes, including those involved in differentiation, invasion and metastasis. Among the growing list of HDAC-inhibitors, the branched-chain fatty acid valproic acid (VPA) has been shown to possess distinct HDAC inhibitory properties and to affect the growth and survival of tumour cells *in vitro* and *in vivo* [4, 5]. VPA is an established drug in the long-term therapy of epilepsy. It can be applied orally, negative side effects are rare and it demonstrates expedient pharmacokinetic properties.

VPA has been investigated in pre-clinical models of skin, breast, colon, liver, prostate, cervix and small cell lung cancer. Currently, the drug is in phase I trials [6, 7] for treatment of solid tumours.

To assess whether VPA might be of benefit in treating RCC patients, the potential of VPA to block growth properties of RCC cell lines *in vitro* and *in vivo* were investigated and the underlying mode of action explored. Also, VPA was combined with low-dosed IFN-α and the effects of the combination regimen compared to single drug application. The experimental strategy was based on earlier reports demonstrating that IFN-α may enhance VPA's potency both *in vivo* and *in vitro* [8–10].

VPA could be shown to potently block RCC tumour cell proliferation *in vitro* and prevent RCC tumour growth *in vivo*. VPA's activity is associated with reduction of HDAC and elevated acetylation of histones H3 and H4. Entry of RCC tumour cells into the S-phase of the cell cycle is delayed and the expression pattern of cell cycle regulating proteins modified by VPA. It is of particular interest that VPA–IFN-α combination induced stronger effects on RCC cell growth *in vitro* than VPA alone. Still, VPA–IFN-α combination and VPA monotherapy induced a similar response *in vivo*. Based on these results, VPA provides potent anti-tumour activity at multiple cellular levels and, therefore, may reveal significant therapeutic benefit in treating advanced RCC.

Materials and methods

Antibodies

Monoclonal antibodies were used, directed against proteins indicated subsequently: Cell cycle: Cdk1/Cdc2 (IgG1, clone 1, dilution 1:2500), Cdk2 (IgG2a, clone 55, dilution 1:2500), Cdk4 (IgG1, clone 97, dilution 1:250), Cyclin A (IgG1, clone 25, dilution 1:250), Cyclin B (IgG1, clone 18, dilution 1:1000), Cyclin D3 (IgG2b, clone 1, dilution 1:1000), Rb (IgG2a, clone 2, dilution 1:250), Rb2 (IgG2a, clone 10, dilution 1:1000), p19 (IgG1, clone 52, dilution 1:5000), p21 (IgG1, clone 2G12, dilution 1:250), p27 (IgG1, clone 57, dilution 1:500) were purchased from BD Biosciences (Heidelberg, Germany).

Apoptosis: Bax (IgG, polyclonal, dilution 1:200) and bcl-2 (IgG, polyclonal, dilution 1:200) were from Santa Cruz Biotechnology, Heidelberg, Germany.

Histones: Anti-histone H3 (IgG, clone Y173, dilution 1:5000), anti-acetylated H3 (IgG, clone Y28, dilution 1:500), anti-histone H4 (polyclonal IgG, dilution 1:250), anti-acetylated H4 (Lys8, polyclonal IgG, dilution 1:500) and anti-HDAC3 (polyclonal IgG, dilution 1:2000) were all from Biomol GmbH, Hamburg, Germany.

Anti-β-actin monoclonal antibody was obtained from Sigma (Taufenkirchen, Germany).

Cell cultures

Kidney carcinoma Caki-1 and KTC-26 cells were purchased from LGC Promochem (Wesel, Germany). A498 cells were derived from CLS (Heidelberg, Germany). Tumour cells were grown and sub-cultured in RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 10% foetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified, 5% CO_2 incubator.

Drug treatment

Tumour cells were treated with VPA (gift from G. L. Pharma GmbH, Lannach, Austria) at a final concentration of 0.25, 0.5 or 1 mM for 3 or 5 days (if not otherwise indicated). Controls remained untreated. In a further experiment, tumour cells were incubated simultaneously with VPA and IFN-α (Roferon A; Roche Pharma AG, Grenzach-Wyhlen, Germany; 200 U/ml), and compared to cells treated with VPA or treated with IFN-α, or which remained untreated.

Tumour cell growth

Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (Roche Diagnostics, Penzberg, Germany). Caki-1, KTC-26 or A498 cells (100 µl, 1×10^4 cells/ml) were seeded onto 96-well tissue culture plates and incubated as described above. After 24 hrs, MTT (0.5 mg/ml) was added for an additional 4 hrs. Thereafter, cells were lysed in a buffer containing 10% SDS in 0.01 M HCl. The plates were allowed to stand overnight at 37°C, 5% CO_2. Absorbance at 570 nm was determined for each well using a microplate ELISA reader. Each experiment was done in triplicate. After subtracting background absorbance, results were expressed as mean cell number.

Cell cycle analysis

Caki-1 cells were grown to 70% confluency and then treated with VPA or with IFN-α or with both VPA + IFN-α (controls remained untreated). Twenty-four hours before starting cell cycle analysis, Caki-1 were synchronized at the G1–S boundary with aphidicolin (1 µg/ml). Caki-1 were resuspended in fresh (aphidicolin free) medium for 2 hrs, harvested, stained with propidium iodide using a Cycle TEST PLUS DNA Reagent Kit (BD Biosciences, Heidelberg, Germany), and then subjected to flow cytometry with a FACScan flow cytometer (BD Biosciences). Ten thousand events were collected from each sample. Data acquisition was carried out using Cell-Quest software and cell cycle distribution calculated using the ModFit software (BD Biosciences). The number of gated cells in G1, G2/M or S-phase was presented as percentage.

