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Valnoctamide, a non-teratogenic amide derivative of valproic acid, inhibits arachidonic acid activation *in vitro* by recombinant acyl-CoA synthetase-4

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Abstract

Objective—Valproic acid (VPA), a mood stabilizer used for treating bipolar disorder (BD), uncompetitively inhibits acylation of arachidonic acid (AA) by recombinant AA-selective acyl-CoA synthetase (Acs1)-4 at K_i of 25 mM. Inhibition may account for VPA's ability to reduce AA turnover in brain phospholipids of unanesthetized rats at a therapeutically relevant dose. VPA is teratogenic. We now tested whether valnoctamide (VCD), a non-teratogenic amide derivative of a VPA chiral isomer, which had antimanic potency in a Phase III BD trial, also inhibits recombinant Acs14.

Methods—Rat Acs14-flag protein was expressed in *E. coli*. We used Michaelis–Menten kinetics to characterize and quantify the ability of VCD to inhibit conversion of AA to AA-CoA by recombinant Acs14 *in vitro*.

Results—Acs14-mediated activation of AA to AA-CoA by Acs14 was inhibited uncompetitively by VCD, with K_i equal 6.38 mM.

Conclusions—VCD's ability to uncompetitively inhibit AA activation to AA-CoA by Acs14, at a lower K_i than VPA, suggests that, like VPA, VCD may reduce AA turnover in rat brain phospholipids. If so, VCD and other non-teratogenic Acs14 inhibitors might be considered further for treating BD.

Keywords

acyl-CoA synthetase 4; anticonvulsant; arachidonic acid; bipolar disorder; brain; cascade; enzyme; inhibition; metabolism; Michaelis–Menten kinetics; mood stabilizer; rat; valnoctamide; valproate

Valnoctamide [valmethamide or 2-ethyl-3-methyl pentanamide (VCD)] (Fig. 1) is an over-the-counter drug available in many European countries as a sedative-hypnotic (1, 2). VCD is a chiral constitutional isomer of valpromide (VPD), the corresponding amide of valproic

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acid (VPA), a mood stabilizer approved by the FDA for treating bipolar disorder (BD) (2–5). Compared to VPA, however, VCD is significantly less teratogenic in mice (2, 6). It is not transformed into its homologous acid, valnoctic acid, or converted to VPA *in vivo* (4, 7, 8). It distributes better than VPA in brain (8), and is three times more potent as an anticonvulsant (3, 9).

VPA is effective in treating bipolar mania, but has a significant risk for birth defects and developmental delay through its epigenetic effects, particularly from inhibition of histone deacetylase (HDAC) (10–12). Having a similarly acting antimanic drug that is not teratogenic would be of clinical relevance. In this regard, in a Phase III trial, VCD was more effective than placebo as an add-on to risperidone for treating bipolar mania (13). On the basis of its clinical antimanic efficacy, metabolic stability, and lack of teratogenicity, VCD has a potential to become a new BD drug.

VPA and the other FDA-approved mood stabilizers, carbamazepine, lithium, and lamotrigine, when given chronically to unanesthetized rats to produce therapeutically relevant plasma concentrations, downregulate markers of the brain arachidonic acid [(AA) 20:4n-6] cascade (14, 15): AA turnover in brain phospholipids or AA influx from plasma, expression of cyclooxygenase (COX)-2, and prostaglandin E (PGE₂) concentration. Since markers of the AA cascade are upregulated in the postmortem BD brain, in association with excitotoxicity, neuroinflammation, apoptosis and synaptic loss (16–18), dampening the cascade by these drugs may contribute to their efficacy in BD (14).

AA undergoes rapid deacylation-reacylation recycling within brain phospholipids (19–21), and it and its products (e.g., prostaglandins, thromboxanes, leukotrienes) have multiple biological effects and participate in neurotransmission and neuroinflammation (14, 15). As part of the deacylation-reacylation cycle, AA is hydrolyzed from membrane phospholipid by AA-selective calcium-dependent cytosolic phospholipase A₂ (cPLA₂) IVA, which is transcriptionally downregulated in rat brain following treatment with the mood stabilizers, carbamazepine and lithium. On the other hand, VPA's downregulation of AA turnover in rat brain has been ascribed to its ability to uncompetitively inhibit activation of AA to AA-CoA by AA-selective acyl-CoA synthetase (Acsl, E.C.6.2.1.3)-4 (22–24). In uncompetitive inhibition, the inhibitor binds to the enzyme-substrate complex [ES] only and not to the free enzyme [E], while in noncompetitive inhibition the inhibitor binds to [E] or [ES]. VPA uncompetitively inhibits recombinant Acsl4 *in vitro* at a K_i of 25 mM (23).

