HDAC inhibition delays cell cycle progression of human bladder cancer cells *in vitro*

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Our aim was to analyze the impact of the histone deacetylase (HDAC)-inhibitor valproic acid (VPA) on bladder cancer cell growth *in vitro*. RT-4, TCCSUP, UMUC-3, and RT-112 bladder cancer cells were treated with VPA (0.125–1 mmol/l) without and with preincubation periods of 3 and 5 days. Controls remained untreated. Tumor cell growth, cell cycle progression, and cell cycle-regulating proteins were investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, flow cytometry, and western blotting, respectively. Effects of VPA on histone H3 and H4 acetylation and HDAC3 and HDAC4 were also determined. Without preincubation, no tumor cell growth reduction was observed with 0.125 and 0.25 mmol/l VPA in TCCSUP, UMUC-3, and RT-112 cells, whereas 0.5 and 1 mmol/l VPA diminished the cell number significantly. VPA (0.25 mmol/l) did exert tumor growth-blocking effects after a 3-day preincubation. To achieve antitumor effects with VPA (0.125 mmol/l), a 5-day preincubation was necessary. A 3-day or 5-day preincubation was also necessary to distinctly delay cell cycle progression, with maximum effects at VPA (1 mmol/l). After the 5-day preincubation, the cell cycle-regulating proteins cdk1, cdk2, cdk4, and cyclins B, D1, and E were reduced, whereas p27 was enhanced. Diminished HDAC3 and 4 expression induced by VPA was accompanied by elevated acetylation of H3 and H4. VPA exerted growth-blocking properties on a panel of bladder cancer cell lines, commensurate with dose and exposure time. Long-term application induced much stronger effects than did shorter application and should be considered when designing therapeutic strategies for treating bladder carcinoma. *Anti-Cancer Drugs* 22:1002–1009 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2011, **22:**1002–1009

Keywords: bladder carcinoma, cdks, cell growth, cyclins, valproic acid

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Received 12 May 2011 **Accepted** 22 June 2011

Introduction

Bladder carcinoma is the second most common malignancy of the genitourinary tract in Western countries, with an incidence of 37.9/100 000 per year for men and 9.6/100 000 per year for women [1]. Approximately 70% of initially diagnosed tumors are superficial and can be treated by transurethral resection, whereby the bladder is preserved. The remaining 30% becomes muscle invasive and is associated with a high risk of metastatic disease [2]. Systemic chemotherapy is a treatment option for patients with locally advanced or metastatic disease, but overall survival can only be extended by 4 months [3].

During recent years, it has been shown that epigenetic aberrations are involved in tumorigenesis. Particularly, an imbalance in the equilibrium between histone acetylation and histone deacetylation has been proposed as a driving force, causing normal cells to become malignant. In fact, analysis of both clinical samples from patients with urinary bladder cancer and tumor tissues from a mouse model have demonstrated a significantly increased histone deacetylase (HDAC) expression compared with surrounding healthy tissue [4]. Therefore, HDAC inhibition might be an innovative and effective option to treat malignant disease.

The branched-chain fatty acid valproic acid (VPA) is an HDAC inhibitor and is a promising candidate for treating malignant disease, as it has been used in clinical practice for more than 40 years. It has a suitable pharmacokinetic profile and negative side effects are moderate and rare. Cinatl *et al.* [5,6] observed that VPA acts as a potent HDAC inhibitor blocking tumor cell growth *in vitro* and *in vivo* 15 years ago. Numerous reports have been published since then, indicating strong induction of differentiation and apoptosis as well as blockage of growth and metastatic spread of various tumor types following VPA exposure [7–9].

VPA has been shown to significantly delay the development of bladder carcinoma in a mouse model and to decrease cell survival *in vitro* [4]. On the basis of these data, it was our purpose to study the growth-blocking potential of VPA on a panel of bladder carcinoma cell lines and to investigate the mechanism of action.

Methods

Cells

RT-4, RT-112, UMUC-3 (ATCC/LGC Promochem GmbH, Wesel, Germany), and TCCSUP (DSMZ, Braunschweig, Germany) bladder carcinoma cells were

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grown and subcultured in RPMI 1640, 10% fetal calf serum, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (20 mmol/l), 1% glutamax, and 1% penicillin/streptomycin (all: Gibco/Invitrogen, Karlsruhe, Germany). The RT-4 cell line is a model for a well-differentiated human papillary bladder cancer (transitional cell carcinoma, grade 1). RT-112 is an invasive (pathological stage T2), moderately differentiated (grade 2/3) model of human bladder cancer, and UMUC-3 is derived from a deeply invasive (pathological stage T3), poorly differentiated (grade 3) human bladder tumor. TCCSUP represents a transitional cell carcinoma, grade 4. Subcultures from passages seven to 24 were selected for experimental use.

VPA

VPA (a gift from G.L. Pharma GmbH, Lannach, Austria) at final concentrations of 0.125, 0.25, 0.5, or 1 mmol/l was added to bladder carcinoma cells. Controls remained untreated. To exclude toxic effects, cell viability was determined by trypan blue (Gibco/Invitrogen). For apoptosis detection, the expression of Annexin V/propidium iodide (PI) was evaluated using the Annexin V–FITC Apoptosis Detection kit (BD Pharmingen, Heidelberg, Germany). Tumor cells were washed twice with PBS and were then incubated with 5 µl of Annexin V–FITC and 5 µl of PI in the dark for 15 min at RT. Cells were analyzed with an FACScalibur (BD Biosciences, Heidelberg, Germany). The percentage of apoptotic cells (early and late) in each quadrant was calculated using CellQuest software (BD Biosciences).

