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Histone deacetylases inhibition and tumor cells cytotoxicity by CNS-active VPA constitutional isomers and derivatives

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Abstract

The tumor cells toxicity of the antiepileptic drug valproic acid (VPA) has been associated with the inhibition of histone deacetylases (HDACs). We have assessed, in comparison to VPA, the HDACs inhibition and tumor cells cytotoxicities of CNS-active VPA's constitutional isomers, valnoctic acid (VCA), propylisopropylacetic acid (PIA), diisopropylacetic acid (DIA), VPA's cyclopropyl analogue 2,2,3,3-tetramethylcyclopropanecarboxylic acid (TMCA) and VPA's metabolites, 2-ene-VPA and 4-ene-VPA, all possessing, as does VPA, eight carbon atoms in their structures. The aim was to define structural components of the VPA molecule that are involved in HDACs inhibition and tumor cells cytotoxicity.

HDACs inhibition by the above-mentioned compounds was estimated using an acetylated lysine substrate and HeLa nuclear extract as a HDACs source. SW620 cells were used for assessing HDACs inhibition in vivo. The cytotoxicity of these compounds was assessed in SW620 and 1106mel cells.

HDAC inhibition potency was the highest for VPA and 4-ene-VPA (IC_{50} = 1.5 mM each). 2-Ene-VPA inhibited HDACs with IC_{50} = 2.8 mM. IC_{50} values of the other tested compounds for HDACs inhibition were higher than 5 mM, 4-ene-VPA and VPA induced histone hyperacetylation in SW620 cells. 4-Ene-VPA and VPA at 2 mM each were also most potent in reducing cell viability, to 59 ± 2.0% and 67.3 ± 5.4%, respectively, compared to control. VCA, PIA, DIA, TMCA, 2-ene-VPA and valpromide (VPD) did not reduce viability to less than 80%. All tested compounds did not significantly affect the cell cycle of SW620 cells.

In conclusion, in comparison to the VPA derivatives and constitutional isomers tested in this study, VPA had the optimal chemical structure in terms of HDACs inhibition and tumor cells cytotoxicity.
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Keywords: Valproic acid; Valproic acid constitutional isomers; Anticancer drugs; Histone deacetylase; Antiepileptic drugs

1. Introduction

Valproic acid (VPA), Fig. 1 is an eight-carbon, branched side chain carboxylic acid with a broad spectrum of antiepileptic activities, which is used also for the treatment of bipolar disorder, neuropathic pain and migraine prophylaxis [1,2]. The anti-proliferative properties of VPA were first demonstrated in 1985 [3]. VPA inhibited at anticonvulsant therapeutic concentrations the mitotic index of murine neuroblastoma and glioma cells. Prolongation of the cell cycle has been attributed to its arrest in the G1 phase [3–5]. Continued exposure to VPA-induced differentiation in various cell lines [6,7] as well as in transformed hematopoietic progenitor cells [8] and leukemic blasts [5]. VPA induced apoptosis in breast carcinoma cells [8], in leukemia cells from patients with acute myeloid leukemia [5] and in vivo, in neuroblastoma xenografts in athymic mice, and this effect may contribute to VPA's effect on proliferation rate [9–11]. Furthermore, administration of

Abbreviations: AED, antiepileptic drug; BuA, butyric acid; HDAC, histone deacetylase; DIA, diisopropylacetic acid; PI, propidium iodide; PIA, propylisopropylacetic acid; TMCA, 2,2,3,3-tetramethylcyclopropanecarboxylic acid; TSA, trichostatin A; VCA, valnoctic acid; VPA, valproic acid; VPD, valpromide