In view of VPA's ability to inhibit recombinant Acsl4 *in vitro*, we thought it of interest to test whether other agents that are not as teratogenic would do so well. As a first approach, we showed that a non-teratogenic constitutional isomer of VPA, propylisopropylacetic acid (PIA), also inhibited recombinant Acsl4 *in vitro*, but with a higher inhibition efficiency than VPA (25) and at a lower K_i, 11.4 mM. In the present study, we used similar *in vitro* Michaelis–Menten kinetics to test whether VCD also would inhibit recombinant Acsl4 activity.

Briefly, we found that VCD inhibited Acsl4-mediated activation of AA to AA-CoA by recombinant Acsl4 *in vitro*, with a lower K_i than reported for VPA and PIA (23, 25).

Butyrate, an analog of VPA and also a histone deacetylase inhibitor (26), had no inhibitory action.

Materials and methods

Reagents

[1-¹⁴C]AA (50 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). Unlabeled AA, sodium butyrate, coenzyme A, and ATP were purchased from Sigma (St. Louis, MO, USA). Racemic VCD was obtained from the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program (Research Triangle Park, NC, USA).

Preparation of bacterial lysate

Proteins were expressed as previously described (23, 25). Briefly, cells containing plasmids that expressed *ACSL4* were grown in Terrific Broth. Protein expression was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside. Cells were pelleted and resuspended in a buffer containing 10 mM HEPES (pH 7.8) and 0.5 mM EDTA, and sonicated. Lysate aliquots were stored at -80°C for enzyme assay. Protein concentrations were determined by the Bradford method (27). As previously reported (23), we demonstrated with Western blotting and a specific anti-Flag M2 monoclonal antibody that the enzyme preparation that we studied was a single *Acsl4* isoenzyme, whereas the empty control showed no immunostaining.

Acsl4 activity assay

The assay mix included 175 mM Tris-HCl pH 7.4, 8 mM MgCl₂, 5 mM dithiothreitol, 10 mM ATP, 0.25 mM CoA, 0.01 mM EDTA, and 5 μM [¹⁴C]AA in 0.5 mM Triton X-100, and increasing concentrations of unlabeled AA in a total volume of 200 μl. PIA (0, 5, 10 or 15 mM in ethanol), PID (10 mM in water) or MTMCD (10 mM in water), was added directly to the reaction mixture during inhibition assays. The drug controls consisted of the respective vehicle without the drug. As an additional negative control, sodium butyrate (a short-chain VPA analog) was added to the reaction mixture at 60 mM. The reaction was started by adding enzyme (1–3 μg protein) and was measured for 5 min at 37°C (23, 25). The reaction was terminated with 1 ml Dole's Reagent (isopropanol:heptane:1M H₂SO₄, 80:20:2, by vol). In a preliminary experiment, the pH of reaction mixtures spiked with VPA and sodium butyrate at concentrations of 60 mM was measured using a pH meter. The pH (7.4) remained constant at these drug concentrations. Unesterified fatty acids were extracted 2–3 times with 2 ml heptane, and [¹⁴C]AA-CoA formed during the reaction was measured by scintillation counting. As a negative control, *Acsl* enzyme activity of the *E. coli* cell lysate lacking a gene coding for *ACSL*-Flag was measured with AA as substrate as described above. The results were corrected for blanks (samples without cell lysates added and samples analyzed in the absence of fatty acids). The negative control (empty vector) activity was compared with *Acsl4* to make sure that signal to noise ratio was adequate between the test and negative control at each concentration of AA.

Analysis and statistics

Initial reaction velocity (V) was plotted against AA concentration for each VCD analogue concentration I₀, and the plots were fitted by least squares to a hyperbolic Michaelis–Menten

model using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA). K_m (μM) and $V_{\max}$ (nmol/min/mg protein) were calculated by the following equation, in which V is reaction velocity (nmol acyl-CoA formed/min/mg enzyme protein, e.g., nmol/min/mg protein) at a given AA substrate concentration, S (μM)

$$V = \frac{V_{\max} S}{K_m + S} \quad (\text{i})$$

The model in which the substrate (i.e. AA) inhibits the reaction velocity can be described as (28),

$$V = \frac{V_{\max} S}{K_m + S \left(1 + \frac{S}{K_s}\right)} \quad (\text{ii})$$

A model that involves both substrate inhibition and uncompetitive inhibition by the inhibitor I_o can be represented as,

$$V = \frac{V_{\max} S}{K_m + S \left(1 + \frac{S}{K_s} + \frac{I_o}{K_i}\right)} \quad (\text{iii})$$

where K_i is the enzyme inhibition constant.