Measurement of tumor 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). Bladder carcinoma cells (100 µl, 1×10^4 cells/ml) were seeded onto 96-well tissue culture plates with the above-mentioned VPA concentrations. After 24, 48, and 72 h, MTT (0.5 mg/ml) was added for an additional 4 h. Thereafter, cells were lysed in a buffer containing 10% SDS in HCl (0.01 mol/l). The plates were allowed to stand overnight at 37°C with 5% CO_2. Absorbance at 570 nm was determined for each well using a microplate ELISA reader. Each experiment was performed in triplicate. After subtracting background absorbance, results were expressed as mean cell number. To evaluate whether the VPA's activity may depend on the drug exposure time, three different sets of experiments were performed. (a) Cell growth analysis without VPA preincubation: Cell cultures were treated with VPA and immediately subjected to the MTT assay for 24, 48, and 72 h; (b) Cell growth analysis with a 3-day VPA preincubation: Cell cultures were pretreated with VPA for 3 days. The medium was then replaced by a new one (including VPA), and cells were subjected to the MTT assay for 24, 48, and 72 h; and (c) Cell growth analysis with a 5-day VPA preincubation: Cell cultures were pretreated with VPA for 5 days. The medium was then replaced by a new one (including VPA), and cells were subjected to the MTT assay for 24, 48, and 72 h. Control cultures did not obtain VPA; however, medium change and MTT assay were carried out in parallel to experiment A, B, or C.

Cell cycle analysis

Tumor cells were grown to 70% confluency and then treated with VPA (controls remained untreated). Cell cycle analyses were carried out after 3 or 5 days. Tumor cell populations were stained with PI, using a Cycle TEST PLUS DNA Reagent Kit (Becton Dickinson) and then subjected to flow cytometry with an FACScan flow cytometer (Becton Dickinson). A total of 10 000 events were collected from each sample. Data acquisition was carried out using CellQuest software and cell cycle distribution was calculated using the ModFit software (Becton Dickinson). The number of gated cells in G_1, G_2/M or S phase was presented as %.

Western blot analysis

To explore cell cycle-regulating proteins following 3-day or 5-day VPA exposure (1 mmol/l), tumor 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 nonfat dry milk for 1 h, the membranes were incubated overnight with monoclonal antibodies directed against cell cycle proteins: cdk1 (IgG_1, clone 1), cdk2 (IgG2a, clone 55), cdk4 (IgG_1, clone 97), cyclin B (IgG_1, clone 18), cyclin D1 (IgG_1, clone $G_1$24–326), cyclin E (IgG_1, clone HE12), Rb2 (IgG2a, clone 10), and p27 (IgG_1, clone 57; all were obtained from BD Biosciences). Horseradish peroxidase-conjugated goat-anti-mouse IgG (Upstate Biotechnology, Lake Placid, New York, USA; dilution 1 : 5. 000) served as the secondary antibody. The membranes were briefly incubated with the enhanced chemiluminescent detection reagent (enhanced chemiluminescent; Amersham/GE Healthcare, München, Germany) to visualize the proteins and were exposed to an radiograph film (Hyperfilm EC Amersham/GE Healthcare). β-actin (1 : 1.000; Sigma, Taufenkirchen, Germany) served as the internal control.

To investigate histone acetylation, tumor cells were treated with VPA for 24 h and cell lysates were marked with anti-HDAC3 (polyclonal IgG, dilution 1 : 2.000), anti-HDAC4 (polyclonal IgG; dilution 1 : 500), antiacetylated H3 (IgG, clone Y28, dilution 1 : 500), and antiacetylated H4 (Lys8, polyclonal IgG, dilution 1 : 500; all from Biomol GmbH, Hamburg, Germany).

Statistics

All experiments were performed three to six 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

