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Increased human cytomegalovirus replication in fibroblasts after treatment with therapeutical plasma concentrations of valproic acid

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Abstract

Valproic acid (2-propylpentanoic acid, VPA), an effective inhibitor of histone deacetylases (HDAC) is used for the treatment of epilepsia. In this study, structure–activity relationships for the action of structurally modified VPA derivatives on human cytomegalovirus (HCMV) replication and HDAC inhibition were defined. Pretreatment of human foreskin fibroblasts with VPA (0.125–1 mM) caused a concentration-dependent increase of HCMV immediate early and antigen late antigen expression. Structure–activity relationships of VPA derivatives for HCMV stimulation were compared to those for teratogenic action and those for HDAC inhibition. Side chain elongation and introduction of a triple bond in 4-position of the other chain caused teratogenicity, stimulated HCMV replication, and increased HDAC inhibition, as demonstrated by enhanced levels of acetylated histones. Teratogenic VPA derivatives with a branched chain in 3-position as well as a non-teratogenic anticonvulsive active VPA derivative did not stimulate HCMV or accumulation of acetylated histones. This demonstrates a strict correlation between inhibition of HDAC and increased HCMV replication.

Keywords: Valproic acid; Human cytomegalovirus; Histone deacetylases; Teratogenicity; Structure–activity relationship

1. Introduction

Valproic acid (VPA), one of the most widely prescribed antiepileptic drugs [1], is increasingly used and investigated for a number of different pathological conditions. This includes bipolar disorders, migraine, different other forms of headache, solid cancers, and leukaemia [2,3].

The substance was already associated with stimulation of replication of different viruses including HIV, human herpesvirus 6, and human herpesvirus 8 [4–7]. Treatment of human fibroblasts (MRC-5 cells) with VPA in hyper-therapeutic concentrations being clearly higher than 1 mM caused increased expression of human cytomegalovirus (HCMV) immediate early antigen (IEA) and early antigen by activation of the immediate early promoter [8]. These results are in concordance with findings showing the HCMV major immediate early promoter (MIEP) to be under the control of histone deacetylases (HDAC) in HCMV non-permissive cells [9] and VPA to inhibit HDAC [10,11].

One major side effect of VPA is its well documented teratogenicity [12]. Although structure–activity relationships for VPA derivatives and anti-cancer activity were much less intensive investigated compared to that for teratogenicity both actions were hypothesised to follow the same rules. Inhibition of HDAC was hypothesised to be one possible mechanism underlying antitumoural effects of VPA since investigation of different VPA derivatives showed that only derivatives that inhibit HDAC were antitumourally active [10]. In contrast to this, anticonvulsive action of VPA derivatives follows different structure–activity relationships, as shown by synthesis of antiepileptic VPA derivatives without teratogenic side effects [12,13].

In this study, we investigated the effects of VPA and structurally modified derivatives on the expression of

Abbreviations: FCS, foetal calf serum; HCMV, human cytomegalovirus; HDAC, histone deacetylases; HFF, human foreskin fibroblasts; IEA, immediate early antigen; LA, late antigen; MIEP, major immediate early promoter; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SDS, sodium dodecyl sulfate; VPA, valproic acid

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HCMV IEA and late antigen (LA) in human foreskin fibroblasts (HFF). We compared structure–activity relationships for HCMV-stimulation, HDAC inhibition, and teratogenic effects caused by VPA derivatives.

2. Materials and methods

2.1. Materials

All cell culture supplements were purchased from Seromed. VPA was obtained from Sigma. The VPA derivatives that were synthesised as described before [12,14] are shown in Fig. 1.

2.2. Cells

Human foreskin fibroblasts (HFF) were cultured as described previously [15].

2.3. Viruses

Strain Hi91 was isolated from the urine of an AIDS patient with HCMV retinitis [16]. The HCMV laboratory strains Towne and AD169 were obtained from American Type Culture Collection. Virus stocks were prepared in HFF grown in MEM with 4% foetal calf serum (FCS). The titres were determined by plaque titration as described previously [15].

