Valproic acid blocks adhesion of renal cell carcinoma cells to endothelium and extracellular matrix

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Abstract

Treatment strategies for metastatic renal cell carcinoma (RCC) have been limited due to chemotherapy and radiotherapy resistance. The development of targeted drugs has now opened novel therapeutic options. In the present study, anti-tumoral properties of the histone deacetylase inhibitor valproic acid (VPA) were tested *in vitro* and *in vivo* on pre-clinical RCC models. RCC cell lines Caki-1, KTC-26 or A498 were treated with various concentrations of VPA to evaluate tumour cell adhesion to vascular endothelial cells or to immobilized extracellular matrix proteins. *In vivo* tumour growth was conducted in subcutaneous xenograft mouse models. VPA was also combined with low dosed interferon- α (IFN- α) and the efficacy of the combination therapy, as opposed to VPA monotherapy, was compared. VPA significantly and dose-dependently prevented tumour cell attachment to endothelium or matrix proteins, accompanied by elevated histones H3 and H4 acetylation. VPA altered integrin- α and - β subtype expression, in particular α_3 , α_5 and β_3 , and blocked integrin-dependent signalling. *In vivo*, VPA significantly inhibited the growth of Caki-1 in subcutaneous xenografts with the 200 mg/kg being superior to the 400 mg/kg dosing schedule. VPA-IFN- α combination markedly enhanced the effects of VPA on RCC adhesion, and *in vivo* tumour growth was further reduced by the 400 mg/kg but not by the 200 mg/kg VPA dosing schedule. VPA profoundly blocked the interaction of RCC cells with endothelium and extracellular matrix and reduced tumour growth *in vivo*. Therefore, VPA should be considered an attractive candidate for clinical trials.

Keywords: HDAC • valproic acid • renal cell carcinoma • adhesion • integrin receptors

Introduction

Renal cell carcinoma (RCC) accounts for 2–3% of adult cancers worldwide, with the highest rates observed in the United States, Australia and Europe. Nearly 40,000 new cases are diagnosed each year in the Western countries, leading to an estimated 20,000 deaths [1]. 30% of RCC patients have metastatic disease at the time of diagnosis. An additional 20% to 30% with clinically localized disease at the time of nephrectomy will subsequently develop metastatic disease [2]. The 5-year survival for those patients with distant metastases is less than 10% [3].

Metastatic RCC is generally resistant to standard chemotherapy, radiotherapy and hormonal therapy. Cytokine-based

immunotherapy has until recently been considered standard care for first-line treatment of metastatic RCC. However, this approach is associated with significant toxicity, only 10–20% of patients experienced objective disease response, and life was prolonged only in selected patients [4].

A novel concept to manage RCC is based on the development of potent anticancer compounds directed against specific and relevant biological targets. 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 [5]. Hence, HDAC inhibitors may be 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 several tumour cells *in vitro* and *in vivo* [6, 7]. VPA is

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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.

Very recently, administration of VPA has been reported to result in a marked decrease in proliferation of RCC cells *in vitro* and a significant reduction in tumour volume *in vivo* [8]. Nevertheless, close communication of RCC cells with vascular endothelium and underlying extracellular matrix proteins is required to allow tumour transmigration and metastatic spread. Since invasion and metastasis are the critical events of malignant tumour progression and the main cause of treatment failure, a potent anticancer compound should not only prevent RCC proliferation but – more importantly – prevent transit of circulating tumour cells from the blood vessel into the target tissue.

To explore whether VPA fulfils this essential criterion, the potential of VPA to block adhesion properties of RCC cells was investigated and the underlying mode of action explored. Also, VPA was combined with low-dosed interferon- α (IFN- α) and the effects of the combination regimen compared to the 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* [9–11].

VPA was shown to potently block RCC tumour cell adhesion *in vitro* and prevent RCC tumour growth *in vivo*. VPA's activity was associated with reduction of HDAC and elevated acetylation of histones H3 and H4. VPA altered integrin- α and - β subtype expression and blocked integrin-dependent signalling. It is of particular interest that VPA-IFN- α combination induced stronger effects on RCC cell adhesion than VPA alone. Based on these results, VPA provides potent anti-tumour activity and, therefore, may reveal significant therapeutic benefit in treating advanced RCC.

