Prostate cancer has become one of the leading lethal cancers in males in many Western industrialized countries, despite progress in diagnosis and treatment. Once tumour cells have spread beyond the tissue capsule or metastasized to local lymph nodes and distal organs, therapy is limited. The genesis of cancer, malignant growth and dissemination of tumour cells are, at least in part, based on the up-regulation of histone deacetylases (HDACs), leading to distinct modifications of histone proteins and silencing of gene expression. Prostate cancer studies have demonstrated that HDAC activity is enhanced in pre-malignant and malignant lesions, with the highest increase in expression in hormone refractory cancer [1]. In vitro analysis of prostate tumour cell lines has revealed cell proliferation and differentiation to be significantly dependent on HDAC overexpression [2]. Therefore, HDAC-inhibition offers an appropriate target for inducing tumour-blocking mechanisms and, consequently, compounds which suppress HDACs may serve as potential antitumoural drugs to treat prostate cancer.

The short-chain fatty acid valproic acid (VPA) has been used for decades in the treatment of seizure disorders in children and adults with minimal side effects but emerged in 1997 as an HDAC inhibitor. The effects of valproic acid (VPA) are limited and concentrations required for exerting anti-neoplastic effects in vitro may not be reached in tumour patients. In this study, we tested in vitro and in vivo effects of two VPA-derivatives (ACS2, ACS33) on pre-clinical prostate cancer models. PC3 and DU-145 prostate tumour cell lines were treated with various concentrations of ACS2 or ACS33 to perform in vitro cell proliferation 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays and to evaluate tumour cell adhesion to endothelial cell monolayers. Analysis of acetylated histones H3 and H4 protein expression was performed by western blotting. In vivo tumour growth was conducted in subcutaneous xenograft mouse models. Tumour sections were assessed by immunohistochemistry for histone H3 acetylation and proliferation. ACS2 and ACS33 significantly up-regulated histone H3 acetylation and proliferation. ACS2 and ACS33 represent novel VPA derivatives with superior anti-tumoural activities, compared to the mother compound. This investigation lends support to the clinical testing of ACS2 or ACS33 for the treatment of prostate cancer.

Keywords: HDAC • valproic acid • VPA-analogues • prostate cancer • adhesion • proliferation
anti-neoplastic agent as well, when findings indicated that the substance inhibited proliferation and induced differentiation of primitive neuroectodermal tumour cells in vitro and in vivo [3]. The anti-cancer characteristics of VPA has meanwhile been investigated in pre-clinical models of skin, breast, colon, prostate and small cell lung cancer. Currently, the drug is in phase I trials [4, 5]. It is now recognized that VPA evoked modulations of tumour cell biology (which not only include cell growth and differentiation, but also metastatic and angiogenic potency) are mediated by HDAC-inhibition [6, 7]. VPA may, therefore, represent an anti-tumoural compound with great pharmaceutical potential. In fact, among a growing list of HDAC-inhibitors, VPA provides several advantages. It is well tolerated, exhibits low toxicity in adults and, with a half-life of 16–17 hrs, has suitable pharmacokinetic properties in vivo [8]. However, effective VPA-concentrations used in in vitro culture models are in the range of 1-5 mM; whereas, due to high-plasma protein binding of the drug, the free, active VPA-levels obtained in patients with epilepsy are in the range of 50–200 μM. Therefore, the differences between desirable and therapeutically achievable plasma concentrations of VPA may limit its application. With respect to this obstacle, evidence has recently been presented that slight changes in the molecular structure of VPA substantially enhance its efficacy, without influencing the pharmacokinetic properties of the drug [9, 10]. Based on these experiments, we speculated that VPA-derivatives may allow drug application in a lower concentration range.