2.4. Virus infectivity assay

Confluent cultures of HFF were incubated with HCMV at a multiplicity of infection of 0.01. After incubation for 1 h, which was required for virus adsorption, cells were washed with PBS and incubated in maintenance medium containing 4% FCS. As described in detail previously [15], cells producing HCMV specific antigens were detected 24 and 72 h postinfection by immunoperoxidase staining using monoclonal antibodies directed against 72 kDa IEA and 67 kDa LA (DuPont), respectively. For control purposes an irrelevant antibody directed against HSV glycoprotein B was used.

The amount of infectious virus was determined by virus yield assay in a single-cycle assay format as described before [17]. Briefly, HFF were infected HCMV Hi91 at a multiplicity of infection of 0.01. After 1 h incubation period cells were washed with PBS. Three days after infection, immediate early forming units (IEFU) were determined as described before [17].

2.5. Cytotoxicity assay

Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay [18]. HFF were seeded onto 96-well microtitre plates, grown to confluency, and incu-

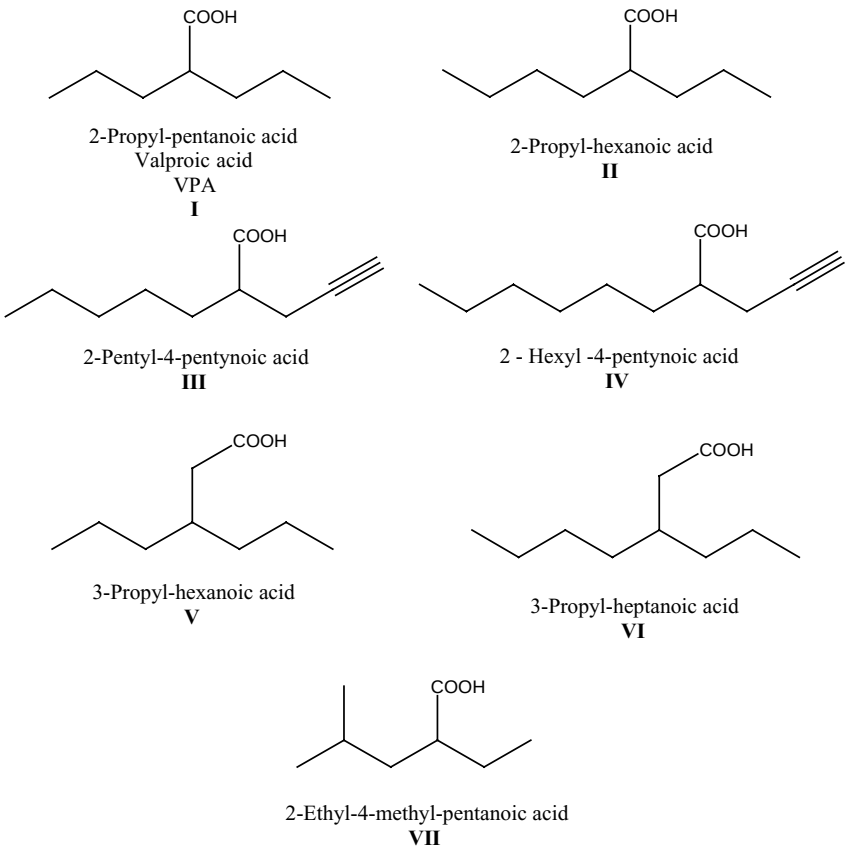

Fig. 1. Chemical structures of investigated valproic acid derivatives.

bated with culture medium containing serial dilutions of VPA and VPA derivatives. Incubation time was analogous to the pretreatment time used for virus experiments. After incubation MTT (1 mg/ml) was added and after an additional 4 h cells were lysed in a buffer containing 20% (w/v) sodium dodecyl sulfate (SDS) and 50% *N*,*N*-dimethylformamide adjusted to pH 4.5. Absorbance at 570 nm was determined for each well using a 96-well multiscanner. After subtracting background absorbance, results are expressed as cell number compared to control cells that were maintained in the presence of solvent.