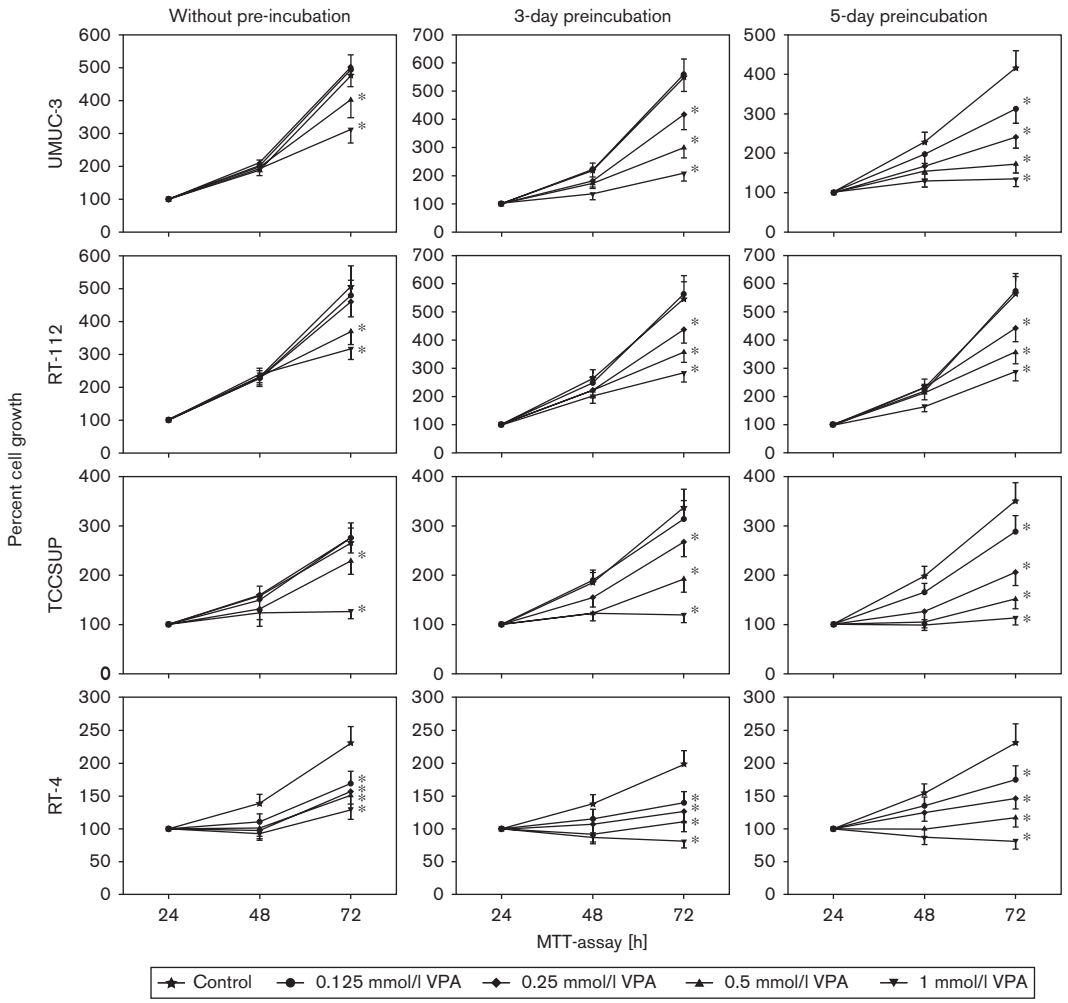
VPA diminishes bladder cancer cell growth

VPA significantly blocked growth of all cell lines investigated (Fig. 1). With respect to UMUC-3 and TCCSUP, the efficacy of VPA strongly depended on the drug exposure time. When UMUC-3 cells were treated with VPA and the MTT assay started immediately thereafter (experiment A, 'without preincubation'), 0.125 and 0.25 mmol/l VPA did not induce any growth reduction. Meanwhile, 0.5 or 1 mmol/l VPA diminished the cell number by 15 ± 2 and $35 \pm 4\%$, respectively (related to 72 h values). However, VPA (0.25 mmol/l) did exert growth-blocking effects when tumor cells were preexposed to this concentration for 3 days (experiment B, '3-day preincubation') and VPA (0.5 mmol/l) decreased UMUC-3 cell growth by $45 \pm 5\%$, and VPA (1 mmol/l) by $62 \pm 6\%$. To achieve antitumor effects with VPA (0.125 mmol/l), a 5-day preincubation was necessary (experiment C, '5 day preincubation'). VPA (0.5 mmol/l) reduced the UMUC-3 cell number by $59 \pm 7\%$, and VPA (1 mmol/l) by $68 \pm 7\%$.

In RT-112 cells, a 3-day VPA pretreatment was superior to direct drug application. However, prolongation of the pretreatment period to 5 days did not further amplify VPA's effects. Both 0.125 and 0.25 mmol/l VPA diminished the cell number of slowly growing RT-4 cells in all experimental settings.