Materials and methods

Antibodies

Integrins: anti- α_1 (IgG1; clone SR84, dilution 1:1000), anti- α_2 (IgG2a; clone 12F1-H6, dilution 1:250), anti- α_3 (IgG1; clone C3II.1, dilution 1:1000), anti- α_4 (IgG1; clone 9F10, dilution 1:200), anti- α_5 (IgG1; clone IIA1, dilution 1:5000), anti- α_6 (IgG2a; clone GoH3, dilution 1:200), anti- β_1 (IgG1; clone MAR4, dilution 1:2500), anti- β_3 (IgG1; clone VI-PL2, dilution 1:2500) or anti- β_4 (IgG2a; clone 439-9B, dilution 1:250) integrins were all from BD Biosciences (Heidelberg, Germany). Anti-integrin-linked kinase (ILK; clone 3, dilution 1:1000), anti-focal adhesion kinase (FAK; clone 77, dilution 1:1000) and anti-phospho-specific FAK (pY397; clone 18, dilution 1:1000) were also derived from BD Biosciences.

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 were derived from CLS (Heidelberg, Germany). Tumour cells were grown and subcultured in RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 10% foetal calf serum (FCS), 100 IU/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified, 5% CO₂ incubator.

Endothelial cells (human umbilical vein endothelial cells) were isolated from human umbilical veins and harvested by enzymatic treatment with chymotrypsin. HUVEC were grown in Medium 199 (Biozol, Munich, Germany), 10% FCS (Gibco, Karlsruhe, Germany), 10% pooled human serum (Blood Bank of The German Red Cross, Frankfurt am Main, Germany), 20 μ g/ml endothelial cell growth factor (Boehringer, Mannheim, Germany), 0.1% heparin (Roche, Basel, Switzerland), 100 ng/ml gentamycin (Gibco) and 20 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (Seromed, Berlin, Germany). To control the purity of HUVEC cultures, cells were stained with fluorescein isothiocyanate labelled monoclonal antibody against factor VIII-associated antigen (Von Willebrand factor; clone F8/86; Dako, Hamburg, Germany) and analysed microscopically or by FACscan (Becton Dickinson, Heidelberg, Germany; FL-1H (log) channel histogram analysis; 1×10^4 cells/scan). Cell cultures with a purity >95% were serially passaged. Subcultures from passages 2–4 were selected for experimental use.

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 (*i.e.* treated with cell culture medium alone). 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 IFN- α , or to those which remained untreated.

Tumour cell adhesion

To analyse tumour cell adhesion, HUVEC were transferred to six-well multiplates (Falcon Primaria; BD Biosciences) in complete HUVEC medium. When 80–100% confluency was reached, Caki-1, KTC-26 or A498 cells were detached from the culture flasks by accutase treatment (PAA Laboratories, Cölbe, Germany) and 0.5×10^6 cells were then added to the HUVEC monolayer for 60 min. Subsequently, non-adherent tumour cells were washed off using warmed (37°C) Medium 199. The remaining cells were fixed with 1% glutaraldehyde. Adherent tumour cells were counted in five different fields of a defined size ($5 \times 0.25 \text{ mm}^2$) using a phase contrast microscope and the mean cellular adhesion rate was calculated.

Attachment to extracellular matrix components

Six-well plates were coated with collagen G (extracted from calfskin, consisting of 90% collagen type I and 10% collagen type III; Seromed; diluted

to 100 µg/ml in (phosphate buffered saline), laminin (derived from the Engelbreth–Holm–Swarm mouse tumour; BD Biosciences; diluted to 50 µg/ml in PBS), or fibronectin (derived from human plasma; BD Biosciences; diluted to 50 µg/ml in PBS) overnight. Plastic dishes served as the background control. Plates were washed with 1% BSA (bovine serum albumin) in PBS to block nonspecific cell adhesion. Thereafter, 0.5×10^6 tumour cells were added to each well for 60 min. Subsequently, non-adherent tumour cells were washed off, the remaining adherent cells were fixed with 1% glutaraldehyde and counted microscopically. The mean cellular adhesion rate, defined by $\text{adherent cells}_{\text{coated well}} - \text{adherent cells}_{\text{background}}$, was calculated from five different observation fields.

