S-2-PENTYL-4-PENTYNIC ACID AND ITS METABOLITE S-2-PENTYL-4-PENTYNIC ACID IN THE NMRI-EXENCEPHALY-MOUSE MODEL: PHARMACOKINETIC PROFILES, TERATOGENIC EFFECTS, AND HISTONE DEACETYLASE INHIBITION ABILITIES OF FURTHER VALPROIC ACID HYDROXAMATES AND AMIDES

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ABSTRACT:

Structure-activity relationship studies of valproic acid (VPA) derivatives have revealed a quantitative correlation between histone deacetylase (HDAC) inhibition and induction of neural tube defects (NTDs) in the NMRI-exencephaly-mouse model, but this correlation has been, so far, limited to congeners with a carboxylic acid function. Whereas the classical HDAC inhibitor trichostatin A is active only as a hydroxamate but not as a carboxylic acid, we found that neither VPA amides nor hydroxamates inhibit HDACs, but can cause NTDs; e.g., 2-pentyl-4-pentynoic hydroxamic acid with its S-enantiomer being the potent teratogen. We therefore investigated the hypothesis that hydroxamic acid derivatives of VPA might be metabolized in vivo and may possibly be pro-teratogenic, as had been shown for valpromide but not valproic hydroxamic acid. We developed two stereoselective quantification methods based on chiral derivatization of VPA hydroxamates with (1R,2S,5R)-(-)-menthylchloroformate and carboxylic acid derivatives with (S)-(−)-1-naphthylethylamine, followed by gas chromatography-nitrogen phosphor detector analysis of biological samples. We then determined the pharmacokinetic profiles of S-2-pentyl-4-pentynoic hydroxamic acid and of S-2-pentyl-4-pentynoic acid in mice. S-2-Pentyl-4-pentynoic hydroxamic acid was found to be extensively metabolized to the corresponding carboxylic acid without affecting the stereochemistry at position C2. Furthermore, the metabolite S-2-pentyl-4-pentynoic acid was found to be very stable in vivo, with an extended half-life of 4.2 h compared with that of VPA, 1.4 h. Comparison of the individual HDAC inhibition abilities of additional VPA amides and hydroxamates, as measured by cellular and enzymatic assays, led us to the conclusion that both classes of VPA derivatives can be pro-teratogenic.

Valproic acid (VPA) has been shown to be an inhibitor of histone deacetylases (HDACs), enzymes which have a fundamental impact on chromatin remodeling and gene expression of cells (Göttlicher et al., 2001; Phiel et al., 2001). HDACs have chiefly been of interest as possible molecular targets for the treatment of cancer diseases (Yoshida et al., 2001). However, in light of reports that further structural derivatives of VPA were HDAC inhibitors only if they were also teratogenic (Gurvich et al., 2004; Eikel et al., 2006), it has been suggested that VPA might also induce embryonic malformations by its inhibition of HDAC (Gurvich et al., 2005; Eikel et al., 2006), and this possibility has led to new interest in HDACs as molecular targets in toxicology. Recently, we used the NMRI-exencephaly-mouse model (Nau et al., 1981) to demonstrate a quantitative correlation between embryonic malformation and the HDAC inhibition potential of a set of 20 structurally diverse VPA derivatives (Eikel et al., 2006). We showed that the structural prerequisites allowing VPA derivatives to inhibit HDACs are unique and have previously not been shown for other classical HDAC inhibitors such as trichostatin A (TSA) or suberoylanilinhydroxamic acid (SAHA).

Both TSA and SAHA have been shown to use their hydroxamic acid function to inhibit HDACs by complexing the catalytically active zinc atom of HDACs (Finnin et al., 1999; Somoza et al., 2004; Vannini et al., 2004). It was also shown that TSA and some structural derivatives are active only as hydroxamic acids but not as carboxylic acids or amidates (Yoshida et al., 1990; Jung et al., 1999), which raises the question whether valproic acid derivatives with hydroxamic acid

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ABBREVIATIONS: VPA, valproic acid; HDAC, histone deacetylase; NMRI, Naval Medical Research Institute; TSA, trichostatin A; SAHA, suberoylanilinhydroxamic acid; VPA-HA, valproic hydroxamic acid; IC_{50}(HDAC), inhibitor concentration with half-maximal HDAC enzyme activity; NPD, nitrogen phosphor detector; GC-MS, gas chromatography-mass spectrometry; ATR IR, attenuated total reflection infrared spectroscopy; LOQ, limit of quantification; LOD, limit of detection; ANOVA, analysis of variance; TBS, Tris-buffered saline; TBS-M, Tris-buffered saline buffer containing 3% nonfat dry milk; BBB, blood-brain barrier.
function might be even more potent HDAC inhibitors than the corresponding carboxylic acids.