2.6. Immunoblotting

Immunoblotting was performed as described before [19]. Cells were lysed in SDS-sample buffer and separated by SDS–PAGE. Proteins were detected using specific antibodies against β-actin (Sigma) or acetylated histone H4 (Upstate Biotechnology), and were visualised by enhanced chemiluminescence using a commercially available kit (Amersham).

2.7. Teratogenicity assay

The experiments used to induce and evaluate exencephaly and embryotoxicity were previously described in detail [20]. In short: female NMRI mice weighing 28–36 g were mated and compounds dissolved in water and adjusted to pH 7.4 injected intraperitoneally on day eight of gestation. On day 18 of gestation the animals were sacrificed, the uteri removed and the number of implants counted. Each foetus was weighed and inspected for external malformations.

2.8. Statistics

Values presented are the mean ± S.D. of at least three experiments. Comparisons between two groups were performed using Student's *t*-test, three and more groups were compared by ANOVA followed by the Student–Newman–Keuls test. *P* values lower than 0.05 were considered to be significant.

3. Results

3.1. Valproic acid increases HCMV strain Hi91 IEA and LA expression in a concentration- and time-dependent manner

VPA pretreatment for 24 h increased HCMV strain Hi IEA expression in a concentration-dependent manner in HFF (Fig. 2A). Addition of VPA after HCMV infection did not affect HCMV IEA expression. The highest tested VPA concentration was 1 mM which is within the range of therapeutically achievable patients' plasma levels [21].

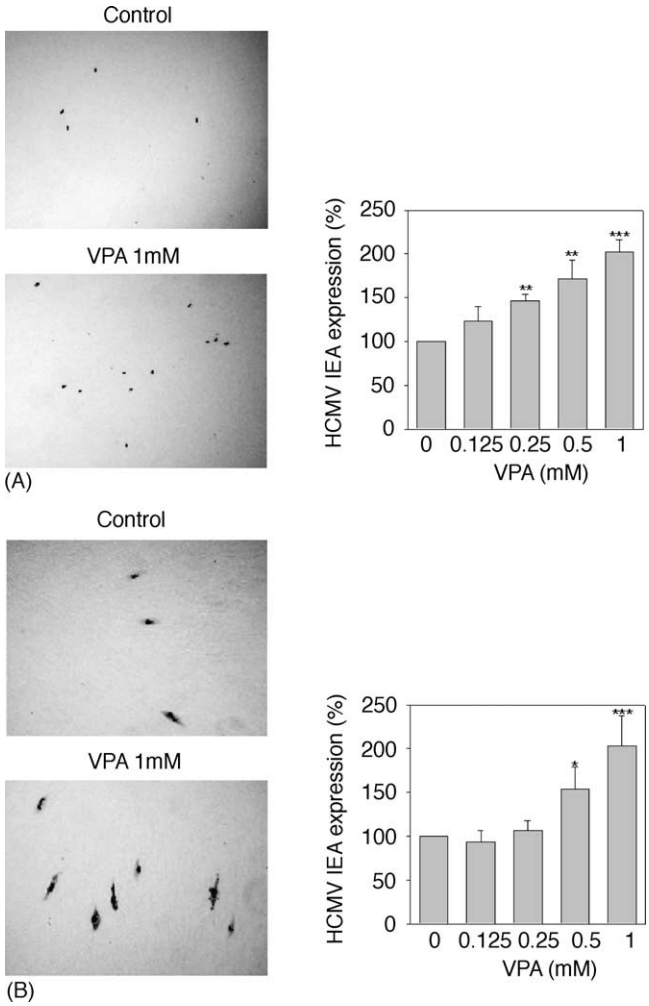

Fig. 2. Effect of valproic acid on HCMV strain Hi91 replication in human foreskin fibroblasts. (A) Human foreskin fibroblasts (HFF) were pretreated with and without valproic acid (VPA) 1 mM for 24 h prior to infection. HFF were stained for 72 kDa immediate early antigen (IEA) 24 h after infection. Histogram shows number of cell expressing IEA after pretreatment for 24 h with different VPA concentrations compared to control (mean ± S.D. of three independent experiments). (B) HFF were pretreated with and without VPA 1 mM for 24 h prior to infection. HFF were stained for 67 kDa late antigen (LA) 72 h after infection. Histogram shows number of cell expressing LA after pretreatment for 24 h with different VPA concentrations compared to control (mean ± S.D. of three independent experiments). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P \leq 0.001$.