Evaluation of integrin surface expression

Tumour cells were washed in blocking solution (PBS, 0.5% BSA) and then incubated for 60 min. at 4°C with phycoerythrin (PE)-conjugated monoclonal antibodies directed against integrin subtypes indicated above. Integrin expression of tumour cells was then measured using a FACscan (Becton Dickinson; FL-1H or FL-2H (log) channel histogram analysis; 1×10^4 cells/scan) and expressed as mean fluorescence units (MFU). A mouse IgG1-PE (MOPC-21) or IgG2a-PE (G155–178; all: BD Biosciences) was used as an isotype control.

Western blotting

Acetylated histones H3 and H4 were evaluated in Caki-1 tumour cells by Western blot analysis. Intracellular integrin subtype level and integrin related signalling 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 antibodies, diluted as listed above. HRP-conjugated goat-antimouse IgG (Upstate Biotechnology, Lake Placid, NY, USA; dilution 1:5000) served as the secondary antibody. The membranes were briefly incubated with enhanced chemiluminescence detection reagent (ECLTM, Amersham/GE Healthcare, München, Germany) to visualize the proteins and exposed to an x-ray-film (HyperfilmTM ECTM, Amersham/GE Healthcare). The 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 GmbH) detecting HDAC 1 and 2 was used according to the manufacturer's protocol. A nuclear extract of HeLa cells containing HDAC 1 and 2 was incubated for 10 min. at 37°C with trichostatin A (TSA, 1 µM 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. To determine the HDAC activity of Caki-1 cells, the cell extracts (50 µg) which had been exposed to VPA, IFN-α, to both VPA + IFN-α, or to control medium, were added to the substrate.

Tumour growth *in vivo*

For *in vivo* testing, 10^7 Caki-1 cells were injected subcutaneously into male NMRI : *nul/nul* 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% PEG 400/saline. It was injected intraperitoneally in doses of 100, 200 or 400 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.

Statistics

All *in vitro* experiments were performed three to six times, *in vivo* experiments were done 8–10 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 adhesion by VPA

RCC cell–HUVEC interaction was explored in a co-culture model. Short-term pre-treatment (1 hr) of tumour cells with VPA did not alter their adhesion behaviour (data not shown). Rather, a 3-day pre-incubation with 0.5 or 1 mM VPA was necessary to significantly prevent tumour cell attachment to endothelium (1 mM VPA > 0.5 mM VPA). A 3-day application of 0.25 mM VPA did not prevent tumour cell attachment to HUVEC. However, extension of the 0.25 mM VPA incubation period to 5 days significantly diminished RCC cell adhesion, compared to the control values (Fig. 1).

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

IFN-α enhances the adhesion blocking properties of VPA

A 3-day pre-incubation of Caki-1 with 1 mM VPA significantly prevented tumour cell adhesion to endothelial cells. More cells detached from HUVEC when VPA and IFN-α were applied in combination, although IFN-α alone did not influence the adhesion behaviour of Caki-1 (Fig. 2, left). With respect to a 5-day pre-treatment, both 0.25 and 1 mM VPA altered HUVEC–Caki-1 interaction

Fig. 1 Adhesion of kidney cancer cells to HUVEC is down-regulated by VPA. KTC-26, Caki-1 or A498 cells were treated with various concentrations of VPA for three or five days, and then added at a density of 0.5×10^6 cells/well to HUVEC monolayers for different time periods. Non-adherent tumour cells were washed off in each sample, the remaining cells were fixed and counted in five different fields ($5 \times 0.25 \text{ mm}^2$) using a phase contrast microscope. Mean values were calculated from five counts. Mean adhesion capacity is depicted as counted cells/mm². * indicates significant difference to controls.

(1 mM > 0.25 mM). The effects observed were drastically enhanced by the VPA-IFN- α combination therapy. Application of IFN- α alone did not result in any adhesion differences, compared to non-treated controls (Fig. 2, right).

The attachment of Caki-1 cells to immobilized laminin, collagen, or fibronectin was investigated next, since RCC transmigration includes both adhesion to endothelial cells and to sub-endothelial matrix components. Attachment rates of treated tumour cells were compared to the attachment rate of non-treated controls which were set to 100%. In doing so, 0.25 mM VPA, given with or without IFN- α for 3 days, did not change tumour cell binding to collagen or laminin. Attachment to fibronectin was diminished by nearly 20%. IFN- α did not further enhance this effect. The 1 mM VPA strongly prevented Caki-1 binding to all matrix proteins. A combination regimen based on 1 mM VPA and IFN- α further diminished the amount of tumour cells which bound to laminin and collagen. However, this was not true with respect to the fibronectin matrix (Fig. 3). Pre-incubation of Caki-1 cells with 0.25 mM VPA for 5 days significantly reduced tumour binding to fibronectin. VPA (0.25 mM)-IFN- α combination

therapy further enhanced this effect and even induced down-regulation of Caki-1 attachment to collagen. Interaction with laminin was not influenced. When 1 mM VPA was applied for 5 days, binding of Caki-1 to collagen, laminin or fibronectin was blocked significantly. The therapeutic response was even stronger when IFN- α was included, although IFN- α alone did not influence tumour cell binding (Fig. 3).