Western blotting

HDAC3, acetylated histones H3 and H4 were evaluated in Caki-1 tumour cells by Western blot analysis. Cell cycle regulating proteins were also explored in treated *versus* non-treated cell populations. Tumour cell lysates were applied to a 7% polyacrylamide gel and electrophoresed for 90 min. at 100 V. The protein was then transferred to nitrocellulose membranes. After blocking with non-fat dry milk for 1 hr, the membranes were incubated overnight with the respective antibodies and dilutions listed above. HRP-conjugated goat-anti-mouse IgG (Upstate Biotechnology, Lake Placid, NY, USA; dilution 1:5000) served as the secondary antibody. The membranes were briefly incubated with ECL detection reagent (ECL™, Amersham/GE Healthcare, München, Germany) to visualize the proteins and exposed to an x-ray film (Hyperfilm™ EC™, Amersham/GE Healthcare). β-Actin (1:1000) served as the internal control.

HDAC activity

For determining the inhibitory activity of VPA and/or IFN-α for HDACs, a cell-free assay (Color de Lys, Biomol) detecting HDAC1 and 2 was used according to the manufacturer's protocol. A nuclear extract of HeLa cells containing HDAC1 and 2 was incubated for 10 min. at 37°C with trichostatin A (TSA, 1μM final concentration) as a positive control of inhibition. The HDAC reaction was initiated by adding a substrate of acetylated peptides, incubated for 15–30 min., and followed by adding a colour developer. The absorbance of triplicate analyses was assayed at 405 nm with a Bio-Tec microtitre-plate reader. For determining the HDAC activity of Caki-1 cells, the cell extracts (50 μg) that had been exposed to VPA, IFN-α, to both VPA + IFN-α, or to control medium, were added to the substrate to determine the HDAC activity as described.

Tumour growth *in vivo*

For *in vivo* testing, 10^7 Caki-1 cells were injected subcutaneously to male NMRI:*nu*/*nu* mice (EPO GmbH, Berlin, Germany). Treatment was initiated when tumours had grown to a palpable size (5–6 mm diameter). VPA was dissolved in 10% polyethylene glycol (PEG) 400/saline. It was injected i.p. in doses of 200 mg/kg/day once daily ($n = 8$). A second group received IFN-α 5×10^5 IU/kg/day once daily ($n = 8$) and a third group both VPA and IFN-α ($n = 8$). The control group of mice was treated with the solvent ($n = 10$). Tumour size was measured with callipers. Tumour volumes, relative tumour volumes (relative to the first treatment day) and treated/control (T/C) values were calculated. Body weight and mortality were recorded continuously to estimate tolerability. Additionally, frozen tissue specimens were generated from nude mice xenografts to evaluate cell cycle protein expression, bax, bcl-2, H3 and HDAC3 by Western blot analysis.

Statistics

All *in vitro* experiments were performed three to six times, *in vivo* experiments were done eight to ten times. Statistical significance was investigated by the Wilcoxon–Mann-Whitney U-test. Differences were considered statistically significant at a *P*-value less than 0.05.

Results

Inhibition of RCC cell proliferation by VPA

Cell proliferation of Caki-1, KTC-26 and A498 cells was quantified 24 and 48 hrs after plating. To clearly interpret and compare cellular growth characteristics, 24-hrs counts were all set at 100%. Growth pattern was similar in the untreated RCC cell lines, cell counts of which all doubled after 24 hrs. Simultaneous addition of VPA into the multiwell plates did not influence cell growth (data not shown). However, a pre-incubation for 3 days with 0.25, 0.5 or 1 mM VPA dose dependently and significantly blocked RCC cell proliferation. The anti-proliferative effect of VPA was distinctly more pronounced when tumour cells were pre-treated with the compound for 5 days, whereby maximum growth reduction was achieved in the presence of 1 mM VPA (Fig. 1).

Further studies concentrated on VPA dosages which induced minimum (0.25 mM) or maximum (1 mM) effects. Because all RCC cell lines showed identical proliferation characteristics, ongoing experiments were restricted to Caki-1 as the representative cell line.

IFN-α enhances the anti-proliferative activity of VPA

A total of 200 U/ml IFN-α given alone for 3 days did not reduce the growth capacity of Caki-1 cells, and 0.25 mM VPA (3-day application) only slightly diminished cell proliferation. However, when both compounds were combined, a significantly stronger reduction of tumour cell growth was seen. In a similar fashion, growth-blocking effects of 1 mM VPA became significantly enhanced in the presence of IFN-α (Fig. 2, left). The same phenomenon was observed after a 5-day pre-incubation period. VPA dose dependently (1 mM > 0.25 mM) reduced Caki-1 proliferation. The 5-day effects were more potent than the 3-day effects and became even stronger when VPA was applied in combination with IFN-α (Fig. 2, right).

VPA and VPA–IFN-α combination increase histone H3 and H4 acetylation

Caki-1 cells were treated with VPA or VPA–IFN-α combination for 12 and 24 hrs, and histone acetylation was assessed by Western blotting. Caki-1 showed distinct reduction of HDAC3 and increase of acetylated H3 and H4 under VPA treatment. Interestingly, VPA's effects were more prominent in presence of IFN-α, although IFN-α alone did not modify histones. Determination of HDAC1 and 2 was also done revealing strong inhibition under VPA, effect of which became much more prominent in the additional presence of IFN-α (Fig. 3).