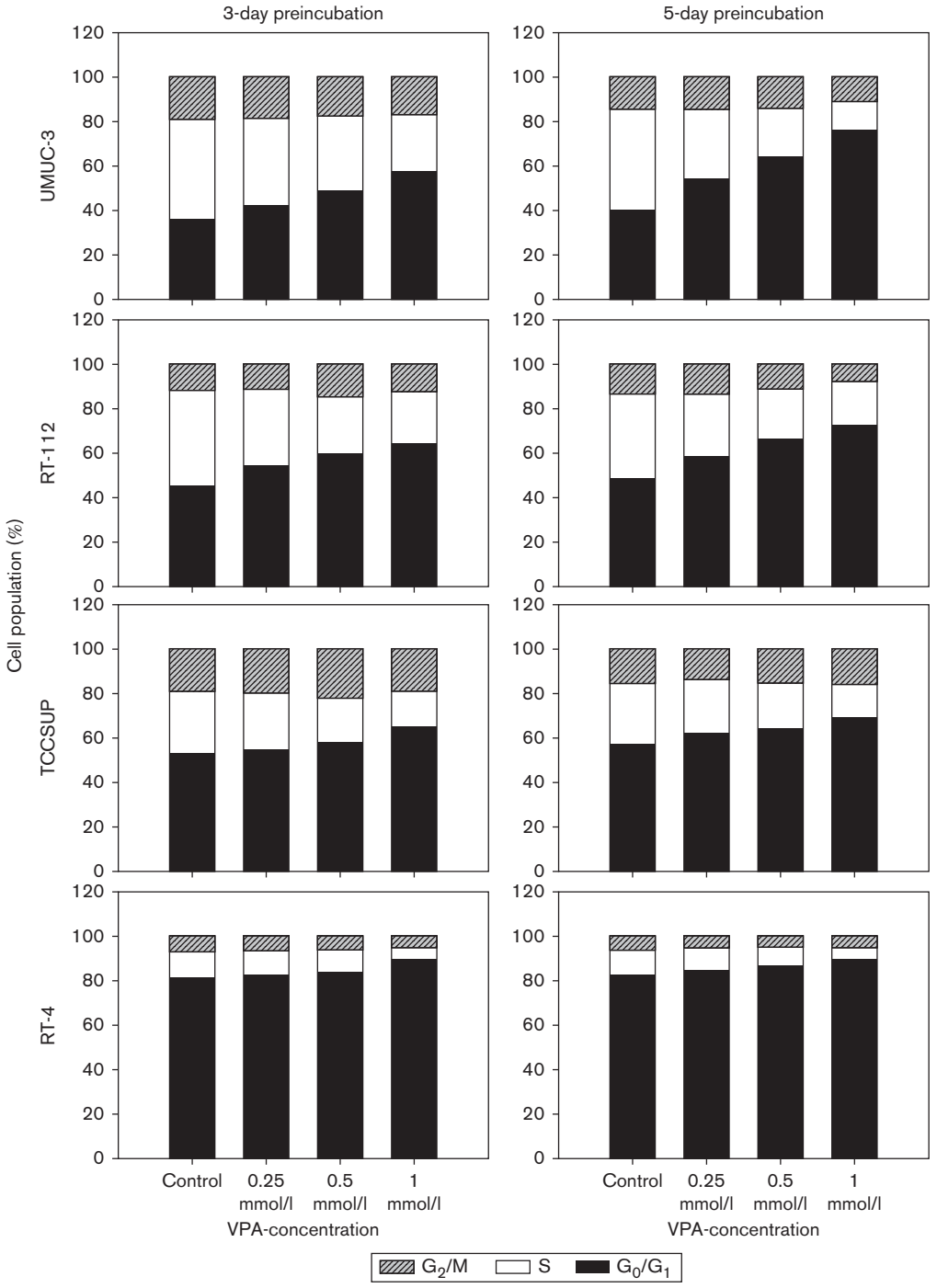
Analysis of apoptotic events was also performed. VPA did not trigger early or late apoptosis in UMUC-3 and TCCSUP cells. Early apoptosis was elevated by $19 \pm 6\%$ in RT-112 and by $20 \pm 7\%$ in RT-4 cells, when VPA (1 mmol/l) was applied for 5 days.

Fig. 1



Growth (percentage) of UMUC-3, RT-112, TCCSUP, and RT-4 cells treated with various valproic acid (VPA) concentrations without and with 3-day or 5-day preincubation as indicated in the 'Methods'. Controls were untreated. Cells were counted after 24, 48, and 72 h using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay. One representative experiment of six is shown. *Significant difference to controls.

Fig. 2



Cell cycle analysis of UMUC-3, RT-112, TCCSUP, and RT-4 cells treated with various valproic acid (VPA) concentrations. The cell populations after 3-day or 5-day VPA incubation are expressed as a percentage (%) of the total cells analyzed. One representative experiment of three is shown.

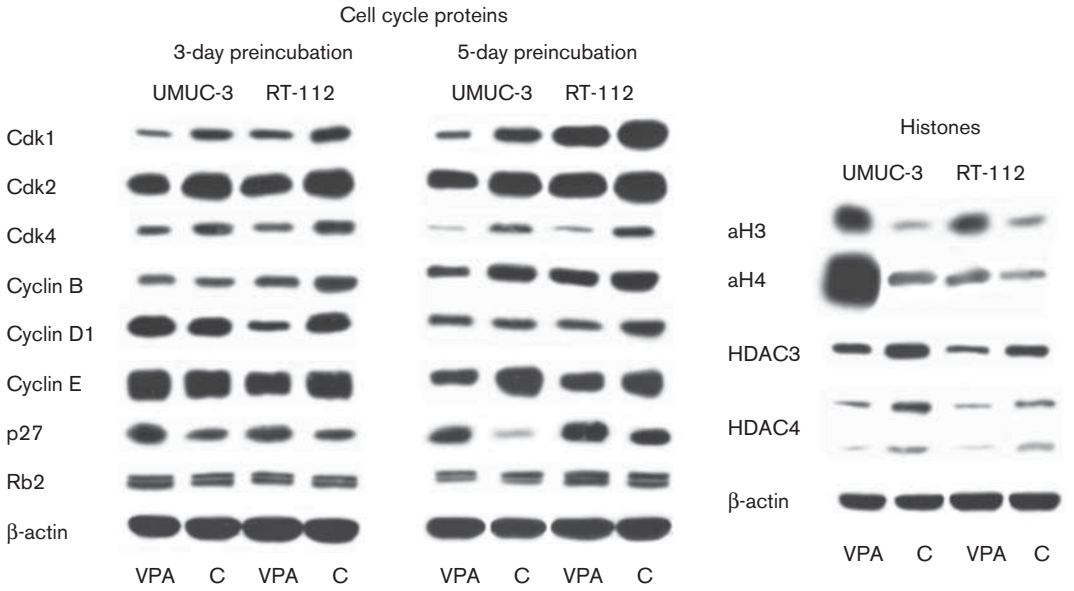
VPA acts on cell cycle progression

VPA dose dependently reduced the amount of S-phase cells and enhanced the amount of G_0/G_1-phase cells (Fig. 2). UMUC-3 responded maximally to VPA when treated for 5 days compared with 3 days, whereas cell cycle progression of RT-112 was similarly altered after 3 or 5 days.