In contrast, it was shown that valproic hydroxamic acid (VPA-HA) was not teratogenic in the NMRI-exencephaly-mouse model, whereas 2-pentyl-4-pentynoic hydroxamic acid induced this specific embryonic malformation (Gravemann, 2002; Volland, 2002). Furthermore, valpromide, the slightly teratogenic amide derivative of VPA, had been shown to be metabolized to VPA in vivo (Radatz et al., 1998), whereas valproic hydroxamic acid was shown not to be hydrolyzed (Levi et al., 1997). This raised the question whether a hydrolysis might be the reason for the teratogenic effects of VPA hydroxamates.

Therefore, we first investigated the HDAC inhibition potential of selected VPA derivatives that have amide and hydroxamic acid functions, and compared these data with the teratogenic potential of these derivatives measured in the NMRI-exencephaly-mouse model. Because no correlation between HDAC inhibition and teratogenic potency was found in our initial trials, we also investigated the pharmacokinetic profile of a pair of most interesting hydroxamic and carboxylic acid derivatives of VPA. Our findings suggest that VPA derivatives with hydroxamic acid function can be extensively metabolized to the corresponding carboxylic acid, thus indicating that both valproic acid amides and valproic hydroxamic acids might be pro-teratogens, depending on the teratogenicity of the corresponding carboxylic acid metabolite.

Materials and Methods

Chemicals and Reagents. All chemicals and reagents used were of analytical grade if not stated otherwise. Valproic acid, Cremophor EL, (1R,2S,5R)-(−)-methylchloroformate, and (S)-(−)-1-naphthylethylamine were obtained from Sigma-Aldrich GmbH (Deisenhofen, Germany). Valpromide was a kind gift from Katwijck Chemie (Katwijck, The Netherlands). The VPA structural derivatives used in this study were synthesized according to methods published elsewhere (Nau, 1985; Levi et al., 1997; Radatz et al., 1998; Gravemann, 2002) or as described below (Fig. 1).

Synthesis of Valproic Hydroxamic Acid (I) and (±)-R, and (S)-2-Pentyl-4-pentynoic Hydroxamic Acid (VI, VIII, X). The hydroxamic acid derivative of VPA and some of its selected congeners were synthesized by activation of the carboxylic acid group to the carboxylic acid chloride and conversion to the corresponding hydroxamate using hydroxylamine as reagent (Levi et al., 1997; Gravemann, 2002). Then, 50 mmol of reactant were dissolved in 15 ml of thionyl chloride and boiled for 5 h at 90°C under reflux. After cooling, the excess thionyl chloride was removed under slight vacuum, and the carboxylic acid chloride was distilled under high vacuum (10 mbar, at between 90 and 110°C). The purified carboxylic acid chloride was then dissolved in 80 ml of dry tetrahydrofuran and added slowly to a 5°C solution of hydroxyl amine hydrochloride (1 mol), triethylamine (140 ml), and water (300 ml). The solution was stirred for another 1.5 h, and the hydroxamic acid product was extracted three times with 150 ml of ethylene chloride. The combined organic solvents were washed with 80 ml of hydrochloric acid solution (1 M) and dried for 12 h by stirring over sodium sulfate. The solution was filtered, the solvent was evaporated to dryness, and the remaining red oil was flash-chromatographed on silica (Si 60 Macherey-Nagel GmbH, Düren, Germany) with a 20:80 (v/v) mixture of ethyl acetate and ligroin. Product control of the eluate was performed using thin-layer chromatography (Alugram Sil G/UV254; Macherey-Nagel GmbH), and solvent fractions containing the product were combined and evaporated to dryness. After recrystallization in a 1:99 (v/v) mixture of ethyl acetate and ligroin, the hydroxamic acid product was obtained as fine colorless crystals.

Results of the Analytical Procedures for the Characterization of the Substances Under Investigation Here. Valproic Hydroxamic Acid (VPA-HA, III). Thin-layer chromatography with a 50:50 (v/v) mixture of ethyl acetate and ligroin as solvent: Rf(VPA-HA) = 0.5, Rf(VPA) = 1.0; melting point: 123°C; elemental microanalysis: C_{8}H_{17}NO_{2}, (M_{w} = 159.23 g/mol) calculated: 60.35% C, 10.76% H, 8.80% N; measured: 60.35% C, 10.62% H, 8.76% N.
Nuclear magnetic resonance $^{13}$C-NMR (100 MHz, δ): 140.0 (C5 and C5'); 20.7 (C4 and C4'); 34.7 (C3 and C3'); 44.0 (C2); 174.2 (C1). Nuclear magnetic resonance $^{1}H$-NMR (400 MHz, δ): 0.89 (6H, δ, J = 7.2 Hz); 1.20–2.02 (m, 9H); 8.20 (bs, 1H). Infrared spectroscopy IR [ATR, ν (cm$^{-1}$)]: 3175 (br); 3027 (br); 2957 (s); 2929 (s); 2874 (s); 1626 (s); 1538 (s); 1464 (s); 1041 (s); 949 (s).