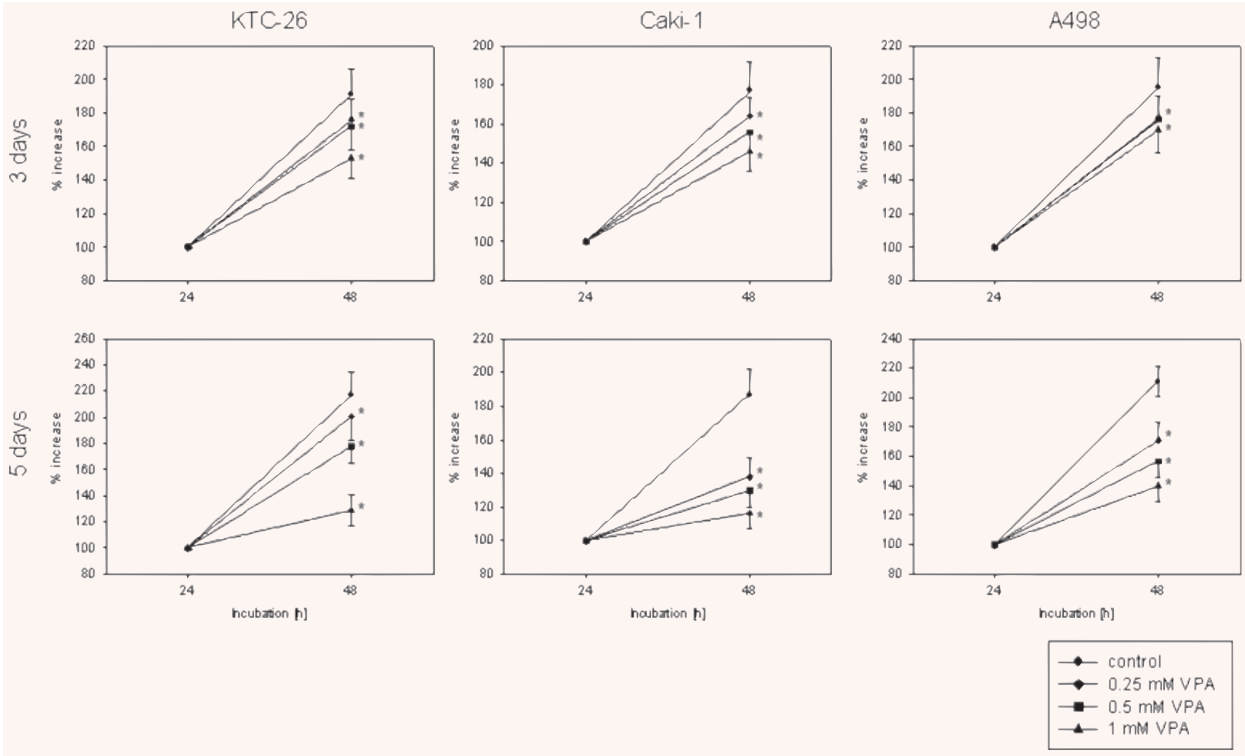

Fig. 1 Effects of VPA on kidney cancer proliferation *in vitro*. KTC-26, Caki-1 or A498 cells were treated with various concentrations of VPA for 3 or 5 days, or remained untreated (control). Cell proliferation was then assessed using the MTT dye reduction assay. Cell numbers at day 2 (48 hrs) were compared to the number of day 1 (24 hrs, as 100%). One representative of six experiments is shown. * Indicates significant difference to controls.

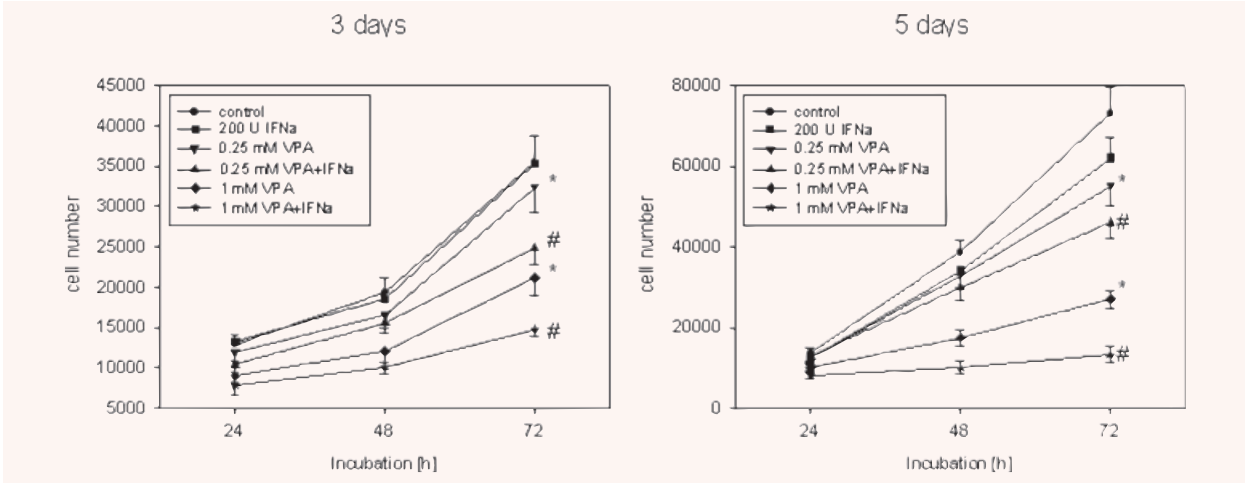

Fig. 2 Effects of VPA *versus* VPA–IFN-α combination on kidney cancer proliferation *in vitro*. Caki-1 cells were treated with 0.25 mM or 1 mM VPA alone or combined with IFN-α. Incubation lasted for 3 or 5 days. Controls remained untreated. Cells were then counted after a further 24, 48 and 72 hrs using the MTT dye reduction assay. One representative of six experiments is shown. * Indicates significant difference to controls. # Indicates significant differences between VPA monotherapy and VPA–IFN-α combination therapy.