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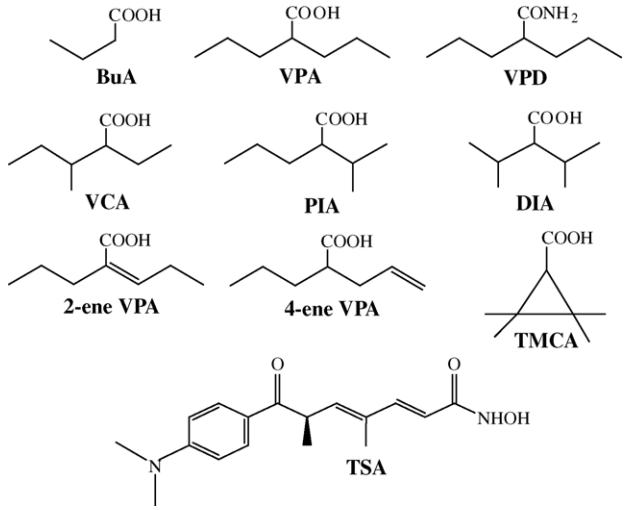

Fig. 1. Chemical structures of the tested compounds. Abbreviations: VPA, valproic acid; VPD, valpromide; VCA, valnoctic acid; PIA, propylisopropylacetic acid; DIA, diisopropylacetic acid; TMCA, 2,2,3,3-tetramethylcyclopropanecarboxylic acid; BuA, butyric acid; TSA, trichostatin A.

VPA to rodents reduced tumor growth and metastasis [8,12]. Based on these results, VPA is currently being used in clinical trials for the treatment of gliomas in children [10,11,13].

More recently it has became evident that similar cellular targets, such as activation of peroxisome proliferator-activated receptor δ (PPARδ) and modulation of the Wnt signaling pathway, may be involved in both VPA's antitumor activity and in its teratogenicity [6,8,14,15]. Both the antitumor and the teratogenic effects of VPA as well as its interaction with some of the aforementioned molecular targets have also been associated with its activity as histone deacetylases (HDACs) inhibitor [8,15,16]. HDACs inhibitors with diverse chemical structures, such as butyric acid (BuA) and trichostatin A (TSA) (Fig. 1), induce histone hyperacetylation and directly alter the transcription of a subset of genes, with resulting antiproliferative, apoptotic and differentiating effects [8,13,17–20]. However, the use of some of these inhibitors is limited by their toxicities or poor pharmacokinetics [17–20]. Compared to other HDACs inhibitors, VPA is generally a well-tolerated drug that is administered orally and has a relatively long half-life [8,17].

Characterization of HDACs inhibition profile and related induction of differentiation of transformed cells by a series of VPA analogues have provided further evidence for the involvement of HDACs inhibition in the antitumor action of VPA [8,15,21]. However, the contribution of specific elements in the structure of VPA molecule for HDACs inhibition has only been partially investigated.

Based on different structural requirements for antiepileptic activity, hepatotoxicity and teratogenicity for VPA and its analogues, second-generation VPA derivatives and analogues have been developed with improved antiepileptic activity and/or reduced teratogenicity and hepatotoxicity [22]. These second-generation drugs include constitutional isomers of VPA, such as propylisopropylacetic acid (PIA) and valnoctic acid (VCA), which exert antiepileptic potencies similar to those of VPA in animal models, but are non-teratogenic [23,24]. In addition, numerous cyclpropyl analogues of VPA have been synthesized which possessed similar or better antiepileptic activities in rodents, compared to VPA [25,26].

The aim of the current work was to assess HDACs inhibition and tumor cell toxicity by CNS-active analogues and constitutional isomers of VPA and two VPA metabolites, 2-ene-VPA and 4-ene-VPA. All tested compounds, including VPA, possess eight carbon atoms in their chemical structures, which enable us to define specific structural components in the aliphatic moiety of the VPA molecule responsible for HDACs inhibition and tumor cell toxicities. We demonstrate here that structural isomers of VPA are distinct from VPA in terms of their potencies as HDACs inhibitors.

2. Materials and methods

2.1. Drugs

VPA was a gift from Teva Pharmaceutical Industries, Petach Tikva, Israel. 2-ene-VPA and 4-ene-VPA were gifts from the Department of Pharmaceutics, University of Washington, Seattle. 2,2,3,3-Tetramethylcyclopropanecarboxylic acid (TMCA), sodium butyrate and TSA were purchased from Sigma–Aldrich, Rehovot, Israel.