Data were plotted as a function of inhibitor concentration I_o , for VCD, and the enzyme inhibition constant (K_i) was derived from the ascending part of the plot. Lineweaver–Burke plots of $1/V$ versus $1/S$ in the presence of different inhibitor concentrations were plotted (28).

Selection of the model

To determine which inhibition model best described the data, we utilized the Akaike Information Criterion (AIC) (29),

$$\text{AIC} = 2k - 2\ln(L) \quad (\text{iv})$$

where k = number of parameters and L = maximized value of the likelihood function of the model. For small sample sizes, the AIC is corrected and is given as AICc (30),

$$\text{AICc} = N \times \ln(\text{ss}/N) + 2K + \frac{2K(K+1)}{N - K - 1} \quad (\text{v})$$

where ss is the sum of squares from the fit, N is the number of experimental observations and K is the number of parameters in the model.

The probability that the model is correct can be determined by the following equation, where Δ is the difference between AIC scores (30)

$$\text{Probability} = \frac{e^{-5\Delta}}{1 - e^{-5\Delta}} \quad (\text{vi})$$

For this study with AA as a substrate, the lowest AICc was found for the *uncompetitive inhibition* model, as reported for VPA and PIA (23, 25, 28)

Data are presented as mean $\pm$ S.D. Linear regression analyses for obtaining K_m , V_{max} , K_i and other parameters were made using GraphPad Prism Version 5.0 (GraphPad Software).

Results

First, we measured conversion of AA to AA-CoA by Acs14 in absence of VCD. As previously described, the kinetics followed a simple Michaelis–Menten model, as illustrated in Figure 2A and showed substrate inhibition (23, 25). The mean apparent K_m was $4.63 \pm 0.67 \mu\text{M}$ and apparent V_{max} was $137.1 \pm 4.80 \text{ nmol/min/mg}$ ($n = 3$ independent experiments), consistent with reported values (23, 25).

To determine the effect of VCD on the kinetics of the reaction, VCD was added to the assay system. As illustrated in Figures 2A and 2B, VCD (1.25, 2.5, and 5.0 mM) inhibited activation of AA to AA-CoA by Acs14, with an apparent K_i of $6.38 \pm 0.63 \text{ mM}$ ($n = 3$). When calculating the Lineweaver–Burke plots in Figure 2C, we considered substrate AA concentrations only in the rising phase of the V (velocity) versus [AA] curves, from 0 to 35 μM AA (Fig. 2B). At higher AA concentrations the enzyme showed substrate inhibition (Fig. 2A). The Lineweaver–Burke plot of Figure 2C showed parallel slopes, which is characteristic of uncompetitive inhibition by VCD (28). The difference between AICc values for the uncompetitive and noncompetitive enzyme inhibition models was 2.595 (Eq. v), which means that the probability that the uncompetitive model was correct was 79%, compared to 21% for the noncompetitive model (Eq. vi). In uncompetitive inhibition, the inhibitor binds to the enzyme-substrate complex [ES] only and not to free enzyme [E], while in noncompetitive inhibition the inhibitor binds to [E] or [ES]. The Lineweaver–Burke plot for a noncompetitive inhibitor shows increases in slopes with the presence of the inhibitor.

As an additional control, we measured Acs14 activity in the presence of sodium butyrate, a 4-carbon analog of VPA that also inhibits histone deacetylase (26). As reported (23, 25), sodium butyrate did not inhibit Acs14 activity at a concentration of 60 mM (data not shown). We also did not find any Acs14 activity with the empty vector, consistent with our earlier observations (data not shown) (23, 25).

Discussion

We examined inhibition of the conversion of AA to AA-CoA by the non-teratogenic VPA analogue, VCD, using rat recombinant Acs14 *in vitro* (23, 25). Similar to VPA and PIA, VCD inhibited Acs14 conversion by an uncompetitive mechanism, whereas butyrate had no measurable inhibitory effect. VCD inhibited Acs14 activity with an apparent K_i of 6.38 mM, much less than the reported K_i of 25 mM for VPA and of 11.4 for PIA (23, 25). These values suggest that VCD would be more effective *in vivo* on an equi-concentration basis than either VPA or PIA.