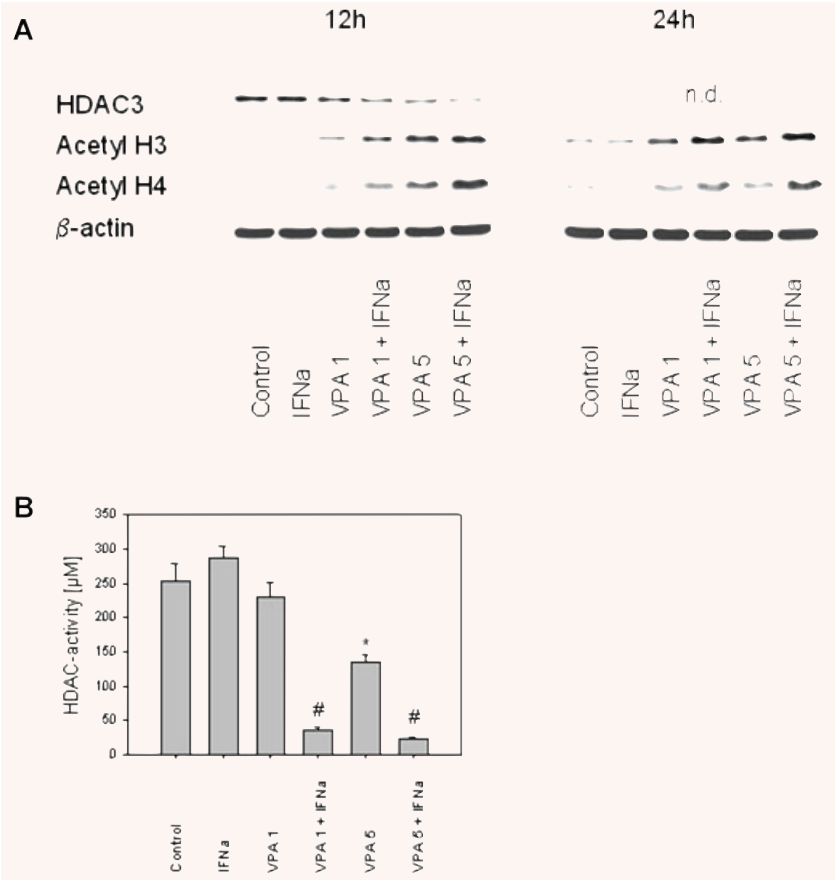

Fig. 3 Western blot analysis of HDAC3 expression and of H3 and H4 acetylation in Caki-1, treated with 1 mM or 5 mM VPA or with VPA–IFN-α combination (**A**). Caki-1 were incubated with VPA/VPA–IFN-α for 12 or 24 hrs. Cell lysates were then analysed by specific antibodies as listed in 'Materials and methods'. β-Actin served as the internal control. One representative experiment of three is shown. n.d.= not done. For determining the inhibitory activity of VPA or VPA–IFN-α combination on HDAC1 and 2, the Color de Lys cell-free assay was used (**B**). HDAC activity is given in µM deacetylated substrate/mg protein. *Indicates significant difference to controls. #Indicates significant differences between VPA monotherapy and VPA–IFN-α combination therapy. One of three experiments is shown.

VPA and VPA–IFN-α combination influences cell cycle regulation

VPA and the VPA–IFN-α combination strongly blocked tumour cell proliferation. Caki-1 cell lines were cultured with low- or high-dosed VPA, with and without IFN-α, and assayed for cell cycle analysis. VPA significantly lowered the amount of tumour cells entering the S-phase (1 mM VPA > 0.25 mM VPA). When combined with IFN-α, 0.25 mM VPA induced much stronger effects than 0.25 mM VPA alone, independently of whether drug treatment lasted for 3 or 5 days. This was also true when the experimental protocol was based on the 1 mM VPA dosage given for 5 days, but not when Caki-1 were pre-treated for 3 days. IFN-α alone did not modify the cell cycle (Fig. 4).

Alterations of cell cycle protein expression depended on the dosing schedule. With respect to the 3-day pre-incubation, 0.25 mM VPA enhanced p21 and p27 and slightly reduced CDK2 and CDK4. One millimolar VPA strongly reduced CDK2 and cyclin B and strongly elevated p21, Rb and cyclin D3.Effects of 1 mM VPA on CDK4 and p27 were similar to the effects seen with 0.25 mM VPA. Combination therapy was more effective than VPA monotherapy concerning the expression of CDK2, CDK4, cyclin D3 (each 0.25 or 1 mM VPA–IFN-α *versus* 0.25 or 1 mM VPA), Rb and p21 (each 0.25 mM VPA–IFN-α *versus* 0.25 mM VPA). Interestingly, IFN-α but not VPA induced up-regulation of CDK1 (Fig. 5). Similar effects were detected after a 5-day pre-incubation period, except for cyclin B which was up-regulated in the presence of 1 mM VPA or 1 mM VPA–IFN-α, and p21, which was reduced by VPA and VPA–IFN-α (VPA–IFN-α > VPA).

VPA treatment inhibits progression of tumour xenografts

Tumour xenografts were established in athymic *nu*/*nu* mice using Caki-1 cells to evaluate the effects of VPA or VPA–IFN-α combination on RCC cell growth *in vivo*. Compared to the untreated animals, application of VPA significantly diminished the tumour volume, with reduction of 70% at day 46, compared to the control (Fig. 6). No body weight loss or diarrhoea was observed and all animals (treated as well as non-treated) survived. Western blot analysis revealed strong accumulation of p21 and bax in tissue specimens of VPA-treated animals (Fig. 6, right). IFN-α treatment led to a moderate reduction of the tumour volume accompanied by a reduction of CDK1 and bcl-2 and elevation of cyclin D3 and Rb.