VPA and VPA-IFN- α combination increase histones H3 and H4 acetylation

Caki-1 cells were treated with VPA (1 and 5 mM) [12] or VPA-IFN- α combination for 12 hrs, and histone acetylation was assessed by Western blotting. Caki-1 showed distinct increase of acetylated H3 and H4 under VPA treatment. Interestingly, VPA's effects were more prominent in the presence of IFN- α , although IFN- α alone did not modify histones (Fig. 4, up). Determination of HDAC activity revealed strong inhibition under VPA, the effect of which became much more prominent in the presence of IFN- α (Fig. 4, down).

Fig. 2 Time dependent adhesion of Caki-1 to HUVEC. Caki-1 cells were treated with low (0.25 mM) or high (1 mM) concentrations of VPA, applied alone or in combination with IFN- α . After a three or five day pre-incubation, tumour cells were added at a density of 0.5×10^6 cells/well to HUVEC monolayers for different time periods. Non-adherent tumour cells were washed off in each sample, the remaining cells were fixed and counted in five different fields ($5 \times 0.25 \text{ mm}^2$) using a phase contrast microscope. Mean values were calculated from five counts. Mean adhesion capacity is depicted as counted cells/mm 2 . * indicates significant difference to controls. # indicates significant differences between VPA monotherapy and VPA-IFN- α combination therapy. If VPA-IFN- α combination therapy was not superior to the VPA monotherapy but evoked significant differences to the untreated controls, respective figure symbols were also marked with *.

VPA modifies integrin expression pattern and blocks integrin activation

The α - and β -integrin subtypes were analysed next, since integrins are deeply involved in tumour cell adhesion and transmigration [13]. With respect to the receptor surface expression, explored by flow cytometry, α_3 was detected most extensively on Caki-1, whereas α_1 , α_2 and α_5 were expressed to a lower extent (Fig. 5). The α_4 and α_6 subtypes were not significantly elevated over the background values (data not shown). The β_1 and β_3 subtypes were found to be highly expressed, β_4 to be moderately expressed on Caki-1. Figure 5 is related to a 1 mM VPA concentration, given for 5 days, and demonstrates enhanced receptor levels, in particular of the α_3 , α_5 and β_3 subtype, which were caused by VPA alone. Comparison between VPA and the VPA-IFN- α combination triggered effects revealed no differences with respect to integrin β_1 , β_3 , β_4 and α_3 surface expression. However, a VPA-IFN- α regimen induced a stronger α_1 , α_2 and α_5 expression than VPA alone.

Intracellular integrin levels were analysed by Western blotting (Fig. 6). The α_1 protein expression increased in the presence of VPA, independently from the VPA concentration. When VPA-IFN- α combination was applied for 5 days, α_1 further increased, whereas α_1 was slightly reduced by the drug combination after 3 days (each compared to VPA alone). IFN- α given alone did not alter α_1 expression. The α_2 became slightly elevated by VPA or VPA-IFN- α after a 5-day pre-incubation. The α_3 was down-regulated equally well by VPA or VPA-IFN- α combination after 3 days. However,

IFN- α did not act on α_3 when given for 5 days. In contrast, the α_5 protein level was altered particularly by IFN- α and VPA-IFN- α combination, but not by VPA alone. With respect to the β_3 and β_4 integrin subunits, 5-day pre-incubation with VPA or VPA-IFN- α was necessary to evoke a distinct (and similar) reduction of these proteins. IFN- α alone did not act on Caki-1 cells in this matter. The influence of the compounds on β_1 was ambiguous. The β_1 was diminished after 3 days by 0.25 mM VPA exclusively. With respect to the 5-day application, β_1 was reduced by IFN- α , 0.25 mM VPA and 1 mM VPA-IFN- α combination, but not by 1 mM VPA alone.