Organosulphur compounds including allyl isothiocyanate have previously been shown to cause increased acetylation of histones and differentiation in mouse erythroleukaemia cells [11]. In addition, the isothiocyanate sulfuraphane has just been identified as an anti-carcinogen that acts on human colon and prostate cancer cells through HDAC-inhibition [12]. Dithiolethiones, like oltipraz (5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione) or anethole trithione (5-(4-methoxyphenyl)-1,2-dithiole-3-thione) are known to exert anti-cancer effects and angiostatic activity [13–15], and S-methyl methanethiosulfonate, isolated from cauliflower, has been demonstrated to inhibit colon tumour incidence when administered to rats during the post initiation phase of carcinogenesis [16]. The findings prompted us to prepare hybrids of VPA with different organosulphur moieties. Of these, 2-propyl-pentanoic acid 4-(3H-1,2-dithiole-3-thione-5-yl)-phenyl ester (ACS2), an ester of VPA with 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione, potently inhibited endothelial cell proliferation and angiogenic responses in muscle and HT29 tumour explants assessed by 3-dimensional collagen matrix assays [17]. To explore if VPA-hybrids may be qualified to treat prostate cancer, we analysed the influence of ACS2 and of another VPA-derivative, 2-propyl-pentanoic acid 2-methanesulfonylsulfanyl-ethyl ester (ACS33), on prostate tumour cells in vitro and in vivo.

Our data show that both compounds are active in the μM range. Growth capacity as well as adhesion of PC3 or DU-145 prostate tumour cell lines to endothelium was significantly prevented at concentrations of 30 μM (ACS2) or 7 μM (ACS33). The effects were accompanied by a distinct increase of histone H3 and H4 acetylation. The prostate tumour xenograft volume was diminished by 70% under ACS33 treatment, and enhanced histone H3 acetylation together with reduced mitosis was found in the tumour tissue. The findings suggest that VPA-derivatives may be of potential therapeutic benefit in the treatment of prostate cancer.

Materials and methods

Cell cultures

Human endothelial cells (HUVEC) were isolated from human umbilical veins and harvested by enzymatic treatment with chymotrypsin. HUVEC were grown in Medium 199 (M199; Biozol, Munich, Germany), supplemented with 10% foetal calf serum (FCS), 10% pooled human serum, 20 μg/ml endothelial cell growth factor (Boehringer, Mannheim, Germany), 0.1% heparin, 100 ng/ml gentamycin and 20 mM HEPES-buffer (pH 7.4). Subcultures from passages 2–6 were selected for experimental use.

Human prostate tumour cell lines PC3 and DU-145 were obtained from DSMZ (Braunschweig, Germany). Tumour cells were grown and subcultured in RPMI 1640 (Gibco/Invitrogen; Karlsruhe, Germany). The medium contained 10% FCS, 2% HEPES-buffer (1M, pH 7.4), 2% glutamine and 1% penicillin/streptomycin. Subcultures from passages 7–11 were selected for experimental use. Cell viability was determined by trypan blue (Gibco/Invitrogen, Karlsruhe, Germany). All cells were maintained in a 37°C incubator in a 5% CO2 humidified atmosphere.

ACS2 and ACS33

ACS2 [17] and ACS 33 [18] were supplied by Sulfidris s.r.l., Milano, Italy (Fig. 1). Both compounds were used in different concentrations and for different time periods, as indicated in results.

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). DU-145 or PC3 (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 hr. 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% CO2. 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.

Tumour cell adhesion to HUVEC

HUVEC were transferred to 6-well multiplates (Falcon Primaria; BD Biosciences, Heidelberg, Germany) in complete HUVEC-medium. When confluency was reached, PC3 or DU-145 cells were detached from the culture flasks by accutase treatment (PAA Laboratories, Cölbe, Germany) in a 37°C incubator. Subsequently, non-adherent tumour cells were washed off using warmed PBS and cell suspension was introduced into bovine type I collagen matrix (65 μl; National Biological Service, Heidelberg, Germany). 20 μl of tumour cell suspension (1 × 10^5 cells/ml; 5 × 10^5 cells/ml) was added on top of the collagen matrix and allowed to polymerize for 40 min. The plates were then washed multiple times (2–3 times) with warm PBS, and tumour cell adhesion was assessed by a modified MTT assay.
In each experimental setting, adherent tumour cells were counted in five different fields of a defined size (5 × 0.25 mm²) using a phase contrast microscope and the mean cellular adhesion rate was calculated.