VPA's constitutional isomers were synthesized according to the synthetic procedures previously described in the following references: PIA, [24]; DIA, [27]; VCA, [28]. Solvents and drugs were purchased from Sigma–Aldrich.

Sodium butyrate, which served as a positive control, was dissolved in either the incubation buffer or cell culture media. VPA, its analogues and derivatives and TSA were dissolved in DMSO, (Sigma–Aldrich) and diluted in buffer or medium up to a final concentration of 1% DMSO, except for HDACs inhibition assay with TMCA, where the highest final DMSO concentration was 10%.

2.2. Cells

SW620 and 1106mel cells were cultured in RPMI 1640 (Biological Industries Ltd., Beit Haemek, Israel) and supplemented with 10% fetal calf serum (Biological Industries Ltd.) and 1% penicillin/streptomycin (Biological Industries Ltd.). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2/95% O_2.

Cells were incubated with 300 nM TSA or the indicated concentrations of other tested compounds in culture medium for the indicated periods. Controls were prepared by incubating cells for the same periods with the medium containing DMSO at the corresponding dilutions. pH

values were adjusted to those of media containing VPA (as the free acid).

2.3. HDACs enzymatic assay

HDACs activity was measured using the HDACs fluorescent activity assay/drug discovery kit (Biomol Research Laboratories, PA, USA), as previously described [29]. In short, HeLa nuclear extracts (0.5 μL of 6–9 mg protein/ml) were incubated in a 96-well microplate with 25 μM acetylated substrate in 50 μL of assay buffer containing or lacking the examined inhibitor at 25 °C for 15 min. The reaction was then stopped by the addition of 50 μL developer containing 2 μM (final concentration 1 μM) TSA. The plate was incubated for an additional 15 min at room temperature. The reaction final product was detected on a fluorimetric reader (Fluostar Galaxy, BMG Labtechnologies GmbH, Germany, excitation at 390 nm and emission at 460 nm). The amount of deacetylated product was calculated relative to a calibration curve using a deacetylated substrate supplied by the kit producer. The assays were repeated at least three times, in duplicates.

2.4. Preparation of nuclear extracts

Treated and control cells were washed with PBS and harvested by scraping; then they were centrifuged at 500 rpm for 10 min twice and resuspended in ice-cold lysis buffer, containing 10 mM HEPES, (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 M HCl, 0.5 mM dithiothreitol and 1.5 mM phenylmethylsulfonyl fluoride, according to instructions of the manufacturer of the antibodies against acetylated histones. Samples were incubated on ice for 30 min. The lysates were centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was dialyzed twice against 0.1 M acetic acid and twice against H_2O, for 1 h each.

2.5. Immunoblotting

The concentration of each protein sample was determined by the Lowry method [30]. Samples were adjusted to equal protein concentrations, mixed with SDS sample buffer and separated by 10–16.5% tricine SDS–polyacrylamide gel electrophoresis. Gels were either stained with Coomassie blue and imaged with BioDocit (Upland, CA, USA) imaging system or immunoblotted with antibodies against acetyl-histone H4, acetyl-histone H3 (1:750 and 1:7500, respectively, Upstate Biotechnology Inc, NY, USA) or β-actin (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, California) at 4 °C overnight. The blots were then incubated with peroxidase-conjugated goat anti-rabbit or rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories Inc., PA, USA, at 1:40,000 and 1:10,000, for anti acetyl histones and anti β-actin antibodies, respectively) for 1.5 h and developed by enhanced chemiluminesence (Santa Cruz). Assays were repeated at least twice.

2.6. Viability assay

Cell viability was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. SW620 or 1106mel cells were incubated with the test compounds for 3 days, followed by medium removal and incubation with MTT (0.5 mg/ml in medium, Sigma–Aldrich) for 2 h at 37 °C. The newly formed formazan precipitate was then extracted from cells with 200 μl DMSO. Absorbance of the resulting solution was measured in a Power Wave microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) at 540 nm. MTT reduction associated with 100% cell viability was determined from cultures subjected to medium exchanges only. Results are expressed as percentage of cell number compared with control cells.