As for VPA and PIA (23, 25), an uncompetitive pattern of inhibition demonstrated by Michaelis–Menten kinetics was consistent with the parallel Lineweaver–Burke plots, and it

had a high probability compared with other mechanisms using the Akaike Information Criterion (AIC). Uncompetitive inhibition implies that VCD binds to the Acsl4-AA substrate complex at a different binding site than does substrate AA, and thereby causes a conformational change that reduces enzyme activity and AA activation to AA-CoA (28).

A therapeutically relevant dose of VPA (200 mg/kg, i.p.) in rats produced a brain VPA concentration of 1.0 to 1.5 mM (31, 32), about 10-fold less than VPA's K_i when inhibiting recombinant Acsl4 *in vitro*. It was suggested that, if VPA inhibition of rat brain AA turnover *in vivo* is due to its inhibition of brain Acsl4, then VPA must be concentrated 10-fold at the enzyme site (23). This is possible, since studies indicate that VPA can accumulate, *via* a short-chain fatty acid transporter, in cellular mitochondria, microsomes and other organelles in which Acsl4 is found (33–38). A similar consideration would apply to VCD, whose brain concentration at its therapeutically relevant dose (20 mg/kg, i.v.) in rats reaches 0.6 mmol/kg (8), also about 10-fold less than its *in vitro* K_i with recombinant Acsl4. Additionally, for each drug, the *in vivo* kinetic inhibition constant for Acsl4 may be less than the measured *in vitro* value, as the latter depends on bath conditions like pH, temperature, salt and ATP concentrations, and on the absence of transporter proteins that are found in the intact brain (39). VCD is retained in the body (in dogs) for a relatively long period of time, and plasma levels of its corresponding acid, valnoctic acid, are much lower than those of VPA (4).

VCD has been shown to be more effective than placebo as an add-on to risperidone in manic patients (13). VPA, approved by the FDA in 1995 for treating bipolar mania, is a popular alternative to lithium for BD. Chronically given VPA to rats reduces AA turnover and downregulates markers of the brain AA cascade as do the other FDA approved mood stabilizers (14, 15, 40). Since markers of the cascade are upregulated in the postmortem BD brain, in association with evidence of excitotoxicity, neuroinflammation, apoptosis and synaptic loss (16, 17), dampening of the brain AA cascade by VPA and other mood stabilizers may contribute to their efficacy in BD (14, 40). Several mechanisms of action for VPA have been proposed such as inhibiting voltage gated Na^+ channels, potentiation of GABAergic activity by GABA-transaminase inhibition, Inhibition of T-type Ca^{2+} channels, suppression of NMDA-mediated excitation, HDAC inhibition and reduced AA turnover. Because it inhibits the chromatin-modifying enzyme, HDAC VPA also is teratogenic (10, 11). As such, it poses a significant fetal risk for pregnant women taking the drug (12), thus justifying the need for a non-teratogenic yet equipotent mood stabilizer that may act by the same mechanism as VPA. Identifying a pharmacological brain target of VPA with regard to BD could lead to rational development of effective VPA-like compounds with fewer side effects, including teratogenicity, such as VCD.

It remains to be determined experimentally whether the observed inhibition by VCD of AA to AA-CoA conversion by recombinant Acsl4 *in vitro* corresponds to VCD's ability to reduce AA turnover within rat brain phospholipids *in vivo*, as we found with VPA. Showing this would suggest that VCD or similar non-teratogenic inhibitors of Acsl4 *in vitro* be considered further for drug trials in BD patients (13) and, furthermore, that the recombinant Acsl4 inhibition assay could be used for screening for new VPA-like mood stabilizers.

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Valproic acid (VPA)

Valnoctamide (VCD)

Fig. 1.
Structure of valproic acid (VPA) and valnoctamide (VCD).

Fig. 2. (A) Initial reaction velocity [V] (nmol/min/mg protein) of acyl-CoA synthetase (Acs14) plotted against increasing arachidonic acid (AA) concentration (0 to 185 μM concentration) [S] in the presence of 0, 1.25, 2.5, or 5.0 mM VCD [I], showing substrate inhibition. (B) Typical plot of V (nmol/min/mg protein) of Acs14 plotted against increasing AA concentration (0 to 35 μM concentration) [S] in the presence of 0, 1.25, 2.5, or 5.0 mM VCD [I]. (C) Typical Lineweaver–Burke plot of the reciprocal of reaction velocity ($1/V$) against inverse of substrate concentration, $1/[S]$ ($1/[\text{AA}]$), within AA concentration from 0 to 35 μM

(see **Results**). The plot is typical of three experiments as indicated in text. Parallel plots are characteristic of uncompetitive inhibition (28).