**Western blotting**

Acetylated histones H3 and H4 in PC3, DU-145 tumour cells, or in frozen tissue specimens generated from nude mice xenografts, were evaluated by western blot analysis. 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 for histone H3 (rabbit monoclonal IgG, clone Y28, dilution 1:500), histone H4 (rabbit polyclonal IgG, dilution 1:250) or acetylated H4 (Lys8, rabbit polyclonal IgG, dilution 1:100), anti-focal adhesion kinase (Fak, clone 77, dilution 1:1000), anti-phospho-specific Fak (pY397, clone 18, dilution 1:1000), anti-integrin-linked kinase (ILK; clone 3, dilution 1:1000), anti-phospho-H3 (rabbit monoclonal IgG, clone Y173, dilution 1:5000), acetylated H3 and H4 in PC3, DU-145 tumour cells, or in frozen tissue specimens generated from nude mice xenografts, were evaluated by immunohistochemistry.

**Statistical analysis**

All studies 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**

**ACS2 and ACS33 down-regulate in vitro cell growth**

Cell proliferation of DU-145 and PC3 cells was quantified 24 hrs and 48 hrs after plating. To clearly interpret and compare the growth characteristics of treated versus untreated tumour cells, 24 hrs counts were all set at 100%. Based on this, the number of untreated DU-145 cells as control increased in the ACS2 study from 100% (2799 ± 186 cells/well) to 160% (Fig. 2A, left) or 155% (Fig. 2A, right), and in the ACS33 study from 100% (2679 ± 302 cells/well) to 172% (Fig. 2B, left) or 166% (Fig. 2B, right).

PC3 control cells increased in the ACS2 study from 100% (2553 ± 161 cells/well) to 178% (Fig. 2A, left) or 169% (Fig. 2A, right). According to earlier observations, simultaneous addition of ACS2 or ACS33 into the multi-well plates did not influence the growth characteristics of DU-145 and PC3 cell lines. However, a pre-incubation for 3 days with ACS2 (prior to plating the cells in multi-well plates for 24 or 48 hrs) at concentrations ≥30 μM significantly blocked the proliferation of both DU-145 and PC3 cells (Fig. 2A). The anti-proliferative effects of ACS2 were even more pronounced when tumour cells were pre-treated with the compound for 5 days and then added into multi-well plates for another 24 or 48 hrs. Already, 15 μM (DU-145) or 7 μM (PC3) were then sufficient to reduce the growth capacity significantly, compared to the controls.
Fig. 2 Effects of ACS2 (2A) or ACS33 (2B) on prostate cancer proliferation in vitro. DU-145 or PC3 cells were treated with various concentrations of ACS2 or ACS33 for 3 or 5 days, or remained un-treated (control). Cell proliferation was then assessed using the MTT dye reduction assay. Cell numbers at day 2 (48 hrs) were normalized to the number of day 1 (24 hrs, as 100%). One representative of six experiments is shown. * indicates significant difference to controls.
Stronger anti-proliferative effects were noted with respect to ACS33. 15 μM (DU-145) or 7 μM (PC3), applied for 3 days, were sufficient to completely block tumour cell growth (Fig. 2B). In contrast to ACS2, extension of the pre-incubation period to 5 days did not further enhance the anti-tumoural potential of ACS33.

In all experiments, the drugs were maintained before transferring the tumour cells into multi-well plates and during the 24 and 48 hrs cell growth evaluation period.