2.7. Cell cycle analysis

Cell cycle progression of SW620 cells following treatments was analyzed by flow cytometry. After drug exposure for 2 days, cells were washed with PBS, centrifuged at 3000 rpm × 3 min and fixed in 100% methanol. The tubes were kept at 4 °C overnight. Cells were then washed with PBS and resuspended in 0.5 ml PBS containing 100 μg/ml RNase A (Sigma–Aldrich). Following incubation for 15 min, propidium iodide (PI, Sigma–Aldrich) was added to cells to a final concentration of 10 μg/ml. Subsequent analysis using a Becton Dickinson FACScan flow cytometer yielded a histogram of DNA content per cell that allowed determination of the fraction of cells in the G1, S and G2 phases of the cell cycle.

2.8. Statistical analysis

Data are expressed as mean ± S.E.M. IC_{50} values for HDACs inhibition by VPA and related compounds were calculated as the concentration of drug resulting in a 50% reduction in enzyme activity compared to control in the absence of an inhibitor. HDACs inhibition, tumor cell toxicity and effects on cell cycle of VPA-related compounds were compared to control (corresponding concentrations of DMSO) using the nonparametric Kruskal–Wallis test followed by Dunn's multiple comparison tests. Statistical analysis was performed using the computer program Graph Pad Prism 3.01 (GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. HDACs inhibition by VPA-related compounds

VPA and some related compounds have recently been demonstrated to inhibit HDACs activity [8,15,16,21]. To

test whether VPA structural isomers and metabolites also inhibit HDACs activity, a fluorescent substrate was deacetylated using HeLa cell nuclear extract as a source of HDACs enzymatic activity at the presence of tested compounds and the results compared to control.

Dose–response curves for HDACs inhibition by the test compounds and their IC_{50} values are shown in Fig. 2A and Table 1, respectively. For the clarity of data, error bars and stars indicating statisticals significance are not indicate Fig. 2A. TSA, which served as a positive control, was used in a single concentration (300 nM), which almost completely abolished HDACs activity (Fig. 2A). BuA, another well-established HDACs inhibitor, significantly inhibited