Mass spectrometry (MS) [70 eV, m/z (%)]: 159 (42) M$^+$; 130 (16) M$^+$ – C2H5; 127 (100) M$^+$ – NOH; 117 (31) M$^+$ – C3H6; 116 (17) M$^+$ – C3H7; 99 (72) M$^+$ – CONHOH; 88 (58); 83 (46); 72 (57). Chemical purity as measured by standard GC-MS analysis of the trimethylsilylether: >99% ($\pm$ 2)-2-Pentyl-4-pentynoic Hydroxamic Acid (VI). Thin-layer chromatography with a 20:80 (v/v) mixture of ethyl acetate and ligrosin as solvent: Rf (2-propyl-4-pentynoic hydroxamic acid) = 0.3. Rf (2-propyl-4-pentynoic acid) = 0.7. Melting point: 66°C. Elemental microanalysis: C$_{10}$H$_{17}$NO$_2$ (M$_r$ = 183.25 g/mol) calculated: 65.54% C, 9.35% H, 7.64% N; measured: 65.48% C, 9.22% H, 7.60% N.

Nuclear magnetic resonance $^{13}$C-NMR (100 MHz, δ): 140.0 (C7); 21.6 (C6); 172.6 (C1). Nuclear magnetic resonance $^{1}H$-NMR (400 MHz, δ): 0.87 (6H, δ, J = 6.9 Hz); 1.29 (m, 6H); 1.51–1.76 (m, 2H); 2.06 (t, 1H, J = 2.6 Hz); 2.23–2.48 (m, 3H); 8.70 (s, 1H); 9.05 (bs, 1H). Infrared spectroscopy IR [ATR, ν (cm$^{-1}$)]: 3279 (m); 3189 (br); 3035 (br); 2919 (s); 2858 (s); 1626 (versus); 1458 (m); 1459 (w); 1111 (m); 1067 (m); 1012 (m); 1003 (m); 953 (m).

Mass spectrometry (MS) [70 eV, m/z (%)]: 183 (23) M$^+$; 164 (17); 151 (100) M$^+$ – NOH; 144 (12) M$^+$ – C$_4$H$_3$; 123 (16) M$^+$ – CONHOH; 113 (32) M$^+$ – C$_4$H$_7$; 112 (26) M$^+$ – C$_4$H$_7$I; 97 (39); 81 (99); 67 (58). Chemical purity as measured by standard GC-MS analysis of the trimethylsilylether: >99% ($\pm$ 2)-2-Pentyl-4-pentynoic Hydroxamic Acid (VIII). The physicochemical parameters of the R-enantiomer were identical to those of the racemate given above. Specific optical rotation $\alpha$ measured at 20°C with c = 10.16 mg/ml in chloroform: +8.8 (589 nm); +9.2 (578 nm); +10.2 (546 nm); +15.7 (436 nm); +21.0 (365 nm); optical purity as measured by GC-NPD analysis after chiral derivatization with (1R,2S,5R,−)-methylchlorofluoromethane showed an enantiomeric excess of >98% ee.

(S)-2-Pentyl-4-pentynoic Hydroxamic Acid (X). The physicochemical parameters of the S-enantiomer were identical to those of the racemate given above. Specific optical rotation $\alpha$ measured at 20°C with c = 10.03 mg/ml in chloroform: −8.6 (589 nm); −8.8 (578 nm); −9.8 (546 nm); −15.6 (436 nm); −21.3 (365 nm); optical purity as measured by GC-NPD analysis after chiral derivatization with (1R,2S,5R,−)-methylchlorofluoromethane demonstrated an enantiomeric excess of >98% ee.

Stereoselective Quantiﬁcation of S-2-Pentyl-4-pentynoic Acid (IX) in Biological Samples. Samples were prepared as above, with the following variations: 30 µl of internal standard solution was added (0.5 µg/µl 3-ethylpentanoic acid in 100 mM disodium hydrogen phosphate adjusted to pH 7.4), and the organic solvents were pipetted into a disposable glass vial in which, due to the volatile nature of the internal standard, the organic solvents were not evaporated to dryness but to a ﬁnal volume of between 50 and 100 µl.

For derivatization, 400 µl of methylene chloride, 200 µl of 1-hydroxybenzo triazole solution [2 mg/ml in a 99:1 (v/v) mixture of methylene chloride and pyridine], 200 µl of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride solution (2 mg/ml in methylene chloride), and 200 µl of S(−)-1-naphthylethylamine (2 mg/ml in methylene chloride) were added to the glass vial, which was then gently shaken for 90 min at room temperature. After derivatization, the solution was evaporated to dryness under a nitrogen stream and resuspended in 100 µl of GC solution [an 80:20 (v/v) mixture of n-hexane and ethyl acetate]; finally, 1 µl of the resuspended solution was injected into the gas chromatograph.

Gas chromatographic separation was performed using an Agilent HP 5 MS column (30 m × 320 µm inner diameter × 0.25 µm film) and the following temperature program: start at 120°C, hold for 2 min; heat at 20°C/min to 220°C, hold for 1 min; heat at 10°C/min to 230°C, hold for 12 min; heat at 60°C/min to 325°C, hold for 6 min. The separation was carried out with helium 5.0 as the carrier gas at a flow rate of 2.0 ml/min, followed by nitrogen-selective detection (50 pA, 20 min equilibration time) on an autosample Agilent GC 6890 gas chromatography station.