**ACS2 and ACS33 block tumour cell adhesion to endothelium**

The number of adherent DU-145 control cells rapidly increased and reached a plateau after 2 hrs (1 hr control values were as follows: ACS2 study: 66.4 ± 6.0 cells/mm² in the 3 day incubation protocol; 98.4 ± 8.8 cells/mm² in the 5 day incubation protocol. ACS33 study: 42.2 ± 7.6 cells/mm² in the 3 day incubation protocol; 86.1 ± 14.9 cells/mm² in the 5 day incubation protocol). PC3 control cell adhesion steadily increased up to 4 hrs (1 hr control values were as follows: ACS2 study: 69.2 ± 8.0 cells/mm² in the 3 day incubation protocol; 80.8 ± 11.8 cells/mm² in the 5 day incubation protocol. ACS33 study: 68.0 ± 12.6 cells/mm² in the 3 day incubation protocol; 101.2 ± 19.7 cells/mm² in the 5 day incubation protocol), and significantly more PC3 cells attached to HUVEC than DU-145 cells (Fig. 3A and B). Similar to the growth blocking effects, a 3 day ACS2 pre-incubation at concentrations ≥ 30 μM significantly decreased (but did not completely prevent) adhesion of both DU-145 and PC3 cells to HUVEC. A 5 day pre-incubation further enhanced the adhesion-blocking effects of ACS2, resulting in very flat adherence curves (Fig. 3A).

ACS33 exerted stronger effects on both tumour cell lines than did ACS2. This was demonstrated by ACS33 concentrations <30 μM being sufficient to significantly decrease PC3 or DU-145 attachment to HUVEC (Fig. 3B). Interestingly, pre-incubating the cells for 5 days with ACS33 did not lead to a more pronounced reduction of tumour cell adhesion and flat adherence curves seen with ACS2. Notably, the effect of ACS33 on PC3 cells was even slightly diminished when the drug was applied after 5 days, compared to the 3 day incubation period.

Figure 4 demonstrates the influence of ACS33 on the endothelial integrity. In all co-culture experiments carried out with DU-145 or PC3 round openings in the endothelial monolayer in the course of tumour cell binding, due to focal disruptions of intercellular endothelial connections, were observed. Treatment of DU-145 or PC3 cells with ACS33 not only prevented tumour cell adhesion, as described above, but also prevented disruption of the HUVEC monolayer. Similar effects were also seen with respect to ACS2 (data not shown).

**ACS2 and ACS33 increase histone H3 and H4 acetylation**

DU-145 or PC3 cells were treated with ACS2 or ACS33 for 24 hrs and then histone acetylation was assessed by western blot. Both cell lines showed distinct increases in acetylated H3 and H4 under drug treatment (Fig. 5). Modifications of acetylated H4 became evident at concentrations ≥ 15 μM independent with both ACS2 or ACS33 application. H3 acetylation strongly increased in presence of 30 μM ACS2 or ACS33, however, slight effects on H3 acetylation were also seen when ACS2 was applied at 15 μM.

**ACS33 treatment inhibits progression of tumour xenografts**

Based on our in vitro data, ACS33 exerted higher adhesion and growth-blocking properties than did ACS2. We, therefore, established tumour xenografts in athymic nu/nu mice using PC3 cells to evaluate the effects of ACS33 on prostate cancer cell growth in vivo. Animals in the treatment arm received ACS33 at a dose of 20 mg/kg body weight daily. Compared to the untreated animals, application of ACS33 significantly diminished the tumour volume, with reductions of 75% (day 17, day 24) and 60% (day 20), compared to the control (Fig. 6). No body weight loss or diarrhea was observed and all animals (treated as well as non-treated) kept alive. Western blot analysis revealed increased histone H3 and H4 acetylation in tissue specimens of treated animals (Fig. 6, right). Furthermore, bcl-2 was down-regulated whereas bax was enhanced, compared to the controls. No differences were seen with respect to integrin α1 adhesion receptors and ILK expression. However, Fak and phosphorylated Fak became strongly reduced in tissue specimens of animals treated with ACS33 (Fig. 6, right). Immunohistochemistry with antibodies against H3 acetylation demonstrated intense nuclear staining in tumour cells derived from ACS33-treated animals, whereas the immunoreactivity for the proliferation marker MIB was diminished remarkably (Fig. 7).