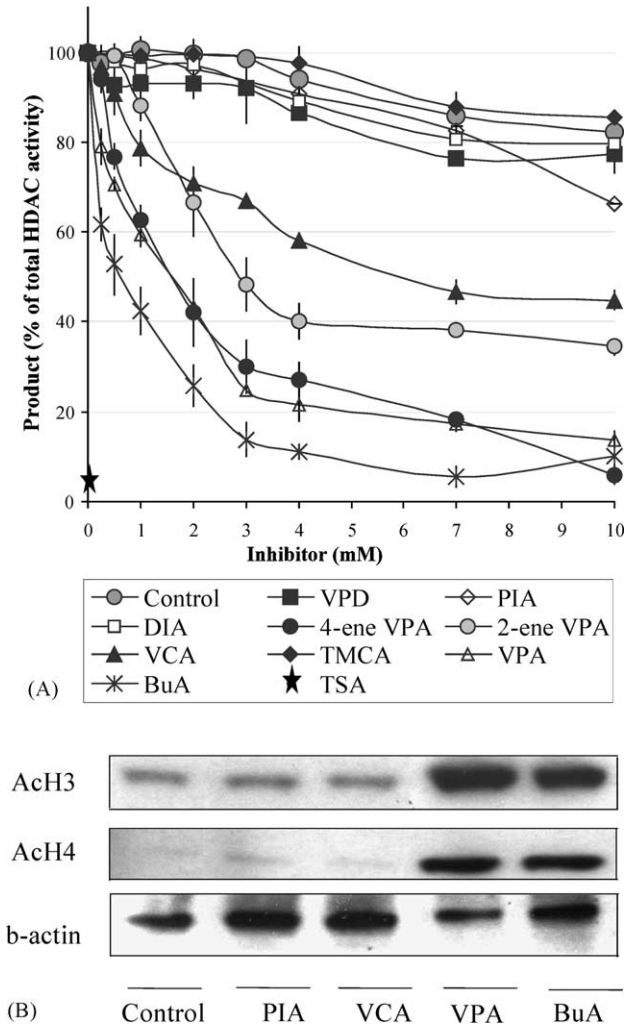

Fig. 2. Inhibition of HDACs by the tested compounds listed in Fig. 1. (A) Inhibition of HDACs from HeLa nuclear extracts. HDAC activity at the presence of the tested compounds is represented as a percentage of the activity in the absence of inhibitors. DMSO, up to 10%, served as the control. Each data point represents the mean from at least three independent experiments. (B) Accumulation of acetylated histones in cultured cells. SW620 cells were treated with 1 mM of each of the tested compounds for 24 h. The cell lysates were immunoblotted for acetylated histones H3 (AcH3) and H4 (AcH4) and β-actin (loading control). Proteins were isolated and immunoblotted with antibodies specific for acetylated histones H3 (AcH3) and H4 (AcH4). Anti β-actin antibody was used for loading control.

Table 1
IC_{50} values for HDACs inhibition by VPA structural isomers and metabolites

Compound tested	IC_{50} (mM)
BuA	0.6
VPA	1.5
4-ene-VPA	1.5
2-ene-VPA	2.8
VCA	5.8
VPD	>10
PIA	>10
DIA	>10
TMCA	>10

In vitro HDACs activity of the tested compounds was assayed as described in Fig. 2. BuA was used as a positive control. Abbreviations: BuA, butyric acid; VPA, valproic acid; VCA, valnoctic acid; VPD, valpromide; PIA, propylisopropylacetic acid; DIA, diisopropylacetic acid; TMCA, 2,2,3,3-tetramethylcyclopropanecarboxylic acid.

HDACs, compared to control, at 1 mM and higher concentrations and had an IC_{50} value of 0.6 mM. Among the tested compounds, VPA and 4-ene-VPA were the most potent HDACs inhibitors and significantly inhibited HDACs (IC_{50} = 1.5 mM), compared to control. 2-ene-VPA had an IC_{50} of 2.8 mM. Among the three constitutional isomers of VPA, VCA was the most potent (IC_{50} = 5.8 mM). VPD had minimal HDACs inhibitory activity, while TMCA did not inhibit HDACs (IC_{50} > 10 mM). Since most of the tested compounds possess low water solubility, they were solubilized in DMSO and diluted in assay buffer up to a final concentration of 1% DMSO (10% for TMCA). 10% DMSO, used for control, reduced fluorescence emission, compared to fluorescence in the absence of this solvent in the incubation mixture. No effect on fluorescence was demonstrated at 1% DMSO (Fig. 2A).

A representative experiment demonstrating the in vivo effects of VPA's constitutional isomers PIA and VCA on HDACs in SW620 cells is shown in Fig. 2B. In contrast to VPA and BuA, which served as the comparative references, and 4-ene-VPA (data not shown), none of VPA's constitutional isomers, as well as 2-ene VPA, induced histone hyperacetylation in SW620 cells.

3.2. Effects of VPA-related compounds on tumor cell viability and cell cycle

The effects on cell viability of VPA and its structural isomers and metabolites, as well as BuA and TSA, were determined by a 3-day incubation of SW620 and 1106mel cells with these test compounds (Fig. 3). The most effective compound in this assay was 4-ene-VPA, at 2 mM reducing SW620 cell viability to 59.0 ± 2.0% of cells treated with the vehicle only ($P < 0.05$, Fig. 3). At the same VPA concentration, cell viability was reduced to 67.3 ± 5.4%. All other VPA isomers and derivatives did not reduce cell viability to less than 80%. BuA, which was used as a positive control, had the highest efficacy among