This concentration caused a two-fold increase in IEA expression. Comparable results were obtained for HCMV LA expression. This demonstrates that HCMV replication cycle continues after IEA expression (Fig. 2B).

To observe the influence of pretreatment time, HFF were incubated with VPA 1 mM for 1, 24 h, and 3 days (Fig. 3). It was shown, that maximum effect was achieved after 3 days of pretreatment, resulting in a more than three-fold increase in HCMV IEA expression. One hour incubation did not result in a change of IEA expression compared to control.

VPA did not affect HFF cell viability in concentrations up to 1 mM.

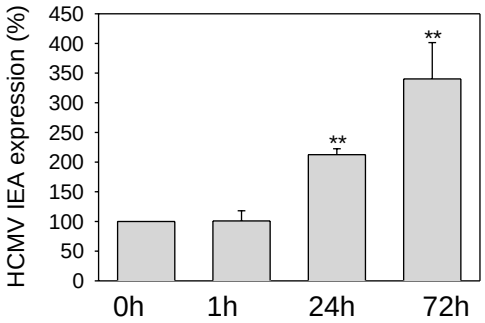

Fig. 3. Time-dependence of effects of valproic acid on HCMV strain Hi91 infection. Histogram showing influence of valproic acid pretreatment period on HCMV immediate early antigen expression. Human foreskin fibroblasts were preincubated with valproic acid for 1, 24, or 72 h prior to HCMV infection. Twenty-four hours after HCMV infection HFF were stained for 72 kDa immediate early antigen (mean ± S.D. of three independent experiments). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P \leq 0.001$.

3.2. Valproic acid increases IEA expression of HCMV strains Towne and AD169

To investigate if VPA pretreatment specifically stimulates HCMV strain Hi91, influence of VPA pretreatment for 24 h on replication of HCMV strain Towne and AD169 was examined. VPA 1 mM increased IEA expression of HCMV strains Towne (Fig. 4A) and AD169 (Fig. 4B)

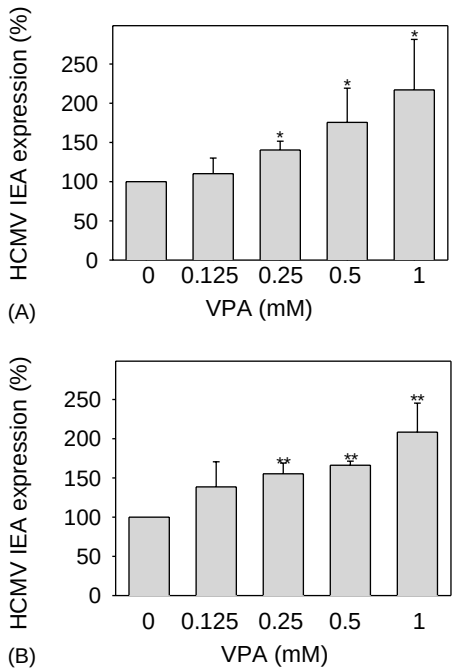

Fig. 4. Effects of valproic acid on HCMV strain Towne and AD169 immediate early antigen expression. Histograms showing influence of valproic acid pretreatment period on HCMV immediate early antigen expression. Human foreskin fibroblasts were pretreated without or with valproic acid in different concentrations prior to infection. Twenty-four hours after infection with HCMV strain Towne (A) or AD169 (B) cells were stained for expression of 72 kDa HCMV immediate early antigen (mean ± S.D. of three independent experiments). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P \leq 0.001$.

IEA expression in a comparable manner like that of strain Hi91.