VPA interferes with cell cycle regulation

UMUC-3 and RT-112 cells were included in subsequent experiments to evaluate the mechanistic background responsible for the cell growth blockade. VPA diminished the cell cycle regulators cdk1, cdk2, cdk4, and cyclins B, D1, and E (Fig. 3). p27 was elevated by VPA, whereas

Fig. 3



Western blot analysis of cell cycle protein and histone modification. UMUC-3 and RT-112 cells were treated with valproic acid (VPA) (1 mol/l) for 3 or 5 days to explore cell cycle-regulating proteins. Controls (C) remained untreated. Cell lysates were then subjected to SDS-PAGE and blotted on the membrane incubated with the respective monoclonal antibodies. β-actin served as the internal control. Histone analysis was performed 24 h after VPA (1 mmol/l) addition. The figure shows one representative of three separate experiments. HDAC, histone deacetylase.

Rb2 remained unchanged. The effects evoked in RT-112 cells were the same when treated for 3 or 5 days with VPA. In contrast, maximum benefit was achieved in UMUC-3 incubated for 5 days with VPA.

Further analysis pointed to a dramatic enhancement of histone H3 and H4 acetylation, accompanied by a loss of HDAC3 and HDAC4. Although this was seen in both RT-112 and UMUC-3, distinct H4 acetylation was observed particularly in UMUC-3 cells.

Discussion

This study demonstrates that VPA alone blocks cell growth in four different bladder cancer cell lines. Efficacy depended on the drug exposure time, as 3-day VPA preincubation evoked stronger effects than without preincubation. Treatment for 5 days did not block growth more than the 3-day treatment in RT-112 and RT-4 cells. Therefore, prolonged VPA treatment seems necessary to significantly alter bladder carcinoma growth dynamics. However, the optimum exposure time may depend on the tumor type. Analysis of gene expression signatures in several tumor cell lines before and after treatment with VPA revealed a close correlation between the HDAC pathway activity and drug response within tumor subtypes [10]. In good accordance with this observation, H3 and H4 acetylation in the low-responding RT-112 cells were found to be moderately upregulated by VPA, whereas the strongly responding UMUC-3 cells expressed a high portion of acetylated H3 and H4 following VPA exposure.

Our data, in line with earlier publications, point to a time-dependent inhibiting action of VPA against bladder cancer cells as has been observed with neuroblastoma, prostate, and kidney cancer cells [8,11,12]. This mode of action does not seem restricted to VPA. Using the structurally unrelated HDAC inhibitors LAQ824, suberoylanilide hydroxamic acid (SAHA) or MS-275, incubation for at least 16 h was required to inhibit growth and to induce apoptosis in several tumor cell lines *in vitro*. Antitumor activity was strongest following a 48-h drug pretreatment [13]. Trichostatin A and depsipeptide (FK228) took 4 days to reduce tumor cell viability by 50%, whereas treatment for 8 or 16 h with both HDAC inhibitors failed to induce significant apoptosis [14,15].

Long-term application of VPA seems necessary to alter the cellular epigenetic environment. Therefore, it was surprising that VPA (1 mmol/l) (but not lower dosages) evoked a rapid response in TCCSUP cell lines as evidenced by the MTT assay. The reason for this is not clear. Recently, Yagi *et al.* [16] observed a rapid effect of high-dose VPA on gastric cancer cells. This was accompanied by a mild modification of the apoptosis-related proteins cleaved caspase 3 and 9, bcl-2, and survivin. In human leukemia cell lines, it has been shown that a low dosage of VPA coupled to a long incubation time is necessary to induce tumor senescence, whereas apoptosis

induction requires a higher VPA dosage, but then appears within 24 h [17]. The annexin V/PI staining did not reveal signs of apoptosis in TCCSUP cells in our test system. However, the apoptotic machinery was not evaluated in detail. Therefore, it is possible that rapid apoptosis was induced in the TCCSUP cell line in the presence of a high VPA concentration and may (partially) account for the reduced tumor cell number.

Cell type-specific differences with respect to VPA's antitumor efficacy are of high clinical relevance. Possibly, only a subgroup of patients may benefit from an HDAC-inhibitor-based therapy. In fact, the overall response rate of patients with cutaneous T-cell lymphoma treated with oral SAHA was approximately 30%, including one complete response [18]. Overall response of patients with myelodysplastic syndrome treated with VPA was 50%, including one complete and one partial response [19]. Thirty-four percent of patients with non-small-cell lung cancer responded to SAHA [20], and integrating VPA into the antitumor regimen led to a response rate of 64% in a cohort of patients with breast cancer [21].