Validation of the Quantification Methods. The linear calibration curves for both quantification methods were computed with the software package Valoo from Analytik-Software GmbH (Leer, Germany), based on the above described procedure and analysis of 20 mouse plasma samples spiked with concentrations ranging from 5 µg/ml to 1000 µg/ml each. All samples were measured in duplicate, and linearity of the calibration curve, as well as limit of quantification (LOQ) and limit of detection (LOD), was computed based on these data. Further validation was done by intraday and interday analysis of spiked mouse plasma and mouse tissue samples as summarized in Table 1.

These samples were analyzed with six (tissues) or eight (plasma) replications in 1 day as intraday analysis of variation, or on six (tissues) or eight (plasma) days in three consecutive weeks as interday analysis of variation. Values shown are the mean of four different concentrations in the range of 30 µg/ml to 800 µg/ml (µg/g for tissues, respectively).

Measurement of the Plasma Protein Binding of S-2-Pentyl-4-pentynoic Acid (IX) in Biological Samples. For plasma protein binding studies, the compound under investigation was incubated for 1 h at 37°C at the indicated concentrations in pooled human or mouse blank plasma (additional control samples were incubated in 100 mM phosphate buffer, pH 7.4, alone). After incubation, one aliquot of each sample was quantified by the above analytical methods, and 500 µl of each plasma or control sample was filtered by centrifugation (1 min, 10,000g, 37°C) in a membrane filter unit (Vivaspin 500, PES membrane, 5-kDa molecular weight cut-off, or Vivaspin 2, Hydroset membrane, 50-kDa molecular weight cut-off, both from Vivasience GmbH, Hannover, Germany). The centrifugation was stopped after 1 min with a filtrate volume of approximately 80 µl in order not to disturb the binding balance. The plasma protein binding was calculated relative to the concentration of the spiked plasma samples with four independent samples at each concentration. The total amount of plasma protein binding was calculated as the average plasma protein binding over the complete concentration range measured.

Measurement of PharmacoKinetic Drug Profiles in Mice. For the analysis of the pharmacokinetic profile of both S-2-pentyl-4-pentynoic hydroxyacid (X) and S-2-pentyl-4-pentynoic acid (IX), female NMRI mice weighing between 28 and 36 g were randomly assigned to groups of three per time of interest. Each animal was weighted and injected s.c. or i.p. with the volume of test solution necessary to yield a dosage of 0.80 mmol/kg or 1.50 mmol/kg, respectively.
respectively. Before injection, the compound under investigation had been dissolved in water to a final concentration of 0.80 mmol/10 ml or 1.50 mmol/10 ml, respectively, and neutralized. The animals were anesthetized with diethyl ether at the indicated times, and blood samples were taken. The animals were then sacrificed to obtain further tissues such as liver, spleen, kidney, and brain. Analysis of the biological samples was performed as above, and the time-dependent concentration curves were analyzed using the WinNonLin 4.0 software package from Pharsight Corporation (Mountain View, CA), with a noncompartmental analysis approach and the liner trapezoidal rule for estimation of the compound half-life.

Reproductive Toxicity Assay in the NMRI-exencephaly-Mouse Model. The reproductive toxicity was determined for selected VPA derivatives and solvent-only samples in the NMRI-exencephaly-mouse model described in detail elsewhere (Nau et al., 1981). In short, female NMRI mice weighing between 28 and 36 g were mated and a y; o nd a y8.25 of gestation, both females were injected intraperitoneally. On day 18 of gestation, the animals were killed, and the fetuses were weighed and inspected for external malformations. The exencephaly rate was calculated relative to the number of living fetuses. All efforts were made to minimize both the suffering and the number of animals used in this study. All procedures conducted were in accordance with the German Animal Welfare Act and were approved by the responsible governmental agency under the license numbers 00/269 and 02/612.

Measurement of the Teratogenic Potency. The exencephaly rate was determined, as described above, as the model parameter for teratogenicity. These values were considered with previously published exencephaly rates for all other VPA derivatives used in this study (Bojic et al., 1998; Radatz et al., 1998; Volland, 2002) to establish an arbitrary range of teratogenic potency, which is shown in Table 2. The decision criteria range from 0 (no detectable teratogenic potency) to +++ (very high teratogenic potency). The corresponding teratogenic potency rating of each VPA derivative used in this study is summarized in Table 3.