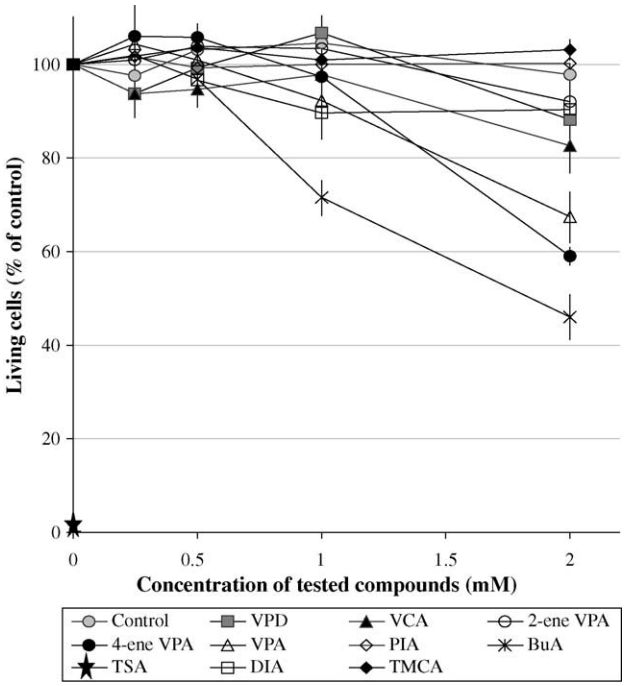

Fig. 3. Effect on cell viability of the tested compounds listed in Fig. 1. SW620 cells were incubated with 0.25–2 mM of each of the analyzed compounds (except TSA, that was tested at 300 nM) for 72 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell viability. BuA and TSA were used as positive controls. Cells used for control were treated with corresponding concentrations of DMSO. Results are expressed as mean ± S.E.M. of percentage viability, compared to untreated cells. Data are representative of two separate experiments.

the carboxylic acids that were tested (46.0 ± 4.8% viability at 2 mM, $P < 0.01$). TSA (300 nM) was toxic to cells, with less than 1% of them surviving following incubation with this compound. The sensitivity of 1106mel cells to VPA-related compounds was similar to that of the SW620 cells.

Table 2
Effects of VPA structural isomers and metabolites on cell cycle

Treatment	%G1	%S	%G2+M
Control	57.2 ± 0.3	15.4 ± 0.6	22.4 ± 1.1
VPA	60.6 ± 1.0	11.8 ± 1.3	20.8 ± 0.8
VPD	54.3 ± 1.8	14.3 ± 1.4	25.4 ± 1.2
VCA	54.2 ± 1.5	15.8 ± 0.5	24.3 ± 0.7
PIA	55.4 ± 1.6	14.8 ± 0.3	23.2 ± 1.7
DIA	53.1 ± 1.3	16.0 ± 0.4	24.8 ± 0.6
TMCA	57.3 ± 0.5	14.1 ± 0.7	20.7 ± 0.9
2-Ene-VPA	56.3 ± 1.6	14.1 ± 1.1	24.0 ± 1.5
4-Ene-VPA	62.1 ± 1.7	13.8 ± 0.6	17.8 ± 2.6
BuA	58.2 ± 2.0	12.7 ± 1.3	21.1 ± 0.9
TSA	62.5 ± 1.4	11.8 ± 0.8	19.1 ± 0.6

SW620 cells were incubated for 48 h in the presence of 2 mM of the tested compounds and then analyzed for cell cycle status using propidium iodide staining. Cells used for control were treated with 1% DMSO. BuA (2 mM) and TSA (50 nM) were used as positive controls. Abbreviations: VPA, valproic acid; VPD, valpromide; VCA, valnoctic acid; PIA, propylisopropylacetic acid; DIA, diisopropylacetic acid; TMCA, 2,2,3,3-tetramethylcyclopropanecarboxylic acid; BuA, butyric acid, TSA, trichostatin A.