**Discussion**

Several studies have pointed to the important role the HDAC-inhibitor, VPA, may play in treating prostate cancer. VPA not only down-regulates prostate-specific antigen to basal levels in LNCaP cells, but also mediates the expression of genes relevant to tumour proliferation and apoptosis [19, 20]. Angiogenesis and senescence in prostate tumour cells have also been shown to be influenced by VPA [21]. The bone-invasive potential of prostate cancer in mice is impaired by VPA [22] and appears particularly effective in controlling refractory stages of this disease [23].

The advantages of VPA include low cost, favourable safety profile and oral dosing. However, the plasma trough concentrations obtained in patients by common anti-epileptic dose regimens are below the concentration required for anti-tumour effects in vitro. It is still not clear if adaptation of the dosing schedule to the anticancer protocol can be carried out with acceptable toxicity [24]. Indeed, dose-limiting toxicities as neurological impairment and febrile neutropenia have been observed in phase I studies [4, 5].
Fig. 3 Adhesion of prostate cancer cells to human endothelial cells (HUVEC) is down-regulated by ACS2 (3A) or ACS33 (3B). DU-145 or PC3 cells were treated with various concentrations of ACS2 or ACS33 for 3 or 5 days, and then added at a density of $5 \times 10^6$ 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. One representative of six experiments is shown. * Indicates significant difference to controls.
VPA has also been associated with reversible hepatotoxicity and, in few cases, with lethal hepatopathies [25].

Circumvention of the dosage problem can be achieved by structurally modifying VPA. Some of these derivatives exceed the HDAC-inhibition potential 40-fold, compared to the mother compound [9].

In this investigation, we report the development of a novel class of HDAC inhibitors, in which VPA was tethered to 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione, or to 2-hydroxyethyl methanesulfonate, both sulfurated moieties through an ester linkage. This structural optimization was based on the hypothesis that inserting an organosulfur group can improve HDAC inhibition. It has previously been shown that several organosulfur compounds inhibit cell proliferation and HDAC, possibly by epigenetically priming cells to respond more effectively to external insults such as oxidative stress or toxins [12].

In fact, treatment of DU-145 or PC3 prostate cancer cells with 15 μM ACS2 or ACS33 caused hyperacetylation of histone H4, and administration of 30 μM ACS2 or ACS33 was sufficient to induce elevation of histone H3 acetylation. Furthermore, micromolar concentrations significantly prevented tumour cell growth in vitro and stopped tumour cell adhesion to HUVEC. With respect to this feature, ACS2 administered for 5 days was more potent than a 3 day application, whereas ACS33, given for 5 days, was not superior to the 3 day incubation period. It is not clear if these differences are caused by a different passage time of the drugs.
Fig. 6 Effect of ACS33 on prostate cancer xenografts. PC3 xenografts were established in male athymic mice. Animals in the treatment arm received 20 mg/kg bw ACS33 each day, or 2 mg cisplatin/kg/day (cis-pt). *Indicates significant difference to the control animals. Western blot analysis of H3 and H4 histone expression (total and acetylated), of bcl-2, bax, ILK and Fak (total and activated) 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. One representative western blot data of three are shown.