Histone Hyperacetylation in Cell Culture. Teratocarcinoma F9 mouse cells (American Type Culture Collection, Manassas, VA) were cultured in Ham’s F-12/Dulbecco’s modified Eagle’s medium containing 2 mM L-glutamine, 10% (v/v) fetal bovine serum, 0.145 mM 2-mercaptoethanol, and 100 U/ml penicillin/streptomycin (medium and supplements from Invitrogen GmbH, Karlsruhe, Germany). For the experimental setup, 10^6 cells were treated in triplicate by incubation at 37°C in a humid air atmosphere with 5% (v/v) CO₂. After 6 h, cells were scraped from the bottom of the well, washed twice with phosphate-buffered saline, dissolved in 100 μl of lysis buffer (62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (w/v), 1% glycerin (v/v), 0.5 μM β-mercaptoethanol, 250 μM phenylmethanesulfonyl fluoride, 0.05 μg/ml aprotinin, 2 μg/ml aprotinin, 0.05 μg/ml leupeptin) and boiled immediately for 5 min at 90°C. Then, 10 μl of this cell lysate was separated by 15% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane by semidry electroblotting. The blotted membrane was washed with TBS buffer (2.4 g/l Tris/HCl, 8 g/l of NaCl, pH 7.6) and blocked with TBS buffer containing 3% nonfat dry milk (TBS-M) for 1 h at room temperature.

TABLE 1

Validation parameters of the quantification methods for both S-2-pentyl-4-pentynoic hydroxamic acid and its metabolite S-2-pentyl-4-pentynoic acid

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Matrix</th>
<th>Sample Amount</th>
<th>Linearity</th>
<th>Coefficient of Variation</th>
<th>μ or mg</th>
<th>μg/ml or μg/g</th>
<th>%</th>
<th>μg/ml or μg/g</th>
<th>%</th>
<th>μ or mg</th>
<th>μg/ml or μg/g</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-2-Pentyl-4-pentynoic hydroxamic acid X</td>
<td>Plasma</td>
<td>50</td>
<td>5–100</td>
<td>5.5</td>
<td>0.997</td>
<td>5</td>
<td>14</td>
<td>103</td>
<td>2–4</td>
<td>103</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Brain</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>50</td>
<td>5–1000</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-2-Pentyl-4-pentynoic acid IX</td>
<td>Plasma</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Brain</td>
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<tr>
<td>Liver</td>
<td>50</td>
<td>5–1000</td>
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<td></td>
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</table>

N.D., not determined.

TABLE 2

Decision criteria for teratogenic potency rating of VPA derivatives investigated in the NMRI-exencephaly-mouse model (Nau, 1981)

<table>
<thead>
<tr>
<th>Teratogenic Potency</th>
<th>Dose Range</th>
<th>Exencephaly Rate</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmol/kg</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>&gt;3.00</td>
<td>0</td>
<td>No teratogenic potency detectable</td>
</tr>
<tr>
<td>+</td>
<td>2.00–3.00</td>
<td>1–5</td>
<td>Low teratogenic potency</td>
</tr>
<tr>
<td>++</td>
<td>2.00–3.00</td>
<td>5–25</td>
<td>Lower teratogenic potency than VPA</td>
</tr>
<tr>
<td>+++</td>
<td>2.00–3.00</td>
<td>25–60</td>
<td>Equal teratogenic potency to VPA</td>
</tr>
<tr>
<td>+++++</td>
<td>1.00–2.00</td>
<td>40–60</td>
<td>Higher teratogenic potency than VPA</td>
</tr>
<tr>
<td>++++++</td>
<td>0.25–1.00</td>
<td>40–60</td>
<td>Very high teratogenic potency</td>
</tr>
</tbody>
</table>

TABLE 3

Teratogenic potency rating, H₂ hyperacetylation in treated F9 cells and HDAC enzyme inhibition ability of the VPA derivatives investigated in this study

<table>
<thead>
<tr>
<th>Compound</th>
<th>Teratogenic Potencyᵃ</th>
<th>IC₅₀(HDAC) ± S.Eᵇ</th>
<th>ΔH₂-Hyperacetylation (0 to +++)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>++</td>
<td>400 ± 50</td>
<td>++</td>
</tr>
<tr>
<td>II</td>
<td>++</td>
<td>&gt;2000</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>0</td>
<td>&gt;2000</td>
<td>+</td>
</tr>
<tr>
<td>IV</td>
<td>+++</td>
<td>35 ± 10</td>
<td>+</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>&gt;2000</td>
<td>0</td>
</tr>
<tr>
<td>VI</td>
<td>++</td>
<td>&gt;2000</td>
<td>0</td>
</tr>
<tr>
<td>VII</td>
<td>+++</td>
<td>870 ± 180</td>
<td>+</td>
</tr>
<tr>
<td>VIII</td>
<td>0</td>
<td>&gt;2000</td>
<td>0</td>
</tr>
<tr>
<td>IX</td>
<td>+++++</td>
<td>50 ± 12</td>
<td>+</td>
</tr>
<tr>
<td>X</td>
<td>+++++</td>
<td>&gt;2000</td>
<td>0</td>
</tr>
</tbody>
</table>

H₂ core histone 4; ΔH₂ acetylated core histone 4.

ᵃ Data from the NMRI-exencephaly-mouse model inferred from Radatz et al. (1998), Bojic et al. (1998), and Volland (2002).

ᵇ S.E. representing the goodness of fit of the mathematically fitted function, not the standard error of the mean.