The reduction of bladder cancer cell growth was accompanied by an increase of the G_0/G_1-phase and a decrease of the S-phase cell fraction. However, treatment of HT1376 bladder cancer cells with VPA (10 mmol/l) was demonstrated to significantly decrease the G2/M and to elevate the sub G_1 population [4]. Therefore, it may be assumed that VPA-triggered cell growth blockade is at least partially caused by delaying the cell cycle progression. Cell cycle modification was particularly apparent in highly malignant cell lines, whereas only moderate effects were exerted on RT-4 cells, originally derived from a nonmuscle invasive papilloma. As RT-4 cells are characterized by a high percentage of G_0/G_1-phase cells *per se*, VPA's action on this cell line should be interpreted with care. Nevertheless, the nonsteroidal, antiinflammatory drug celecoxib has been shown to evoke a heterogeneous molecular response in the low-grade RT-4 versus high-grade UMUC-3 bladder cancer cells [22]. This supports speculation that VPA may provide the optimum benefit in treating highly aggressive tumors.

Normal progression through the cell cycle requires sequential activation of cyclin-dependent kinases. Analysis of normal urothelial cells revealed that cyclin-dependent kinases of the early G_1 phase, such as cdk4 and cdk2, were readily detectable, whereas cell cycle proteins of the later G_1 and S phases, such as cdk1, cyclin E, and cyclin A, were absent, thus preventing the cell from progressing through the cell cycle. In contrast, carcinoma *in situ* of the bladder induced by SV40 large T antigen showed a dramatic increase in post-G_1 cell cycle regulators, including cyclin A and E and cdk1 and cdk2 [23]. Therefore, reduced expression of cdk1, cdk2, and cdk4 by VPA might explain how this compound acts on bladder carcinoma cells. The same is true with respect to cyclin B and E expression levels, which were also reduced by VPA. Downregulation of cyclin D1, which was observed in RT-112 but not in UMUC-3 cells, is difficult to interpret. As a central target of mitogenic signals, cyclin D1 is frequently overexpressed in a variety of human cancers, particularly during the early stages of tumorigenesis [24]. Bladder cancer is no exception with up to 30% of cases having increased expression of cyclin D1 [25]. Several clinical studies confirm the relation between cyclin D1 elevation, urothelial proliferation, and worse patient prognosis [26–28]. In this context, the loss of cyclin D1 seen in our model provides a further link to the growth-blocking properties of VPA. Garcia-España *et al.* [23] noted a negative correlation of cyclin D1 between tumor and normal tissue, whereas cyclin D1 immunoreactivity was not associated with any pathologic characteristics or clinical outcomes by others [29]. Further investigation is therefore necessary to resolve the conflicting reports of cyclin D1's role in bladder cancer development and progression.

P27, considered to be a tumor suppressor protein, was strongly upregulated by VPA. This effect is desirable, as loss of p27 has been significantly correlated to tumor grade, presence of distant metastasis, and patient survival [30,31]. Kamai *et al.* [32] have provided evidence that decreasing the p27 protein level is associated with progression from superficial to invasive bladder cancer. Consequently, VPA-evoked upregulation of p27 may counteract this process and delay the time to progression. Contrary findings have also been reported, demonstrating an increased p27 expression in high-grade tumors [33,34]. However, this phenomenon probably represents accumulation of a nonfunctional protein [33].

Finally, histone acetylation and HDAC expression were evaluated, as they represent the VPA target structures. VPA enhanced the acetylation of histones H3 and H4 and decreased HDAC3 and HDAC4. This has been confirmed by others who showed that VPA inhibits HDAC activity and increments histone H3 and H4 acetylation in T24 transitional cell carcinoma cells [35]. We conclude that the effects exerted by VPA are specific, triggered by an epigenetic mechanism. This is important as HDACs have been associated with many types of cancers and affect cancer development [36,37]. Deactivation of HDAC and simultaneous reactivation of histone acetylation might therefore be an effective strategy in combating bladder cancer. Several trials have been initiated to evaluate the benefit of HDAC inhibition in the treatment of solid tumors [38–40].

VPA has been chosen in this study as a prototype HDAC inhibitor. It provides distinct therapeutic advantage from a pharmacokinetic as well as from an economic point of view. Nevertheless, further HDAC inhibitors (e.g. vorinostat and depsipeptide) are under investigation in

clinical trials, either as monotherapies or in conjunction with other treatments, such as chemotherapy, biologic therapy, or radiation therapy. The new HDAC inhibitors are characterized by a high specificity and potency, which however might be coupled to stronger side effects and to a higher risk to develop resistance. Careful weighting against risks and benefits is necessary to assess which type of HDAC inhibitor should be used in future to treat certain groups of patients with cancer.

In summary, VPA exerted growth-blocking properties on bladder cancer cells in our study, both in a dose-dependent and exposure time manner. Long-term application induces much stronger effects than does shorter application and should be considered when designing novel therapeutic strategies for treating bladder carcinoma.

Acknowledgements

We would like to thank Karen Nelson for critically reading the manuscript. This study was supported by the 'Jung-Stiftung', Hamburg, Germany.

Conflicts of interest

There are no conflicts of interest.