Fig. 7 Histological examination of the PC3 tumour xenografts growing s.c. in male athymic mice. Representative pictures are shown. Immunostaining of MIB (dark red reaction product) documents a decreased tumour cell proliferation. Enhanced immunostaining of acetylated H3 indicates HDAC-inhibitory activity of ACS33.
through the cellular membrane or by a different cellular response towards both compounds. It has been demonstrated that long-term application of VPA is necessary to delay tumour cell growth and block metastatic processes [26, 27]. Xia et al. supposed that chronic administration of VPA is required to achieve therapeutic benefits [28]. This may also be the case for VPA-analogues. Significant tumour reduction was not seen until 1 week after starting chronic ACS33 application in the PC3 xenograft model.

Concerning the animal data, 20 mg/kg ACS33 induced distinct antitumoural activity in the xenograft model. This is 10-fold lower than the optimum concentration of VPA required to exert similar effects. Shabbeer et al. treated male nu/nu mice with 200 mg/kg bw VPA daily to evoke significant growth blockade of xenografted prostate tumour cells [21]. The same dosing schedule was recommended by Xia and coworkers [28] and Gao et al. [29] to diminish prostate cancer xenograft growth by 40–70%. We did not repeat the same experiments; however, 200 mg/kg bw VPA was necessary to significantly reduce the in vivo growth of kidney carcinoma cells whereas 20 mg/kg bw VPA was without any effect (data not shown).

No body weight loss or overt toxic effects in the treated animals, which could be related to the drug treatment, were observed. Therefore, ACS33 seemed well tolerated and safe. Still, this conclusion is made with caution since side effects in humans may develop during protracted treatment. It is notable that our in vivo data are based on the hormone-independent prostate tumour cell line PC3, since it is the androgen-independent prostate cancer that ultimately leads to patient death. Consequently, ACS2 and ACS33 may become particularly important for patients with recurrent and progressive disease.

The present investigation demonstrates that potent HDAC-inhibitors can be derived from the VPA mother compound. Studies which deal with structure-activity relationships of VPA are limited. Based on a mouse model, it has been found that the α-carbon atom of VPA must be tetrahedral (sp3 hybridization) and connected to a free carboxyl function to express significant anti-tumoural potential [9, 30]. Two alkyl groups, branched on the carbon atom C-2 are also obligatory to evoke anti-tumoural effects [31]. However, structural modifications such as derivatization of the carboxylic function, side chain elongation and further branching, introduction of double and triple bonds and enantiomeric derivatives with the chiral center at position C-2 allowed distinct modifications of the pharmacokinetic profile of VPA. In particular, side chain elongation of VPA derivatives with a triple bond in position C-4 resulted in decreasing IC50-HDAC values, while further branching of one side chain diminished the HDAC inhibition effect [9].

Lu and colleagues rationalized that the weak potency of VPA may be, in part, attributable to its inability to access the Zn2+-chelation in the HDAC active-site pocket, which plays a pivotal role in histone deacetylation catalysis. Tethering VPA with Zn2+-chelation via an aromatic linker led to the development of novel compounds that showed inhibition of HDAC activity and cancer cell proliferation in the micromolar range. The finding that removing the valproyl group completely abrogates the inhibitory activity of the conjugate underscores the importance of the acyl moiety in interacting with the active-site pocket [32].

This is the first report that demonstrates potential anti-tumoural effects of VPA-derivatives in a prostate cancer model. In vivo, western blot analysis and immunohistochemistry revealed an association between ACS33-induced growth inhibition and intra-tumour histone H3 acetylation. These pharmacodynamic data suggest that ACS33, as administered, reached the molecular targets that are, however, still not pinpointed. Aside from the direct biological effects on tumour cells, an indirect mode of action should also be considered. We have previously reported that the mother compound, VPA, modulates angiogenesis and tumour immunity [8, 33]. An antiangiogenetic activity has already been found for ACS2 [17].

From a clinical viewpoint, molecular-targeted therapies including both HDAC and angiogenesis inhibition provide promise. Further studies are necessary to understand the molecular background of post-transcriptional modifications and to design specific VPA derivatives to exploit the potential of this class of agents in the treatment of prostate cancer.

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