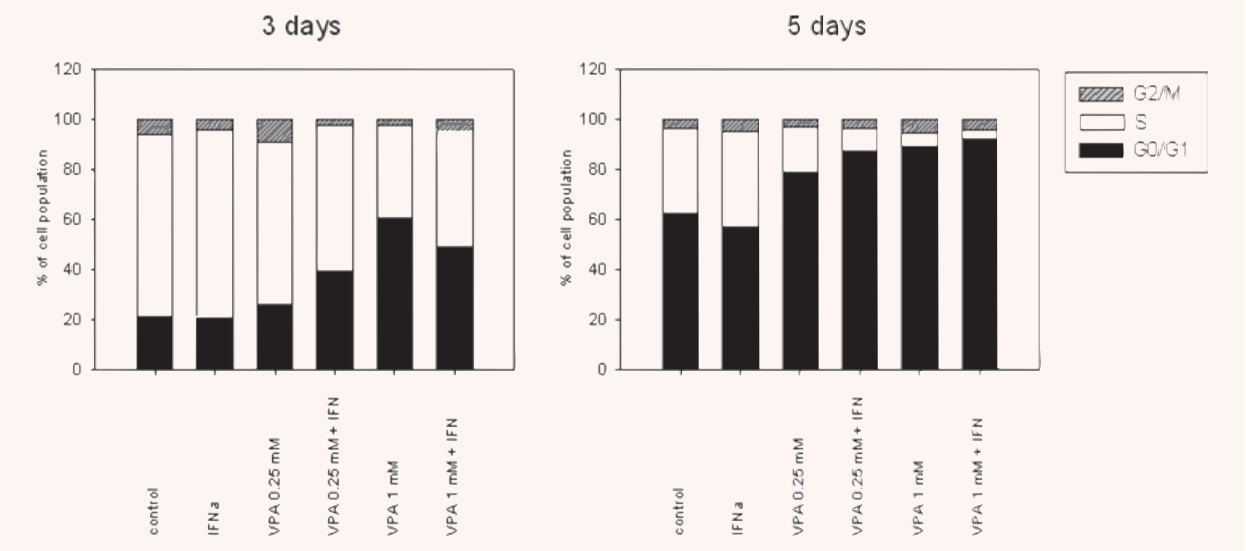

Fig. 4 Cell cycle analysis of synchronized Caki-1 cells, treated with IFN-α, with low- (0.25 mM) or high-dosed (1 mM) VPA or with VPA–IFN-α combination. The phrase '3 days' is related to a 3-day VPA incubation, the phrase '5 days' is related to a 5-day VPA incubation. Controls remained untreated. The cell population at each specific checkpoint is expressed as percentage of the total cells analysed. One representative experiment out of three is shown.

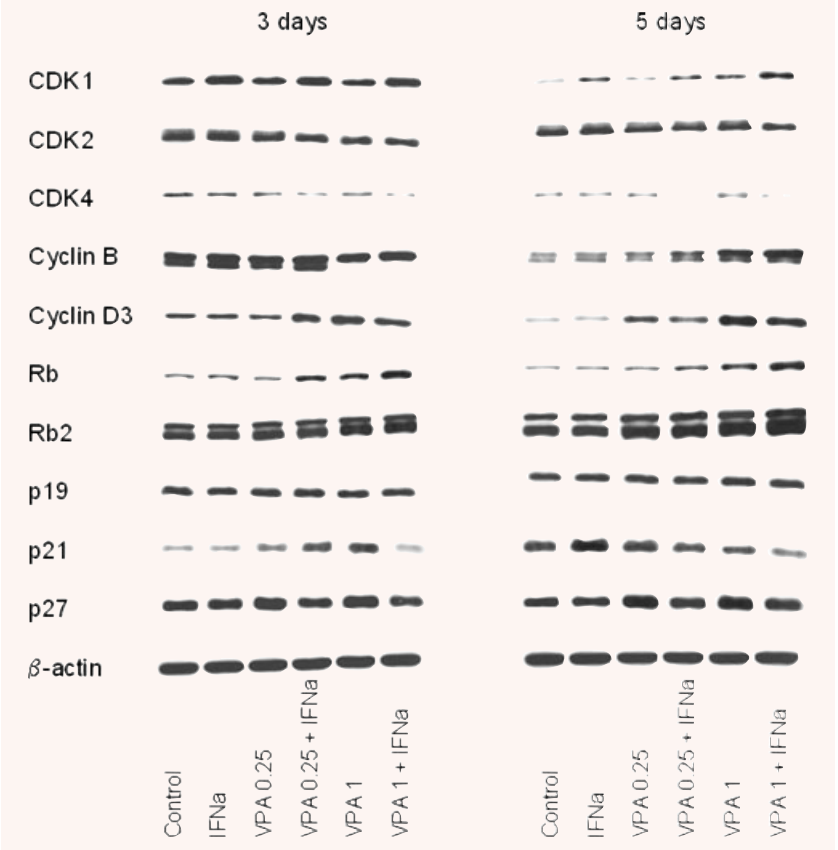

Fig. 5 Western blot analyses of cell cycle proteins, listed in 'Materials and methods'. Synchronized Caki-1 cells were treated with IFN-α, with low- (0.25 mM) or high-dosed (1 mM) VPA, with VPA–IFN-α combination or remained untreated (control). The phrase '3 days' is related to a 3-day VPA incubation, the phrase '5 days' is related to a 5-day VPA incubation. Cell lysates were subjected to SDS-PAGE and blotted on the membrane incubated with the respective monoclonal antibodies. β-Actin served as the internal control. The figure shows one representative from three separate experiments.

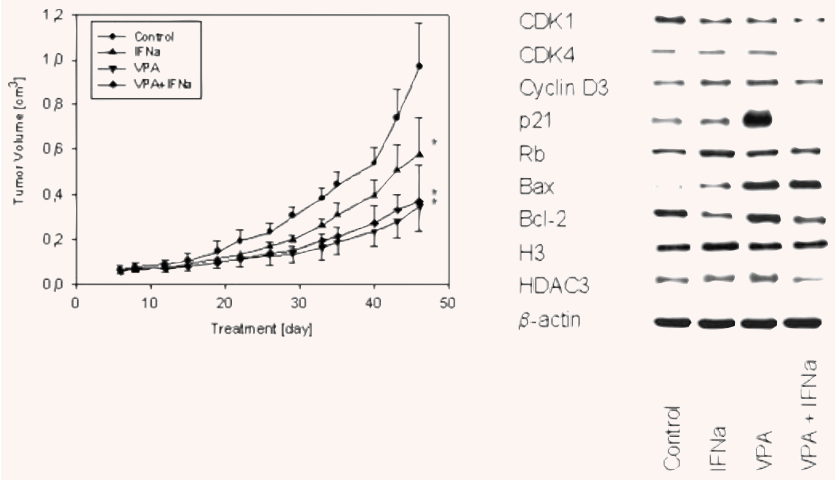

Fig. 6 Effect of VPA and VPA–IFN-α combination on RCC xenografts. Caki-1 xenografts were established in male athymic mice. Animals in the treatment arm received 200 mg/kg VPA or IFN-α 5×10^5 IU/kg once daily ($n = 8$), and a third group received both VPA and IFN-α ($n = 8$). The control group of mice was treated with the solvent ($n = 10$). * Indicates significant difference to the control animals. Western blot analysis of cell cycle regulating proteins, bax, bcl-2, H3 and HDAC3 was carried out on the tissue specimens using specific antibodies as listed in 'Materials and methods' (fig 6, right). β-Actin served as the internal control. Western blot data from one of three representative experiments are shown.