Quantitative alterations of integrins may not necessarily be coupled with reduced receptor activity. We, therefore, explored ILK, FAK and phosphorylation of FAK (pFAK) which are involved in the regulation of integrin function [14]. Figure 7 demonstrates that both FAK and pFAK were markedly down-regulated in Caki-1 cells when the combination regimen, but not the VPA monotherapy, was applied for 3 days. Moderate effects were also seen on ILK protein expression. When the incubation time was extended to 5 days, pFAK was nearly lost, this effect being caused by both VPA monotherapy as well as VPA-IFN- α combination.

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

Fig. 3 Adhesion of RCC cells to extracellular matrix proteins is down-regulated by VPA or VPA-IFN- α . Caki-1 cells were pre-treated with low (0.25 mM) or high (1 mM) concentrations of VPA, applied alone or in combination with IFN- α . Non-treated cells served as the controls. Cells were then added to immobilized fibronectin, laminin or collagen at a density of 0.5×10^6 cells/well for 60 min. Plastic dishes were used to evaluate unspecific binding (background control). Non-adherent tumour cells were washed off in each sample, the remaining cells were fixed and counted in five different fields ($5 \times 0.25 \text{ mm}^2$) using a phase contrast microscope. Mean values were calculated from the five counts. Specific adhesion capacity (background adhesion on plastic surface was subtracted from adhesion to matrix proteins) is depicted as % binding and related to non-treated controls which were set to 100%. * indicates significant difference to controls. # indicates significant differences between VPA monotherapy and VPA-IFN- α combination therapy. If VPA-IFN- α combination therapy was not superior to the VPA monotherapy but evoked significant differences to the untreated controls, figure symbols were also marked with *.

on RCC cell growth *in vivo*. Compared to the untreated animals, application of 200 mg/kg VPA significantly diminished the tumour volume, with reduction of 70% at day 46, compared to the control (Fig. 8). 400 mg/kg VPA induced minor effects with a reduction of 40% at day 46, whereas 100 mg/kg VPA did not influence tumour growth. Simultaneous application of both 400 mg/kg VPA and IFN- α led to a further reduction of the tumour volume, compared to 400 mg/kg VPA injected alone (mean tumour volume 0.60 ± 0.08 versus $0.47 \pm 0.07 \text{ cm}^3$, day 46). However, VPA (200 mg/kg schedule) – IFN- α combination treatment was not superior to the 200 mg/kg VPA monotherapy (data not shown).

Discussion

The results presented here provide evidence that the HDAC inhibitor VPA potently blocks RCC cell adhesion to endothelial

cells and to extracellular matrix proteins. The attachment of circulating RCC cells to vascular endothelium and subsequent disruption of the basement membrane are crucial steps in haematogenous metastasis [15, 16]. Thus, the blocking characteristics of VPA could serve to optimize metastatic RCC treatment by suppressing tumour transmigration, thereby slowing RCC progression.

Earlier data have already demonstrated that VPA alters RCC cell growth dynamics [8]. Furthermore, VPA inhibited hypoxia-inducible factor 1 α in RCC cells which plays a critical role in transcriptional gene activation involved in tumour angiogenesis [17]. Obviously, VPA exerts multi-targeted effects on cancer cells and thus may represent an attractive candidate for therapeutic intervention.

In the *in vitro* system, pre-treatment of RCC cells with VPA for several days was necessary to induce a significant adhesion blockade. Effects of 0.25 mM VPA were not seen before a 5-day pre-incubation, whereas 1 mM VPA modified adhesion of RCC cells

Fig. 4 Figure 4A: Western blot analysis of H3 and H4 acetylation in Caki-1, treated with 1 mM or 5 mM VPA or with VPA-IFN- α combination. 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. The β -actin served as the internal control. One representative experiment of three is shown. 4B: For determining the inhibitory activity of VPA or VPA-IFN- α combination on HDAC activity, the Color de Lys cell-free assay was used. Amount of HDAC is given in μ M. * indicates significant difference to controls. # indicates significant differences between VPA monotherapy and VPA-IFN- α combination therapy. One of three experiments is shown here.

already after a 3-day pre-treatment period. This observation concurs with earlier studies dealing with the influence of VPA on RCC cell growth *in vitro* and *in vivo* [8]. Prolonged VPA exposure was also necessary to modify neuroectodermal tumour cells [18, 19], and Xia *et al.* has suggested that chronic administration of VPA is required to achieve therapeutic benefits with prostate carcinoma [12]. In our RCC xenograft model significant tumour reduction was not seen until 10 days after starting chronic VPA application. We, therefore, propose that long-term application of VPA is necessary to delay tumour cell growth and block metastatic processes.