3.3. Teratogenic effects of valproic acid derivatives

The structures of the VPA derivatives that were investigated within this study are presented in Fig. 1. The results from teratogenicity testing are shown in Table 1. A number of different VPA (**I**) derivatives was examined for their teratogenic effects in mice. As shown before, side chain elongation leads to enhanced teratogenic effects of saturated and unsaturated VPA derivatives compared to VPA itself [14,22,23]. To study the effect of side chain elongation of saturated compounds 2-propyl-hexanoic acid **II** was chosen. Moreover, introduction of a triple bond in 4-position of the second side chain further enhanced teratogenic effects. From this group 2-pentyl-4-pentynoic acid **III** and 2-hexyl-4-pentynoic acid **IV** were investigated as model compounds. Our study demonstrates that VPA derivatives with side chain in 3-position instead of 2-position (3-propyl-hexanoic acid **V**, 3-propyl-heptanoic acid **VI**) also cause teratogenic effects. 2-Ethyl-4-methyl-pentanoic acid **VI** was investigated as a non-teratogenic, anticonvulsive active VPA derivative.

3.4. Influence of valproic acid derivatives on HCMV IEA and LA expression

Compound **VII** (2-ethyl-4-methyl-pentanoic acid) as well as VPA derivatives with side chains in 3-position (3-propyl-hexanoic acid **V**, 3-propyl-heptanoic acid **VI**) did not influence HCMV IEA or LA expression compared to virus control or cell viability compared to cell controls in concentrations up to 1 mM. 2-Pentyl-4-pentynoic acid **III** and 2-hexyl-4-pentynoic acid **IV** slightly decreased cell viability in concentrations of 0.5 mM and higher (Fig. 5). Therefore, a concentration of 0.25 mM was chosen being a

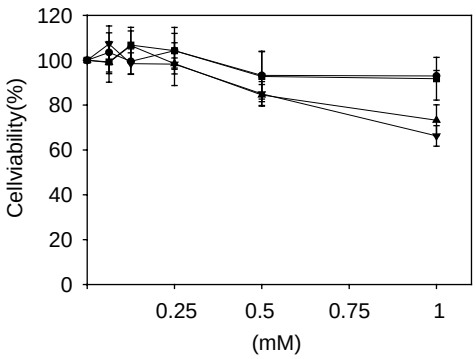

Fig. 5. Influence of valproic acid derivatives on cell viability. Confluent human foreskin fibroblasts were incubated with valproic acid derivatives in concentrations ranging from 0.0625 to 1 mM for 5 days. Cell viability (determined using MTT assay) is shown for valproic acid (●), 2-propylhexanoic acid (■), 2-pentyl-4-pentynoic acid (▲), and 2-hexyl-4-pentynoic acid (▼). 2-Ethyl-4-methylpentanoic acid, 3-propylhexanoic acid, and 3-propylheptanoic acid did not influence cell viability of human foreskin fibroblasts (not shown).

Table 1
Influence of valproic acid derivates on embryo development

Substance	Dose (mg/kg)	Dose (mmol/kg)	Number of dams (*n*)	Live fetuses (*n*)	Fetal weight (g)	Embryo-lethality (%)	Exencephaly (%)
Valproic acid (**I**)	430	3.00	8	63	1.09 ± 0.11	32	37
2-Propyl-hexanoic acid (**II**)	470	3.00	6	65	1.02 ± 0.17	29	52
2-Pentyl-4-pentynoic acid (**III**)	210	1.25	6	44	1.02 ± 0.09	40	60
2-Hexyl-4-pentynoic acid (**IV**)	230	1.25	7	29	0.96 ± 0.06	67	79
3-Propyl-hexanoic acid (**V**)	320	2.00	6	52	0.97 ± 0.13	20	44
3-Propyl-heptanoic acid (**VI**)	220	1.25	8	54	1.04 ± 0.14	49	26
2-Ethyl-4-methyl-pentanoic acid (**VII**)	430	3.00	4	46	1.23 ± 0.07	4	0
Controls (NaCl)	–	10	6	126	−1.14 ± 0.05	6	0

non-toxic dose for comparison of effects of VPA and its derivatives on HCMV IEA and LA expression. The use of 2-propyl-hexanoic acid **II** resulted in similar IEA and LA expression compared to VPA, whereas the use of 2-pentyl-4-pentynoic acid **III** and 2-hexyl-4-pentynoic acid **IV** led to a clear increase in HCMV IEA and LA expression (Fig. 6). Statistical significant differences were detected between VPA and 2-hexyl-4-pentynoic acid **IV** treatment (IEA: $P = 0.001$; LA: $P = 0.033$) as well as between