References

1 Edwards BK, Ward E, Kohler BA, Eheman C, Zauber AG, Anderson RN, *et al*. Annual report to the nation on the status of cancer, 1975–2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates. *Cancer* 2010; **116**:544–573.
2 Kaufman DS, Shipley WU, Feldman AS. Bladder cancer. *Lancet* 2009; **374**:239–249.
3 Goebell PJ, Vom Dorp F, Rödel C, Frohneberg D, Thüroff JW, Jocham D, *et al*. Noninvasive and invasive bladder cancer: diagnostics and treatment. *Urologe A* 2006; **45**:873–884.
4 Ozawa A, Tanji N, Kikugawa T, Sasaki T, Yanagihara Y, Miura N, *et al*. Inhibition of bladder tumour growth by histone deacetylase inhibitor. *BJU Int* 2009 [Epub ahead of print]
5 Cinatl J Jr, Cinatl J, Driever PH, Kotchetkov R, Pouckova P, Kornhuber B, *et al*. Sodium valproate inhibits in vivo growth of human neuroblastoma cells. *Anticancer Drugs* 1997; **8**:958–963.
6 Cinatl J Jr, Cinatl J, Scholz M, Driever PH, Henrich D, Kabickova H, *et al*. Antitumor activity of sodium valproate in cultures of human neuroblastoma cells. *Anticancer Drugs* 1996; **7**:766–773.
7 Blaheta RA, Michaelis M, Driever PH, Cinatl J Jr. Evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. *Med Res Rev* 2005; **25**:383–397.
8 Jones J, Juengel E, Mickuckyte A, Hudak L, Wedel S, Jonas D, *et al*. The histone deacetylase inhibitor valproic acid alters growth properties of renal cell carcinoma in vitro and in vivo. *J Cell Mol Med* 2009; **13 (8B):**2376–2385.
9 Jones J, Juengel E, Mickuckyte A, Hudak L, Wedel S, Jonas D, *et al*. Valproic acid blocks adhesion of renal cell carcinoma cells to endothelium and extracellular matrix. *J Cell Mol Med* 2009; **13 (8B):**2342–2352.
10 Cohen AL, Soldi R, Bild A. Use of genomic signatures to predict response to histone deacetylase inhibitors and correlation with tumor subtypes and molecular pathways. *J Clin Oncol* 2010; **28 (suppl):**e13596.
11 Wedel S, Hudak L, Seibel JM, Juengel E, Oppermann E, Haferkamp A, *et al*. Critical analysis of simultaneous blockage of histone deacetylase and multiple receptor tyrosine kinase in the treatment of prostate cancer. *Prostate* 2010 [Epub ahead of print]
12 Blaheta RA, Michaelis M, Natsheh I, Hasenberg C, Weich E, Relja B, *et al*. Valproic acid inhibits adhesion of vincristine- and cisplatin-resistant neuroblastoma tumour cells to endothelium. *Br J Cancer* 2007; **96**:1699–1706.
13 Atadja P, Gao L, Kwon P, Trogani N, Walker H, Hsu M, *et al*. Selective growth inhibition of tumor cells by a novel histone deacetylase inhibitor, NVP-LAQ824. *Cancer Res* 2004; **64**:689–695.
14 Byrd JC, Shinn C, Ravi R, Willis CR, Waselenko JK, Flinn IW, *et al*. Depsipeptide (FR901228): a novel therapeutic agent with selective, in vitro activity against human B-cell chronic lymphocytic leukemia cells. *Blood* 1999; **94**:1401–1408.
15 Inoue S, MacFarlane M, Harper N, Wheat LM, Dyer MJ, Cohen GM. Histone deacetylase inhibitors potentiate TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in lymphoid malignancies. *Cell Death Differ* 2004; **11 (Suppl 2):**S193–S206.
16 Yagi Y, Fushida S, Harada S, Kinoshita J, Makino I, Oyama K, *et al*. Effects of valproic acid on the cell cycle and apoptosis through acetylation of histone and tubulin in a scirrhous gastric cancer cell line. *J Exp Clin Cancer Res* 2010; **29**:149.
17 Elknerova K, Myslivcova D, Lacinova Z, Marinov I, Uherkova L, Stöckbauer P. Epigenetic modulation of gene expression of human leukemia cell lines – induction of cell death and senescence. *Neoplasma* 2011; **58**:35–44.
18 Kavanaugh SM, White LA, Kolesar JM. Vorinostat. A novel therapy for the treatment of cutaneous T-cell lymphoma. *Am J Health Syst Pharm* 2010; **67**:793–797.
19 Candelaria M, Herrera A, Labardini J, González-Fierro A, Trejo-Becerril C, Taja-Chayeb L, *et al*. Hydralazine and magnesium valproate as epigenetic treatment for myelodysplastic syndrome. Preliminary results of a phase-II trial. *Ann Hematol* 2010 [Epub ahead of print]
20 Ramalingam SS, Maitland ML, Frankel P, Argiris AE, Koczywas M, Gitlitz B, *et al*. Carboplatin and Paclitaxel in combination with either vorinostat or placebo for first-line therapy of advanced non-small-cell lung cancer. *J Clin Oncol* 2010; **28**:56–62.
21 Munster P, Marchion D, Bicaku E, Lacevic M, Kim J, Centeno B, *et al*. Clinical and biological effects of valproic acid as a histone deacetylase inhibitor on tumor and surrogate tissues: phase I/II trial of valproic acid and epirubicin/FEC. *Clin Cancer Res* 2009; **15**:2488–2496.
22 Gee JR, Burmeister CB, Havighurst TC, Kim K. Cyclin-mediated G1 arrest by celecoxib differs in low-versus high-grade bladder cancer. *Anticancer Res* 2009; **29**:3769–3775.
23 Garcia-España A, Salazar E, Sun TT, Wu XR, Pellicer A. Differential expression of cell cycle regulators in phenotypic variants of transgenically induced bladder tumors: implications for tumor behavior. *Cancer Res* 2005; **65**:1150–1157.
24 Weinstein IB. Disorders in cell circuitry during multistage carcinogenesis: the role of homeostasis. *Carcinogenesis* 2000; **21**:857–864.
25 Lee K, Jung ES, Choi YJ, Lee KY, Lee A. Expression of pRb, p53, p16 and cyclin D1 and their clinical implications in urothelial carcinoma. *J Korean Med Sci* 2010; **25**:1449–1455.
26 Lopez-Beltran A, Luque RJ, Alvarez-Kindelan J, Quintero A, Merlo F, Requena MJ, *et al*. Prognostic factors in survival of patients with stage Ta and T1 bladder urothelial tumors: the role of G1-S modulators (p53, p21Waf1, p27Kip1, cyclin D1, and cyclin D3), proliferation index, and clinicopathologic parameters. *Am J Clin Pathol* 2004; **122**:444–452.
27 Urakami S, Shiina H, Enokida H, Kawakami T, Tokizane T, Ogishima T, *et al*. Epigenetic inactivation of Wnt inhibitory factor-1 plays an important role in bladder cancer through aberrant canonical Wnt/beta-catenin signaling pathway. *Clin Cancer Res* 2006; **12**:383–391.
28 Yurakh AO, Ramos D, Calabuig-Fariñas S, López-Guerrero JA, Rubio J, Solsona E, *et al*. Molecular and immunohistochemical analysis of the prognostic value of cell-cycle regulators in urothelial neoplasms of the bladder. *Eur Urol* 2006; **50**:506–515.
29 Shariat SF, Ashfaq R, Sagalowsky AI, Lotan Y. Correlation of cyclin D1 and E1 expression with bladder cancer presence, invasion, progression, and metastasis. *Hum Pathol* 2006; **37**:1568–1576.
30 Abd Elazeez TA, AL El-Balshy, Khalil MM, El-Tabye MM, Abdul-Halim H. Prognostic significance of P27 (Kip 1) and MUC1 in papillary transitional cell carcinoma of the urinary bladder. *Urol Ann* 2011; **3**:8–13.
31 Shariat SF, Zlotta AR, Ashfaq R, Sagalowsky AI, Lotan Y. Cooperative effect of cell-cycle regulators expression on bladder cancer development and biologic aggressiveness. *Mod Pathol* 2007; **20**:445–459.
32 Kamai T, Takagi K, Asami H, Ito Y, Oshima H, Yoshida KI. Decreasing of p27 Kip1 and cyclin E protein levels is associated with progression from superficial into invasive bladder cancer. *Br J Cancer* 2001; **84**:1242–1251.
33 Brunner A, Verdorfer I, Prelog M, Mayerl C, Mikuz G, Tzankov A. Large-scale analysis of cell cycle regulators in urothelial bladder cancer identifies p16 and p27 as potentially useful prognostic markers. *Pathobiology* 2008; **75**:25–33.