ᶜ Not taking into account that valpromide is metabolized to the corresponding valproic acid (Radatz et al., 1998) and, therefore, probably activated and intrinsically not active (also see Results and Discussion).

ᵈ Assumed that the solvent enhancer Chemoprop EL 25% (v/v) is reducing the teratogenic effect of the compound (also see Results and Discussion).
many) in TBS-M at 4°C for 12 h. The membrane was washed once with TBS buffer and again incubated with a 1:5000 dilution of an anti-rabbit antibody (ECL detection kit, GE Healthcare, Solingen, Germany) in TBS-M for 1.5 h at room temperature. Blots were washed three times with TBS buffer, once with a mixture of 0.05% (v/v) Tween 20 and TBS buffer, and again three more times with TBS buffer before antibodies were detected with the ECL detection kit according to the manufacturer’s instructions.

**HDAC Inhibition Human Enzyme Assay.** HDAC activity was measured by using an HDAC fluorescence activity assay kit (Biomol GmbH, Hamburg, Germany). Because the enzymatic test system is pH-dependent, the compounds to be measured were first dissolved in water and neutralized before preparation of further dilution series with HDAC assay buffer. The dose-activity samples were tested with at least three repeats according to the manufacturer’s instructions. In short, HeLa nuclear extracts (1 µl of between 6 and 9 mg/ml total protein) were incubated with 500 µM acetylated Fluode-Lys substrate in 50 µl of assay buffer in the presence or absence of the respective valproic acid analog. The HDAC inhibitor TSA, at a concentration of 5 µM, served as positive control. The deacetylation reaction was carried out at 37°C for 3 h and stopped by addition of 50 µl of Fluode-Lys developer solution containing 2 µM trichostatin A. After 15 min, fluorescence activity was measured with a Victor 1420 fluorescence reader (PerkinElmer LAS GmbH, Rodgau-Jügesheim, Germany) at 355 nm excitation and 535 nm emission. The enzyme activity was calculated relative to the measured fluorescence activity of four negative controls (HDAC assay buffer only) on each 96-well plate. Determination of the IC₅₀(HDAC) value was computed, fitting at least six data points to a mathematical enzyme inhibition function with the pharmacodynamic module of the WinNonLin 4 software package (Pharsight Corporation) to give IC₅₀ values in micromoles per liter, with a standard error (S.E.) in micromoles per liter, which represents the goodness of fit between the computational model and the experimental data.

**Results**

The method used here to synthesize hydroxamic acids from the chemically and optically pure carboxylic acid derivatives of VPA resulted, in all cases, in excellent yields of between 80% and 90%, and with chemical and optical purities >99% (98% ee). We further developed two quantification methods for the stereoselective determination of VPA hydroxamates and carboxylic acids via chiral derivatization, followed by GC-NPD analysis. These methods were used to investigate the pharmacokinetic profile of S-2-pentyl-4-pentynoic hydroxamic acid and its potential metabolite, S-2-pentyl-4-pentynoic acid.

Both analytes were obtained from biological samples by ethyl acetate liquid-liquid extraction at pH 5.0 (Hauck et al., 1992). The carboxylic acid was then derivatized in a classical approach using a chiral amine, (S)(−)-1-naphthylethylamine. The hydroxamate was derivatized using (2R,3S,5R)(−)-menthylchloroformate as the derivatization reagent, which resulted in a stable carbonate. Both analytes were separated from their corresponding enantiomer by GC and determined by NPD detection (Fig. 2). Validation of both methods in

![Fig. 2. GC separation and NPD detection of the R- and S-enantiomers of both 2-pentyl-4-pentynoic hydroxamic acid (top; retention times 19.98 min and 20.04 min) and 2-pentyl-4-pentynoic acid (bottom; retention times 16.01 min and 16.48 min) after spiking of mouse plasma samples with 300 µg/ml, extraction process, and derivatization with either (2R,3S,5R)(−)-menthylchloroformate (MCF) or (S)(−)-1-naphthylethylamine (NEA).](image-url)
a variety of biological matrices showed that kidney samples could not be tested for S-2-pentyl-4-pentynoic hydroxamic acid because of an unknown by-product of the derivatization process, which crystallized inside the GC liner and blocked the injection port immediately after sample injection. Furthermore, there was a reproducible and significant signal enhancement of approximately 30% when liver samples were analyzed for S-2-pentyl-4-pentynoic acid, but these effects were not further investigated in this study. The overall evaluation of the two analytical procedures is summarized in Table 1 and shows the good reliability of both quantification methods.

Before the investigation of the pharmacokinetic profile of these two VPA derivatives, we measured the plasma protein binding property of both S-2-pentyl-4-pentynoic hydroxamic acid and its hypothetical metabolite S-2-pentyl-4-pentynoic acid (Table 4). It could be shown that plasma protein binding of the carboxylic acid was between 20% and 25% higher than those of the hydroxamic acid, which supports the assumption that protein binding increases with increasing ionization at physiological pH. The average plasma protein binding of S-2-pentyl-4-pentynoic acid is 91% in human and 69% in spiked mouse plasma, values that are clearly higher than the plasma protein binding of VPA [80% and 12%, respectively (Löschler, 1999)]. Both compounds also exert higher plasma protein binding in human plasma than mouse plasma, which is in agreement with plasma protein binding data reported for VPA in both species (Löschler, 1999).