Surprisingly, VPA–IFN-α combination treatment was not superior to VPA monotherapy. Western blot data indicated cooperative processes with respect to CDK1 and CDK4 down-modulation and HDAC3 was nearly lost by the combined use of VPA and IFN-α. On the other hand, combination regimen reverted the strong p21 and bcl-2 accumulation caused by VPA alone.

Discussion

HDAC are critically important in regulating gene and protein expression and offer a target for therapeutic intervention. The results presented here provide evidence that the HDAC inhibitor VPA potently blocks RCC cell growth both *in vitro* and *in vivo*. No experiments have been carried out so far dealing with this issue. However, VPA has recently been shown to inhibit hypoxia-inducible factor 1 α in RCC cells which plays a critical role in transcriptional gene activation involved in tumour angiogenesis [11].

VPA administered for 5 days was more potent than a 3-day application. Prolonged VPA exposure has been shown to be necessary to modify neuroectodermal tumour cells [12, 13], and Xia *et al.* has suggested that chronic administration of VPA is required to achieve therapeutic benefits with prostate carcinoma [14]. In the RCC xenograft model significant tumour reduction was not seen until 10 days after starting chronic VPA application. It seems therefore, that long-term application of VPA is necessary to delay tumour cell growth.

VPA decreased the proportion of cells in the S-phase and increased the proportion of cells in the G0-/G1-phase of the cell cycle. Simultaneously, the expression pattern of cell cycle regulating proteins became modified. In particular, cyclin D3, p27 and Rb were up-regulated by VPA. The up-regulation was not dependent on the exposure time.

Data indicate that p27 is involved in cell-cycle control, and the loss of p27 expression is a risk factor for disease recurrence and the strongest predictor of cancer-specific survival [15, 16]. VPA may, therefore, interfere with the mitotic cycle of RCC tumour cells by enhancing the p27 expression level. The role of cyclin D3 in tumour progression is discussed controversially. Based on an immunohistochemical study, high cyclin D3 expression has been associated with high proliferation in RCC, although this was related to only 16% of the tumours [17, 18]. In another study, cyclin D3 levels were significantly higher in ovarian tumours of low malignant potential than in ovarian adenocarcinomas, and absent cyclin D3 expression was an indicator of poor survival in the patient cohort [19]. Suppressive activity of cyclin D3 has also been reported in skin carcinogenesis [20], in pancreatic adenocarcinomas [21] and prostate carcinoma [22]. In good accordance with our data, exposure of C6 glioma cells to VPA induced a marked up-regulation of cyclin D3 and decreased expression of the proliferating cell nuclear antigen, both of which are detectable in the G1-phase [23].

Evidence has been provided that cyclin D3 expression positively correlates with Rb protein expression [19]. In fact, hypophosphorylated Rb is strongly involved in the repression of proliferation-associated genes [24]. Although Rb expression has not been analysed in an RCC cell culture model, aberrant Rb levels were detected in tissue specimens taken from RCC patients [18]. Recently, the short-chain fatty acid HDAC inhibitors tributyrin and sodium butyrate were demonstrated to enhance expression levels of hypophosphorylated Rb in prostate cancer [25], and VPA was shown to induce G1 arrest in association with Rb up-regulation in human melanoma cells [26]. We, therefore, postulate that VPA's effects on RCC cell mitosis may also be caused by modified Rb and cyclin D3 protein levels.

Although cyclin D3, p27 and Rb elevation seem to be a general feature of VPA's mode of action, modifications of further cell signalling proteins are difficult to interpret. Notably, cyclin B was down-regulated after 3 days, but up-regulated after a 5-day VPA exposure. The p21 was enhanced after 3 days, but lowered after 5 days by VPA. Interestingly, control values were also modified time

dependently, cyclin B being diminished, p21 enhanced after 5 days, compared to the 3-day values. Possibly, periodic alterations of p21 and cyclin B levels take place, which may account for the persistent proliferation of cancer cells [27]. If this is the case, the effects of VPA might be due to its dampening this oscillatory behaviour, returning the cells to normal dissipative dynamics. However, this concept is purely speculative. The existence of cyclin B double bands may also be interpreted in this way: There is no difference between control and the various therapies in the 3-day experiment but a loss of the lower band for 1 mM VPA (with or without IFN-α). In case of the 5-day experiment, the lower (bona fide unspecific) band is unaltered and the (bona fide specific) cyclin B band increases from control through increasing VPA concentrations and addition of IFN-α.

A total of 200 mg/kg bw VPA significantly reduced the growth of xenografted RCC cells which clearly confirmed our *in vitro* data. The effect was concomitant with altered expression of proteins related to the malignant phenotype, including a massive increase of p21 and bax. Interestingly, the same dosing schedule has been recommended by others to diminish prostate cancer xenografts [28], and to suppress tumour angiogenesis *in vivo* [29]. However, a different VPA regimen may be required to treat other tumour types. Daily i.p. injections of 366 mg/kg VPA were necessary to inhibit gastrointestinal tumour growth in *nu/nu* mice [30], and neuroblastoma xenograft studies were based on 400 mg/kg VPA [8, 31]. The present data are related to RCC, and it may be possible that different tumour types require different VPA regimens.

Finally, whether IFN-α added to VPA in low concentrations could offer an advantage over VPA monotherapy was investigated. It has recently been shown that IFN-α, when used together with VPA, significantly potentiates the anti-tumoural activity of VPA on the human N-myc amplified cell line BE(2)-C, whereas IFN-α on its own has little or no effect [9]. Most strikingly, VPA plus IFN-α synergistically inhibited growth of UKF-NB-3 xenograft tumours in nude mice and induced complete cures in two out of six animals, whereas single treatment merely inhibited tumour growth [8]. Furthermore, IFN-α has been documented to enhance the anti-angiogenic action of HDAC-inhibitors in neuroblastoma bearing transgenic mice [10], and to potentiate the influence of HDAC-inhibitors on growth and invasion of lung and liver cancer cells [32, 33].