34 Fu TY, Tu MS, Tseng HH, Wang JS. Overexpression of p27kip1 in urinary bladder urothelial carcinoma. *Int J Urol* 2007; **14**: 1084–1087.
35 Segura-Pacheco B, Avalos B, Rangel E, Velazquez D, Cabrera G. HDAC inhibitor valproic acid upregulates CAR in vitro and in vivo. *Genet Vaccines Ther* 2007; **5**:10.
36 Weichert W, Röske A, Gekeler V, Beckers T, Ebert MP, Pross M, *et al*. Association of patterns of class I histone deacetylase expression with patient prognosis in gastric cancer: a retrospective analysis. *Lancet Oncol* 2008; **9**:139–148.
37 Mariadason JM. HDACs and HDAC inhibitors in colon cancer. *Epigenetics* 2008; **3**:28–37.
38 Yong WP, Goh BC, Soo RA, Toh HC, Ethirajulu K, Wood J, *et al*. Phase I and pharmacodynamic study of an orally administered novel inhibitor of histone deacetylases, SB939, in patients with refractory solid malignancies. *Ann Oncol* 2011 [Epub ahead of print]
39 Razak AR, Hotte SJ, Siu LL, Chen EX, Hirte HW, Powers J, *et al*. Phase I clinical, pharmacokinetic and pharmacodynamic study of SB939, an oral histone deacetylase (HDAC) inhibitor, in patients with advanced solid tumours. *Br J Cancer* 2011; **104**:756–762.
40 Ramalingam SS, Maitland ML, Frankel P, Argiris AE, Koczywas M, Gitlitz B, *et al*. Carboplatin and Paclitaxel in combination with either vorinostat or placebo for first-line therapy of advanced non-small-cell lung cancer. *J Clin Oncol* 2010; **28**:56–62.