Using the above analytical quantification methods, we analyzed the pharmacokinetic profiles of both selected VPA derivatives (Table 5). Concentration-time curves of S-2-pentyl-4-pentynoic hydroxamic acid in mice after i.p. dosage of 0.8 mmol/kg revealed that the compound is rapidly transported into the brain, where concentrations were even higher than plasma concentrations 1 h after injection (Fig. 1).
3a). Assuming that only the free drug could penetrate the blood-brain barrier (BBB), and taking into account the 45% plasma protein binding of 69%, it appears that there were approximately 40% of plasma concentrations. Again, keeping in mind the elevated plasma protein binding with respect to the exencephaly endpoint. On the contrary, average embryonic weight was significantly increased. But even at this low concentration, the corresponding S-enantiomer led to a significant increase in embryo lethality of up to 19% and to an exencephaly rate of 2%, which is well above the spontaneous exencephaly rate of approximately 0.5% in this mouse strain (Bojic et al., 1998; Radatz et al., 1998). With Cremophor EL (25% v/v) as the solubility enhancer, it was possible to extend the dosage to 1.50 mmol/kg. The solvent enhancer did not increase the spontaneous exencephaly rate of the NMRI mouse strain (Table 7). Cremophor EL 25% (v/v) is also maternally nontoxic but increases embryo lethality up to 13%, compared with 5% embryo lethality when dosing saline solution as control. With this solubility enhancer, a dose of 1.50 mmol/kg S-2-pentyl-4-pentynoic hydroxamic acid induced a significantly higher rate of 5% exencephalies, whereas the corresponding R-enantiomer did not cause this form of malformation. The embryo lethality of the S-enantiomer was also higher than that of the R-enantiomer, but not significantly different from that of the control group. Since valproic acid and its teratogenic derivatives normally lead to quite steep increases on exencephaly rates, depending on the dose (Nau, 1985) it is likely that Cremophor EL reduced the teratogenic effects of S-2-pentyl-4-pentynoic hydroxamic acid. Although the dose was nearly doubled from 0.80 to 1.50 mmol/kg, the exencephaly rate increased only slightly, from 2% to 5%. Such an effect might be caused by influences of the solvent enhancer on both the biological availability and pharmacokinetics of the dissolved test compound.

We compared the above exencephaly rates for both enantiomers of 2-pentyl-4-pentynoic hydroxamic acid with previously published rates for all VPA derivatives used in this study and converted all rates into the arbitrary range of teratogenic potency. We also measured the HDAC inhibition ability of each compound, both in an F9 cell culture assay and an HDAC human enzyme inhibition assay as described above, and compared the properties of these two compounds (Table 3). It was apparent that, regardless of the optical conformation, both amide and hydroxamic acid derivatives of VPA were not HDAC inhibitors at concentrations up to 2000 μM, and they did not induce H-4 hyperacetylation in the F9 cell system. Nevertheless some members of both groups of VPA derivatives had been rated as slightly teratogenic (II, V, VI, and X).

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**TABLE 6**

Pharmacokinetic parameters of S-2-pentyl-4-pentynoic hydroxamic acid, its metabolite S-2-pentyl-4-pentynoic acid, and valproic acid (Radatz et al., 1998) in the NMRI mouse model

<table>
<thead>
<tr>
<th>Dosage (mmol/kg)</th>
<th>S-2-Pentyl-4-pentynoic Hydroxamic Acid X</th>
<th>S-2-Pentyl-4-pentynoic Acid IX</th>
<th>Valproic Acid I</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.80</td>
<td>30</td>
<td>630</td>
<td>360</td>
</tr>
<tr>
<td>2.00</td>
<td>80</td>
<td>630</td>
<td>600</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Plasma and brain concentrations of S-2-pentyl-4-pentynoic hydroxamic acid after i.p. dosage of 0.80 mmol/kg (top) and of S-2-pentyl-4-pentynoic acid after s.c. dosage of 1.50 mmol/kg (bottom) in the NMRI mouse model.
In light of the above-mentioned findings that VPA hydroxamates can be metabolized to the corresponding acids, we conclude that both amides (Radatz et al., 1998) and hydroxamates can be pro-teratogens if the in vivo biotransformation leads to corresponding VPA carboxylic acids that are teratogenic.