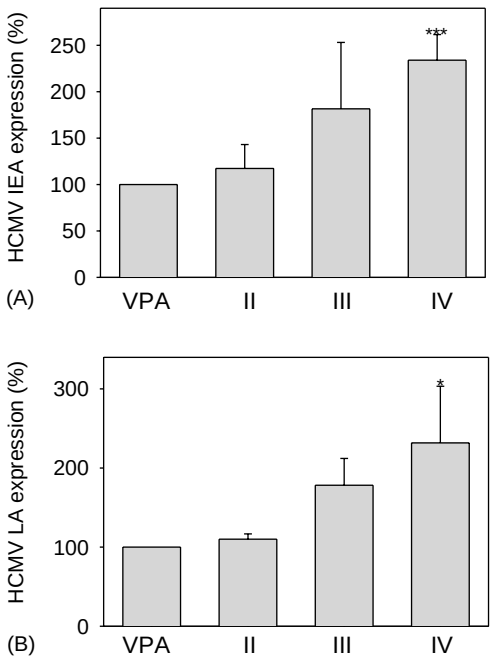

Fig. 6. Influence of valproic acid derivatives on HCMV immediate early and late antigen expression. (A) Human foreskin fibroblasts (HFF) were pretreated with and without valproic acid (VPA) derivatives 0.25 mM for 24 h prior to infection. HFF were stained for 72 kDa immediate early antigen (IEA) 24 h after infection. Histogram shows number of cell expressing IEA after pretreatment for 24 h with VPA derivatives compared to control (mean ± S.D. of three independent experiments). (B) HFF were pretreated with and without VPA derivatives 0.25 mM for 24 h prior to infection. HFF were stained for 67 kDa late antigen (LA) 72 h after infection. Histogram shows number of cell expressing LA after pretreatment for 24 h with VPA derivatives compared to control (mean ± S.D. of three independent experiments) (II, 2-propyl-hexanoic acid; III, 2-pentyl-4-pentynoic acid; IV, 2-hexyl-4-pentynoic acid). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P \leq 0.001$.

2-propyl-hexanoic acid and 2-hexyl-4-pentynoic **IV** acid treatment (IEA: $P = 0.006$; LA $= 0.043$). This shows that structure–activity relationships for HCMV stimulation follow at least in part different rules than those defined for teratogenic action as demonstrated by the teratogenic VPA derivatives 3-propyl-hexanoic acid **V** and 3-propyl-heptanoic acid **VI** that do not stimulate HCMV.

3.5. Influence of valproic acid derivatives on production of infectious virus

To determine the influence of VPA on production of infectious virus, virus yield assay was performed in HCMV Hi91-infected HFF. Twenty-four hours preincubation with VPA 1 mM resulted in titres of $(4.1 \pm 0.24) \times 10^3$ IEFU/ml compared to $(1.6 \pm 0.19) \times 10^3$ IEFU/ml in untreated cells. Twenty-four hours pretreatment of HFF with VPA 0.25 mM or 2-pentyl-4-pentynoic acid 0.25 mM resulted in titres of $(2.6 \pm 0.22) \times 10^3$ IEFU/ml and $(4.7 \pm 0.41) \times 10^3$ IEFU/ml, respectively. This indicates that increased IEA and LA expression of HCMV-infected HFF is followed by increased production of infectious virus and that derivatives with higher potency further increase infectious virus production.