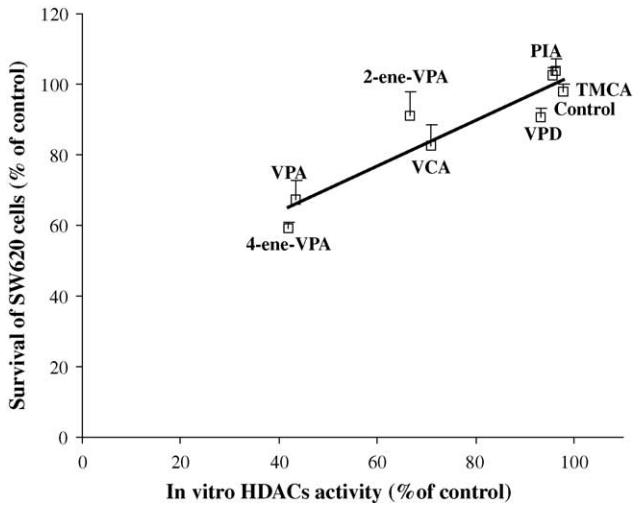

Fig. 4. Correlation between HDACs inhibition by the compounds listed in Table 1 and survival of SW620 cells at 2 mM of the tested compounds. Data are represented as mean ± S.E.M. ($r = 0.936$, $y = 0.65x + 37.9$, $P < 0.01$). VPA, valproic acid; VPD, valpromide; VCA, valnoctic acid; DIA, diisopropylacetic acid; PIA, propylisopropylacetic acid; TMCA, 2,2,3,3-tetramethylcyclopropanecarboxylic acid.

We also determined the effects of the test compounds on cell cycle kinetics of SW620 cells (Table 2). Compared to control cells, none of these compounds significantly affected cell cycle behaviour. TSA was assessed at 50 nM, as higher concentrations were toxic to cells (Fig. 3).

3.3. Correlation between antiproliferative effects of VPA structural isomers and derivatives and their HDACs inhibition potency

We subsequently tried to determine to what extent the cytotoxic effects of VPA-related compounds are associated with HDACs inhibition. We found a good correlation between HDACs inhibition by 2 mM of these compounds and their effects on the viability of SW620 cells (Fig. 4, $r = 0.94$, $y = 0.65x + 37.9$, $P < 0.01$). A significant correlation between HDACs inhibition and tumor cell toxicities by these compounds was demonstrated also at 1 mM ($r = 0.77$, $y = 0.20x + 82.6$, $P < 0.05$). This correlation could not be established for concentrations lower than 1 mM concentrations.

4. Discussion

We examined HDACs inhibition and tumor cell cytotoxicity by a series of CNS-active structural isomers and derivatives of VPA on SW620 and 1106mel cells and compared their effects to those of VPA. All test compounds, like VPA, possess eight carbons in their chemical structures. We have demonstrated that structural modifications, as shown in Fig. 1, of the alkyl side chains in the VPA molecule, reduce the potency of these compounds as

HDACs inhibitors as well as their in vitro tumor cell cytotoxicity.

VPA was used as the positive reference in the cellular assays, as it is currently an established HDACs inhibitor undergoing clinical trials in patients with cancer and hematologic disorders [17,18]. In our study VPA inhibited nuclear HDACs activity with IC50 = 1.5 mM (Table 1). These values are comparable to those obtained by Phiel et al. [15], who demonstrated that 50% inhibition of HDACs activity by VPA in HeLa nuclear extracts is obtained between 0.5 and 2 mM. VPA's IC_{50} for HDACs inhibition in HeLa nuclear extracts are higher than VPA therapeutic plasma concentration range associated with its antiepileptic activity (0.3 ± 0.7 mM) [31]. However, HeLa nuclear extracts contain several isoforms of HDAC [21,32–35] and at least some of them are inhibited by therapeutic concentrations of VPA [8,21]. VPA has low potency compared to typical HDACs inhibitors, such as TSA and suberoylanilide hydroxamic acid (SAHA), which have optimal structures with regard to HDACs binding and inhibition [17,18]. VPD was used in our study as a comparative reference for compounds with low potencies in HDACs inhibition and cytotoxicity [8,15,21].