To analyse the mechanistic background responsible for VPA's adhesion blocking properties, integrin receptor expression was

explored, since these molecules play key roles in cancer metastasis by controlling tumour cell targeting, arrest, adhesion and migration [20, 21]. Incubation of Caki-1 with VPA evoked a distinct receptor increase on the cell surface, notably of the α integrin subtypes and β_3 integrins. It is still not clear which subtypes are involved in RCC transmigration and overall malignancy and how they contribute to these processes. RCC specimens taken from higher grade tumours showed decreased α_3 , α_5 and α_6 expression [22]. It has recently been demonstrated that down-regulation of α_2 , α_3 and α_5 surface levels is necessary to allow transendothelial RCC migration [23]. Presumably, enhanced presentation of integrins at the cell surface, caused by VPA, impairs RCC motile behaviour. In line with this assumption, novel findings suggest that integrin internalization contributes to tumour metastasis and, conversely, integrin translocation from the cytoplasm to the cell membrane may prevent tumour cells from crossing the endothelial barrier [24–26]. Indeed, up-regulation of integrin surface expression by VPA was paralleled by a down-regulation of intracellular integrin proteins of the α_3 , β_2 and β_3 subtypes in the RCC *in vitro* model. Nevertheless, whether α_3 , β_2 and β_3 subtype translocation or improper localization of all integrins contributes to the diminished adhesion capacity of RCC cells requires further investigation.

Beside quantitative integrin alterations, phosphorylated FAK became strongly reduced in Caki-1 by VPA, pointing to a specific de-activation of the integrin receptors. It is generally accepted that FAK promotes cell adhesion, and in fact, integrin-stimulated cell adhesion requires FAK and FAK activity [27, 28]. Satoh and coworkers demonstrated that FAK constitutes a functional unit which may be essential in determining malignant properties of RCC cells. [29]. We reported recently that FAK phosphorylation modifies RCC migration [30]. Consequently, loss of FAK activity seen under VPA therapy may be (at least in part) responsible for the diminished adhesion capacity of RCC cells. It has not been elucidated yet how VPA contributed to FAK de-activation. Lee and coworkers demonstrated loss of FAK and FAK activity in colon cancer cells following exposure to the HDAC-inhibitor butyric acid. They hypothesized that beside transcriptional regulation of HDAC-mediated pathways down-regulation of FAK might also be achieved by events other than histone acetylation and deacetylation since HDAC inhibitors could also acetylate non-histone targets as well [31]. A similar scenario may also hold true in our RCC model.

Xenograft studies have been included in our experimental design. Although they may not be fully predictive of the therapeutic efficacy of cancer therapies in clinical trials, they may offer additional information to cell culture studies. 200 mg/kg body weight VPA significantly reduced the growth of xenografted RCC cells. An altered expression of proteins related to the malignant phenotype, including a massive increase of p21 and bax in this model has been reported [8]. The 200 mg/kg dosing schedule has been recommended by others to diminish prostate cancer xenografts [32], and to suppress tumour angiogenesis *in vivo* [33]. However, a different VPA regimen may be required to treat other tumour types. Daily i.p. injections of 366 mg/kg VPA were

Fig. 5 FACS analysis of integrin surface expression on Caki-1 cells. Caki-1 cells were incubated with 1 mM VPA (VPA), with 1 mM VPA-IFN- α (V-IFN) combination, or remained untreated (control). Cells were then washed in blocking solution and stained with specific monoclonal antibodies as listed in materials and methods. A mouse IgG1-PE or IgG2a-PE was used as the isotype control. Fluorescence was analysed using a FACScan flow cytometer, and a histogram plot was generated to show PE-fluorescence. MFU (mean fluorescence units) values are given below each histogram. One of three independent experiments is shown here.

necessary to inhibit gastrointestinal tumour growth in *nu/nu* mice [34], and neuroblastoma xenograft studies were based on 400 mg/kg VPA [9, 35]. On the other hand, the 400 mg/kg VPA dosage induced only minor effects in our RCC model. The reason for the lower response in the 400 mg/kg protocol compared to the 200 mg/kg application is not clear. Speculatively, tumours may activate feedback or compensatory pathways to promote RCC growth and survival. Further studies are necessary to concentrate on this issue.