3.6. Influence of valproic acid derivatives on histone acetylation in HFF

VPA is under investigation as anti-cancer drug [2]. Its antitumoural activity was associated with its potential to inhibit HDAC [10,11,24]. Therefore, the influence of VPA on accumulation of acetylated histone H4 in HFF was investigated to examine the influence of VPA on HDAC. Concentration-dependent accumulation of acetylated histones indicated concentration-dependent inhibition of HDAC by VPA in HFF (Fig. 7).

To investigate the influence of different VPA derivatives on HDAC, acetylated histone H4 was observed after 24 h treatment of HFF. Results clearly show a correlation between increase of HCMV IEA or LA expression and accumulation of acetylated histone H4 caused by VPA derivatives (Fig. 7). The derivatives that did not increase HCMV antigen expression (2-ethyl-4-methyl-pentanoic

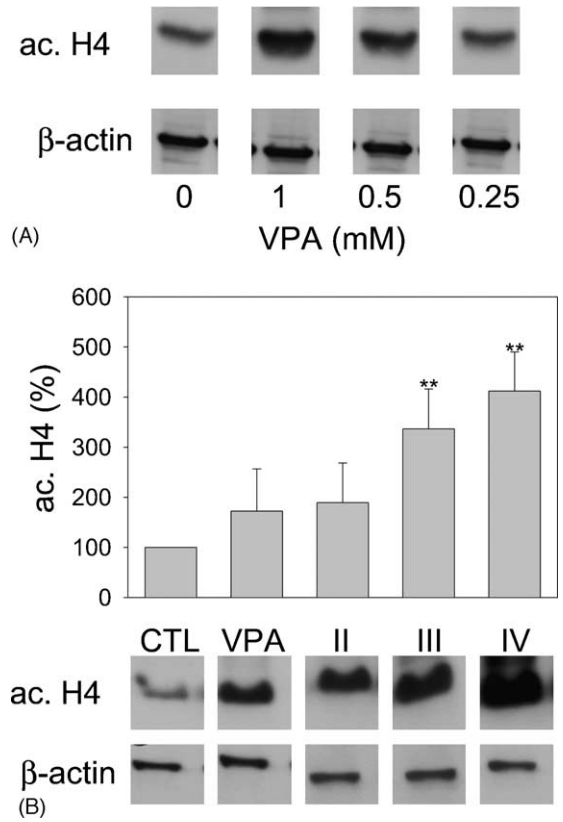

Fig. 7. Influence of valproic acid and derivatives on histone H4 acetylation in human foreskin fibroblasts. (A) Human foreskin fibroblasts were incubated with different valproic acid (VPA) concentrations for 24 h. Three independent experiments were performed with similar results. The representative Western blot shows concentration-dependent effect on histone H4 acetylation from one experiment. (B) Human foreskin fibroblasts were incubated with different VPA derivatives 0.25 mM for 24 h. The densitometric analysis (mean ± S.D. of three independent experiments) and the representative Western blot from one experiment show effects of derivatives on histone H4 acetylation (II, 2-propyl-hexanoic acid; III, 2-pentyl-4-pentynoic acid; IV, 2-hexyl-4-pentynoic acid). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P \leq 0.001$.

acid, 3-propyl-hexanoic acid, 3-propyl-heptanoic acid) did not affect histone acetylation.

4. Discussion

Our data show that pretreatment with VPA in concentrations up to 1 mM increases HCMV infection as indicated by determination of HCMV IEA expression in HFF in vitro. VPA treatment after HCMV infection was without effect. The used VPA concentrations are within the range of plasma levels of VPA-treated patients [21]. Similar results were obtained with HCMV strain Hi91, a patient's isolate, and HCMV laboratory strains Towne and AD169, indicating that the observed effects are not limited to a specific HCMV strain. These results are in contrast to a previous report showing that VPA increases HCMV IEA expression only in hyper-therapeutic concentrations in human fibroblasts (MRC-5 cells) [8]. A reason for this differences might be, that influence of VPA on HCMV replication depends on the cell lines used and even differs between different fibroblasts. The idea of cell-specific differences of VPA action is further supported by differences in the time-dependence of VPA effects on HCMV IEA expression. Whereas the report of Kuntz-Simon and Obert [8] showed that pretreatment with VPA for 24 h caused the maximal effect, we found maximal effect after 72 h pretreatment with VPA 1 mM.