Inhibition potencies for HDACs were explored recently for a non-branched chain carboxylic acid (4-pentenoic acid) and 2-methyl-pentenoic acid, 2-ethylhexanoic acid and 2-methyl-2*n*-propylpentanoic acid, representing branched chain carboxylic acids. In nearly all assays, VPA had the highest potencies compared to these compounds [21]. Our results further indicate that structural modification in the aliphatic moiety of VPA structure, as represented by the constitutional VPA isomers PIA, DIA, VCA and its analogue, TMCA, might impair the compound's ability to place the carboxylic function into the metal binding domain of HDACs active-site pocket [36,37]. The introduction of a double bond into the structure of the VPA molecule can also affect HDACs inhibitory activity. The position of the double bond in these derivatives of VPA is also important for HDACs inhibitory activity. This is reflected by the two VPA metabolites possessing a double bond in their structures. 2-Ene-VPA was less potent than VPA in inhibiting HDACs in vitro, however, 4-ene-VPA was as potent as VPA (Fig. 2A, Table 1). Hence, the results obtained in this study provide additional evidence that there are specific structural requirements for a VPA analogue to become a potent HDACs inhibitor [8,15,21].

Recently, specific HDACs inhibition potencies, induction of p21 expression (in K562 cells) and associated differentiation (in U937 and K562 cells), and activation of mitogen-activated protein kinase (MAPK) by a series of short-side chains carboxylic acids were reported [21]. This study further supports the suggested role of HDACs in the observed antitumor effects of VPA. Our results also indicate that the tumor cells cytotoxicities of VPA analogues and derivatives (Fig. 1) might be at least partially mediated by HDACs inhibition. We found a correlation between the HDACs inhibition potencies of VPAs isomers and analogues at 2 mM and their effects on cell viability (Fig. 4). The correlation was also found at 1 mM of the tested compounds. However, a significant correlation could not be established for lower than 1 mM concentrations of the tested compounds, because their cytotoxicities for SW620 cells were not different from control at those concentrations. While high concentrations of tested compounds were used in HDACs inhibition assays in order to establish their IC_{50} values (Fig. 2, Table 1), their in vivo effects on histone acetylation and their cytotoxic activities for SW620 cells were assessed in concentrations comparable to those which are used in antiepileptic therapy with VPA [31].

The exact pathway for the tumor cell toxicity of VPA has not yet been identified, but a few targets have been suggested, including activation of peroxisome proliferator-activated receptor δ (PPAR-δ), deregulation of β-catenin, and the induction of the cell cycle inhibitor p21 [8,15,21,38]. Some of these mechanisms are also shared by other HDACs inhibitors, like TSA, which increases β-catenin in SW620 cells [39]. These effects may be associated with a G1 phase arrest induced by VPA in certain cell lines, which may result in prolongation of the cell cycle and decreased cellular proliferation, as well as VPA-induced apoptosis [10,21,40]. The sensitivity of SW620 colon carcinoma cells to VPA-induced cell cycle arrest was limited, resulting in a slight, statistically non-significant tendency towards cell accumulation in the G1 phase, induced by VPA and 4-ene-VPA, as well as the positive controls, BuA and TSA (Table 2).

We analyzed two unsaturated metabolites of VPA and demonstrated that the teratogenic 4-ene metabolite of VPA [41,42] is a more potent HDACs inhibitor than the non-teratogenic metabolite, 2-ene VPA. Since these metabolites are always present during therapeutic use of VPA, evaluation of their tumor cell toxicity is required.

It has been shown that structural modifications of the alkyl chains of VPA to form its constitutional isomers, usually retain its anticonvulsant activity but significantly reduce teratogenicity in mice embryos [24,28,43–45]. The non-teratogenic derivatives, VPD and 2-methyl-2-propylpentanoic acid, were also less potent than VPA as HDACs inhibitors [15]. In addition, only the stereoisomer 4-yn VPA with (S)-configuration of directly inhibits HDACs and induces the accumulation of acetylated histones in F9 cells [8].

The structure activity relationship described here indicates the important limitations on pharmacological activity imposed by the side chains of VPA molecule. In agreement with previously published results [21], the main conclusion from our study is that VPA and not its constitutional isomers has the optimal chemical structure, in term of HDACs inhibition and tumor cells cytotoxicity.

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