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-tumoral 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 [10]. 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, while single treatment merely inhibited tumour growth [9]. 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 [36, 37].

A combination VPA-IFN- α regimen was more effective than a VPA monotherapy *in vitro*. IFN- α alone did not act on RCC adhesion, showing that IFN- α boosts VPA's anti-tumoral properties. The conclusion is corroborated by histone analysis, since VPA-IFN- α combination induced much stronger alterations on HDAC activity and histone acetylation than VPA alone, whereas IFN- α given separately was without any effect. A similar phenomenon has been observed in neuroblastoma cell cultures. Treatment with VPA alone decreased the ability of BE(2)-C cells to adhere to and penetrate human endothelium. All these effects of VPA were significantly enhanced when combined with IFN- α which on its own had little or no effect [10].

The mode of action of IFN- α is not clear regarding this matter. However, a similar behaviour has recently been noticed on melanoma cell lines, where IFN- α alone did not induce apoptosis but drastically enhanced the pro-apoptotic effect of VPA [38]. Intriguingly and consistent with further reports, treatment of these cells with IFN- α evoked strong increase of the Stat1 protein level which optimized the response of the melanoma cell lines to VPA [38, 39]. Although purely speculative, enhancement of Stat1 by IFN- α might also render RCC cells to become more susceptible to VPA treatment. Nevertheless, it may not be logical to exclude any specific activity of IFN- α . Indeed, IFN- α

Fig. 6 VPA or VPA-IFN- α modifies intracellular integrin proteins. Caki-1 cells were incubated with IFN- α , with low- (0.25 mM) or high-dosed (1 mM) VPA, with VPA-IFN- α combination or remained untreated (control). Incubation lasted for three or five days. Cell lysates were then analysed by specific antibodies against integrin subtypes as listed in materials and methods. The β -actin served as the internal control. One representative experiment of three is shown.

Fig. 7 Influence of VPA or VPA-IFN- α on integrin-dependent signalling. Caki-1 cells were incubated with IFN- α , with low- (0.25 mM) or high-dosed (1 mM) VPA, with VPA-IFN- α combination or remained untreated (control). Incubation lasted for three or five days. Intracellular signalling was evaluated using the appropriate monoclonal antibodies recognizing ILK, FAK or phosphorylated FAK (pFAK). The β -actin served as the internal control. One representative experiment of three is shown.

Fig. 8 Effect of VPA on RCC xenografts. Caki-1 xenografts were established in male athymic mice. Animals in the treatment arm received 100, 200 or 400 mg/kg VPA ($n = 8$). The control group of mice was treated with the solvent ($n = 10$). *indicates significant difference to the control animals.

but not VPA triggered alterations of α_5 integrin expression in the experiments presented here. Recently, IFN- α but not VPA was shown to reduce bcl-2 expression *in vivo* [8], and Kaneko *et al.* reported down-regulation of matrix metalloproteinase activity in a hepatocellular carcinoma invasion model which was caused by IFN- α [37].

Surprisingly, the *in vitro* RCC data were not always confirmed by the *in vivo* model, since the 200 mg/kg VPA-IFN- α combination was not superior to the 200 mg/kg VPA monotherapy. However, when interpreting the results, 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. It should also be considered that optimum therapeutic response may have already been achieved with 200 mg/kg VPA and, therefore, additional drugs may provide no further benefit. The latter hypothesis might explain why additive effects became obvious when the sub-optimal concentration of 400 mg/kg VPA was used in combination with IFN- α . Nevertheless, the molecular background of VPA-IFN- α interaction *in vivo* has not been evaluated in detail. Thus, this assumption remains speculative.

In summary, administration of VPA resulted in a marked decrease in adhesion of RCC cells *in vitro* and significant reduction in tumour volume *in vivo*. We postulate that VPA's effects are (partially) based on the alterations of integrin expression and signalling. For the future, primary tumour cells should be tested, because they more closely reflect the clinical situation than the

established cell lines we used. Since VPA has been approved by the U.S. Food and Drug Administration, with an established safety profile, and since drug concentrations used in the present study are within the therapeutic range, it can be considered an attractive candidate for clinical trials.

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