AIDS progression may be associated with HCMV reactivation and HCMV-retinitis is a common complication of AIDS patients [25–27]. Moreover, HCMV infection causes frequently a number of pathologies as well as transplant rejection in transplant recipients [28,29]. Therefore, it is important to investigate whether VPA may influence HCMV-replication in patients.

In order to receive more information concerning the mechanism of HCMV stimulation by VPA, we investigated the influence of structurally modified VPA derivatives on HCMV replication. From teratogenicity studies it was shown that elongation of one side chain as well as introduction of a triple bond in 4-position lead to an increased teratogenic potential [12,14]. We used 2-propyl-hexanoic acid **II** as example for a saturated chain elongation whereas 2-pentyl-4-pentynoic acid **III** and 2-hexyl-4-pentynoic **IV** acid are examples for derivatives with a triple bond in 4-position. Moreover, 3-propyl-hexanoic **V** acid and 3-propyl-heptanoic acid **VI** were found to be teratogenic within this study. 2-Ethyl-4-methyl-pentanoic acid **VII** was the model compound for non-teratogenic but still anticonvulsive active derivatives [12,14]. 2-Ethyl-4-methyl-pentanoic acid, 3-propyl-hexanoic acid, and 3-propyl-heptanoic acid did not affect HCMV replication. The other derivatives increased HCMV replication. Whereas 2-propylhexanoic acid **II** had a similar influence compared to VPA, introduction of a triple bond strongly increased HCMV IEA and LA expression. This shows that the HCMV stimulation by VPA derivatives differs in part from the structure-activity relationships detected for teratogenic effects and hypothesised for anti-cancer activity [10]. However, it remains to be determined if teratogenicity caused by VPA derivatives with a branched chain in 3-position shares the same molecular targets than those involved in teratogenicity caused by VPA derivatives with a branched chain in 2-position.

VPA was already demonstrated to be an inhibitor of HDAC [10,11] and to induce proteasomal degradation of HDAC2 [30]. Moreover, HDAC3 and possibly additional HDAC were shown to control HCMV major immediate early promoter (MIEP) [9]. However, not all effects caused by short chain fatty acids appear to be associated with HDAC inhibition [31]. Therefore, we investigated the influence of VPA and its derivatives on acetylation of histone H4. We found a strict correlation between HCMV IEA expression and accumulation of acetylated histones after treatment with the different VPA derivatives. This

suggests inhibition of HDAC to contribute to increased HCMV IEA. This result is in concordance with results showing the HDAC inhibitor trichostatin A to activate the HCMV MIEP [9]. Moreover, a strict correlation between production of HCMV LA and accumulation of acetylated histones was found indicating that viral replication cycle continues. This finding is in accordance with a recent report showing that mouse cytomegalovirus replication is increased by treatment with the HDAC inhibitor trichostatin A in mouse embryo fibroblasts [32]. The available data concerning influence of VPA on HCMV replication as well as reports showing influence of VPA on replication of different other viruses [4–7] suggest to investigate influence of VPA on replication of a greater collective of viruses. Moreover, it is known that numerous viral proteins from different viruses depend on cellular acetylation or deacetylation and that different viruses control acetylation-dependent gene-expression through the modulation of histone acetyltransferases and HDAC [33]. Mouse cytomegalovirus immediate early 1 protein was recently shown to decrease deacetylation activity of HDAC2 [32]. Consequently, HDAC inhibitors like VPA might interact with replication of different viruses in various ways [33].

In conclusion, we show in this report that VPA increases HCMV replication in HFF in concentrations that are achieved in plasma of VPA treated patients. The increased HCMV replication appears to be associated with inhibition of HDAC. VPA should be used with care for patients for whom increased HCMV replication might represent a risk factor such as immuno-compromised.

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