**Discussion**

Currently, there is speculation about the possibility that both the teratogenic and anticancer effects of VPA are mediated by the inhibition of HDACs, an enzyme class crucially important for chromatin remodeling and expression of specific genes (Göttlicher et al., 2001; Phiel et al., 2001; Blaheta et al., 2005; Gurvich et al., 2005; Menegola et al., 2005). Support for the hypothesis that there is an interrelation between HDAC inhibition and teratogenic effects is provided by structure-activity relationship studies on VPA derivatives demonstrating that teratogenic congeners are, in fact, at the same time HDAC inhibitors (Gurvich et al., 2004; Eyal et al., 2005; Eikel et al., 2006). Here we demonstrated that VPA hydroxamides and amides are not HDAC inhibitors, unlike TSA and SAHA, which are HDAC inhibitors only as hydroxamates, but not as amides or carboxylic acids (Yoshida et al., 1990; Jung et al., 1999). We detected neither any hyperacetylation in F9 teratocarcinoma mouse cells at concentrations up to 1 mM nor any functional inhibition of human HDAC enzymes at concentrations up to 2 mM. This fact suggests that VPA derivatives have a unique property for binding to HDAC enzymes not yet described for other groups of known HDAC inhibitors.

But we also demonstrated that hydroxamic acid derivatives of VPA can, indeed, be teratogenic. The S-enantiomer of the chiral pair of VPA hydroxamates investigated here can induce teratogenic effects in the NMRI-exencephaly-mouse model, whereas the corresponding R-enantiomer does not. This is in good agreement with results of other studies of VPA carboxylic acid derivatives (Göttlicher et al., 2001; Eikel et al., 2006). Similarly, valpromide, the amide derivative of VPA, has teratogenic effects in vivo (Radatz et al., 1998) but is not able to inhibit HDAC. At first, both these observations somewhat challenge the validity of the hypothesis that VPA exerts its teratogenic effects by inhibiting HDAC.

But in light of previous reports of the in vivo metabolism and biotransformation of valpromide to valproic acid (Radatz et al., 1998), we hypothesized that VPA hydroxamides are also metabolized to the corresponding acids and, therefore, may be metabolically activated in vivo. So far, it has been reported that valproic hydroxamic acid is not metabolized to its carboxylic acid in vivo (Levi et al., 1997).

To investigate our hypothesis, we first developed two quantification methods that enabled us to detect both the S-2-pentyl-4-pentynoic hydroxamic acid and the theoretical metabolite S-2-pentyl-4-pentynoic acid. Additionally, we were able to investigate the possible conversion of the chiral center at position C2. The latter is theoretically possible because of the close proximity of the chiral center to the reactive hydroxamate function. Both quantification methods were successfully validated, so that we were able to monitor the parent compound and its hypothetical metabolite in a variety of murine tissues.

Investigation of the pharmacokinetic profile of S-2-pentyl-4-pentynoic hydroxamic acid itself revealed that the stereogenic center at C2 is stable in vivo with no chiral conversion detectable. This VPA derivative is rapidly and extensively distributed into the brain, so that brain concentrations are equal to plasma concentrations within the first 2 h after application. In light of the measured plasma protein binding of 45%, S-2-pentyl-4-pentynoic hydroxamic acid shows an excellent passage of the BBB. This observation is in agreement with previous findings that the anticonvulsant activities of VPA hydroxamates are equal or even superior to that of valproic acid (Levi et al., 1997; Volland, 2002).

Our results regarding the metabolization of S-2-pentyl-4-hydroxamic acid (X) to its corresponding acid are different from previously reported results obtained with valproic hydroxamic acid (III) in dogs.
A study of the pharmacokinetic profile of this metabolite, S-2-pentyl-4-pentynoic acid, revealed that its half-life in mice is 4.2 h, which is nearly 4 times longer than that of VPA (Löscher, 1999). Nor was any conversion of the chiral center at position C2 detected in that study. Both these properties (long half-life, nonconversion) demonstrate the metabolic stability of this VPA derivative. Such stability might be caused by the triple bond in position C4-C5 (Hauck et al., 1992), which possibly withdraws the compound from the fatty acid metabolism pathways.

S-2-Pentyl-4-pentynoic acid can also be shown to be a very potent HDAC inhibitor with an IC_{50}(HDAC) of 50 μM, indicating that its activity is 10 times higher than that of valproic acid itself. Due to metabolism, the accumulated amount of S-2-pentyl-4-pentynoic acid after i.p. dosage of 0.8 mmol/kg of the parent hydroxamic acid was approximately 15% to 20% of the plasma concentrations reached after direct s.c. dosage of 1.5 mmol/kg. With respect to the fact that the metabolite S-2-pentyl-4-pentynoic acid is a remarkably potent HDAC inhibitor, we conclude that the teratogenic effects of S-2-pentyl-4-pentynoic acid hydroxamic acid are induced by its metabolite.

Overall, we conclude that VPA hydroxamates can be metabolized to their corresponding acids in vivo and are therefore potential pro-teratogens, depending on the teratogenic potential of their corresponding carboxylic acid metabolite. This metabolic process can be held responsible for the teratogenic effects of S-2-pentyl-4-pentynoic acid hydroxamic acid in the NMRI-exencephaly-mouse model, thus suggesting that VPA hydroxamates are not intrinsically teratogenic but can be activated in vivo. This study is further evidence of HDAC inhibition as a molecular target for the induction of embryonic malformation and makes hydroxamate derivatives of VPA interesting model compounds for further mechanistic studies of teratogenicity.

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References


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