A combination VPA–IFN-α regimen was more effective than a VPA monotherapy. IFN-α alone did not act on RCC proliferation, showing that IFN-α boosts VPA's anti-tumoural properties. The same was true with respect to several cell cycle regulating proteins, particularly CDK2, CDK4, cyclin B, Rb, and (partially) p21.

Nevertheless, IFN-α was also found to counteract some effects of VPA. Notably, p21 which was strongly up-regulated by 1 mM VPA after 3 days was reduced by the VPA–IFN-α combination. In a similar fashion, cyclin D3 was altered more distinctly by 1 mM VPA alone than by the VPA–IFN-α combination therapy. Kuljaca and coworkers observed a similar phenomenon. Although combination therapy with IFN-α and the HDAC inhibitor TSA exerted a synergistic action on prostate and breast cancer cell growth, p21 elevation was much more pronounced by TSA alone than by the TSA–IFN-α combination [10]. The authors concluded that slight inhibition of p21 sensitizes p21-expressing cancer cells to the combination therapy. However, it is not clear if this may also hold true for the RCC model or if the discrepancies are irrelevant side effects.

Surprisingly, the *in vitro* data were not confirmed by the *in vivo* model, because the VPA–IFN-α combination was not superior to VPA monotherapy. However, when interpreting the data, we should be aware that different experimental protocols have been used. VPA and IFN-α were administered once in the *in vitro* system whereas animals were treated chronically over a prolonged time period. Therefore, drug concentrations reaching the target cells may vary *in vitro* and *in vivo* and, consequently, different tumour responses may be evoked. Based on a human breast cancer cell line, the concomitant presence of the HDAC inhibitor sodium butyrate and IFN-α significantly improved the antiproliferative effect of IFN-α alone, however sequential treatment sodium butyrate-IFN-α did not enhance the inhibitory activity seen with either drug alone [34]. This issue should be considered, because nude mice were treated first with VPA and afterwards with IFN-α, whereas RCC cell cultures were incubated with VPA and IFN-α simultaneously. Further reports suggest that IFN-α enhances the anti-tumoural potential of VPA when applied in sub-therapeutic concentrations, *i.e.* when IFN-α alone exerts no anti-cancer effects [35, 36].

This (still hypothetical) assumption might explain the discrepancy between the *in vitro* and *in vivo* results. Indeed, synergistic combinational anti-cancer effects became evident when IFN-α alone exerted no biological activity on RCC cells, *i.e. in vitro* but not *in vivo*. The molecular background of VPA–IFN-α interactions *in vivo* has not been evaluated in detail. However, though a synergy was seen with respect to CDK1, CDK4 and HDAC3 expression, VPA–IFN-α combination strongly counteracted the effects of VPA on bcl-2 and, most impressively, on p21 expression. Presumably, counter-regulation of p21 and bcl-2 was (at least in part) responsible for the loss of synergism in treated animals. Ongoing studies dealing with this issue are underway.

In summary, administration of VPA resulted in a marked decrease in proliferation of RCC cells *in vitro* and significant reduction in tumour volume *in vivo*. We postulate that VPA's effects are based on the induction of cell cycle arrest and alterations of cell cycle regulating proteins. Still, the protein data are somewhat descriptive and, therefore, experiments using transfected RCC cells to knock down a particular protein may further deepen our knowledge about VPA's mode of action. Because VPA has been approved by the U.S. Food and Drug Administration, with an established safety profile, and because drug concentrations used in the present study are within the therapeutic range, it can be considered an attractive candidate for clinical trials.

Acknowledgements

We would like to thank Karen Nelson for critically reading the manuscript. This work was supported by the Horst Müggenburg-Stiftung, Jung-Stiftung, Walter Schulz Stiftung, Ebert-Stiftung and Held-Hecker-Stiftung.

References

1. **Jacobsohn KM, Wood CG.** Adjuvant therapy for renal cell carcinoma. *Semin Oncol.* 2006; 33: 576–82.
2. **Rubagotti A, Martorana G, Boccardo F.** Epidemiology of kidney cancer. *Eur Urol Suppl.* 2006; 5: 558–65.
3. **Motzer RJ, Russo P.** Systemic therapy for renal cell carcinoma. *J Urol.* 2000; 163: 408–17.
4. **Cinatl J Jr, Cinatl J, Scholz M, *et al.*** Antitumor activity of sodium valproate in cultures of human neuroblastoma cells. *Anticancer Drugs.* 1996; 7: 766–73.
5. **Blaheta RA, Michaelis M, Driever PH, *et al.*** Evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. *Med Res Rev.* 2005; 25: 383–97.
6. **Munster P, Marchion D, Bicaku E, *et al.*** Phase I trial of histone deacetylase inhibition by valproic acid followed by the topoisomerase II inhibitor epirubicin in advanced solid tumors: a clinical and translational study. *J Clin Oncol.* 2007; 25: 1979–85.
7. **Atmaca A, Al-Batran SE, Maurer A, *et al.*** Valproic acid (VPA) in patients with refractory advanced cancer: a dose escalating phase I clinical trial. *Br J Cancer.* 2007; 97: 177–82.
8. **Michaelis M, Suhan T, Cinatl J, *et al.*** Valproic acid and interferon-alpha synergistically inhibit neuroblastoma cell growth *in vitro* and *in vivo*. *Int J Oncol.* 2004; 25: 1795–9.
9. **Cinatl J Jr, Kotchetkov R, Blaheta R, *et al.*** Induction of differentiation and suppression of malignant phenotype of human neuroblastoma BE(2)-C cells by valproic acid: enhancement by combination with interferon-alpha. *Int J Oncol.* 2002; 20: 97–106.
10. **Kuljaca S, Liu T, Tee AE, *et al.*** Enhancing the anti-angiogenic action of histone deacetylase inhibitors. *Mol Cancer.* 2007; 6: 68.
11. **Qian DZ, Kachhap SK, Collis SJ, *et al.*** Class II histone deacetylases are associated with VHL-independent regulation of hypoxia-inducible factor 1 alpha. *Cancer Res.* 2006; 66: 8814–21.
12. **Blaheta RA, Michaelis M, Natsheh I, *et al.*** Valproic acid inhibits adhesion of vincristine- and cisplatin-resistant neuroblastoma tumour cells to endothelium. *Br J Cancer.* 2007; 96: 1699–706.
13. **Beecken WD, Engl T, Ogbomo H, *et al.*** Valproic acid modulates NCAM polysialylation and polysialyltransferase mRNA expression in human tumor cells. *Int Immunopharmacol.* 2005; 5: 757–69.
14. **Xia Q, Sung J, Chowdhury W, *et al.*** Chronic administration of valproic acid inhibits prostate cancer cell growth *in vitro* and *in vivo*. *Cancer Res.* 2006; 66: 7237–44.
15. **Pertia A, Nikoleishvili D, Trsintsadze O, *et al.*** Loss of p27(Kip1) CDKI is a predictor of poor recurrence-free and cancer-specific survival in patients with renal cancer. *Int Urol Nephrol.* 2007; 39: 381–7.
16. **Langner C, von Wasielewski R, Ratschek M, *et al.*** Biological significance of p27 and Skp2 expression in renal cell carcinoma. A systematic analysis of primary and metastatic tumour tissues using a tissue microarray technique. *Virchows Arch.* 2004; 445: 631–6.
17. **Hedberg Y, Davoodi E, Ljungberg B, *et al.*** Cyclin E and p27 protein content in human renal cell carcinoma: clinical outcome and associations with cyclin D. *Int J Cancer.* 2002; 102: 601–7.
18. **Hedberg Y, Roos G, Ljungberg B, *et al.*** Cyclin D3 protein content in human renal cell carcinoma in relation to cyclin D1 and clinico-pathological parameters. *Acta Oncol.* 2002; 41: 175–81.
19. **Levidou G, Korkolopoulou P, Thymara I, *et al.*** Expression and prognostic significance of cyclin D3 in ovarian adenocarcinomas. *Int J Gynecol Pathol.* 2007; 26: 410–7.
20. **Rojas P, Cadenas MB, Lin PC, *et al.*** Cyclin D2 and cyclin D3 play opposite roles in mouse skin carcinogenesis. *Oncogene.* 2007; 26 : 1723–30.
21. **Tsutsumida H, Swanson BJ, Singh PK, *et al.*** RNA interference suppression of MUC1 reduces the growth rate and metastatic phenotype of human pancreatic cancer cells. *Clin Cancer Res.* 2006; 12: 2976–87.
22. **Olshavsky NA, Groh EM, Comstock CE, *et al.*** Cyclin D3 action in androgen receptor regulation and prostate cancer. *Oncogene.* 2008; 27: 3111–21.
23. **Bacon CL, Gallagher HC, Haughey JC, *et al.*** Antiproliferative action of valproate is associated with aberrant expression and nuclear translocation of cyclin D3 during the C6 glioma G1 phase. *J Neurochem.* 2002; 83: 12–9.
24. **Tan J, Zhuang L, Jiang X, *et al.*** Apoptosis signal-regulating kinase 1 is a direct target of E2F1 and contributes to histone deacetylase inhibitor-induced apoptosis through positive feedback regulation of E2F1 apoptotic activity. *J Biol Chem.* 2006; 281: 10508–15.
25. **Kuefer R, Hofer MD, Altug V, *et al.*** Sodium butyrate and tributyrin induce *in vivo* growth inhibition and apoptosis. *Br J Cancer.* 2004; 90: 535–41.
26. **Valentini A, Gravina P, Federici G, *et al.*** Valproic acid induces apoptosis, p16INK4A upregulation and sensitization to chemotherapy in human melanoma cells. *Cancer Biol Ther.* 2007; 6: 185–91.
27. **Wolfrom C, Martin OC, Laurent M, *et al.*** Sinusoidal swinging dynamics of the telomere repair and cell growth activation functions of telomerase in rat liver cancer cells. *FEBS Lett.* 2007; 581: 125–30.
28. **Shabbeer S, Kortenhorst MS, Kachhap S, *et al.*** Multiple Molecular pathways explain the anti-proliferative effect of valproic acid on prostate cancer cells *in vitro* and *in vivo*. *Prostate.* 2007; 67: 1099–110.
29. **Gao D, Xia Q, Lv J, *et al.*** Chronic administration of valproic acid inhibits PC3 cell growth by suppressing tumor angiogenesis *in vivo*. *Int J Urol.* 2007; 14: 838–45.
30. **Greenblatt DY, Vaccaro AM, Jaskula-Sztul R, *et al.*** Valproic acid activates notch-1 signaling and regulates the neuroendocrine phenotype in carcinoid cancer cells. *Oncologist.* 2007; 12: 942–51.
31. **Yang Q, Tian Y, Liu S, *et al.*** Thrombospondin-1 peptide ABT-510 combined with valproic acid is an effective antiangiogenesis strategy in neuroblastoma. *Cancer Res.* 2007; 67: 1716–24.
32. **Yamamoto-Yamaguchi Y, Okabe-Kado J, Kasukabe T, *et al.*** Induction of apoptosis by combined treatment with differentiation-inducing agents and interferon-alpha in human lung cancer cells. *Anticancer Res.* 2003; 23: 2537–47.
33. **Kaneko F, Saito H, Saito Y, *et al.*** 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.* 2004; 24: 837–45.

34. **Biffi A, Coradini D, Pellizzaro C, *et al.*** Simultaneous but not sequential treatment with sodium butyrate improves the antiproliferative effect of alpha- or beta-interferon on a breast cancer cell line. *Anticancer Res.* 1998; 18: 4109–14.

35. **Lucero Gritti MF, Beviacqua M, Bordenave RH, *et al.*** Interferon-alpha 2b modulation of doxorubicin sensitivity in a multidrug resistant cell line. *J Exp Clin Cancer Res.* 2001; 20: 393–400.

36. **Okamoto E, Kinne RK, Sökeland J.** Interferons modify *in vitro* proliferation of human bladder transitional cell carcinoma in the presence of doxorubicin and mitomycin C. *J Urol.* 1996; 156: 1492–5.