BIPOLAR DISORDERS
AN INTERNATIONAL JOURNAL OF PSYCHIATRY AND NEUROSCIENCES

Bipolar Disorders 2015: 17: 615–625

BIPOLAR DISORDERS

Original Article

Comparative teratogenicity analysis of valnoctamide, risperidone, and olanzapine in mice

Wlodarczyk BJ, Ogle K, Lin LY, Bialer M, Finnell RH. Comparative teratogenicity analysis of valnoctamide, risperidone, and olanzapine in mice.
Bipolar Disord 2015: 17: 615–625. © 2015 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd.

Objectives: Based on the recent findings from animal studies, it has been proposed that the therapeutic use of valnoctamide, an anxiolytic drug developed in the early 1960s, be extended to treat other neurological disorders such as epilepsy and bipolar disease. Given the scarcity of adequate data on its prenatal toxicity, a comparative teratogenicity study of valnoctamide and two of the most commonly used drugs to treat bipolar disorder, risperidone and olanzapine, was carried out in a mouse model system.

Methods: Pregnant dams were treated with the aforementioned three drugs at the dose levels calculated as an equal proportion of the respective LD_{50} values of these drugs. The main reproductive indices examined included the numbers of implantations and resorptions, viable and dead fetuses, and fetal gross, visceral and skeletal abnormalities.

Results: The outcomes of the present study indicated that olanzapine was the most teratogenic of the three drugs, inducing maternal-, embryo-, and fetotoxicity. Risperidone also exerted a significant prenatal toxicity, but its adverse effect was less pronounced than that induced by olanzapine. Valnoctamide did not show any teratogenic effect, even when used in relatively higher dosages than olanzapine and risperidone. The observed increased skeletal abnormalities in one of the valnoctamide treatment groups were nonspecific and, as such, signaled a modest developmental delay rather than an indication that the compound could induce structural malformations.

Conclusions: Under our experimental conditions, valnoctamide demonstrated the lowest prenatal toxicity of the three tested drugs.

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doi: 10.1111/bdi.12325

Key words: comparative teratogenicity – mice – olanzapine – risperidone – valnoctamide

Received 20 February 2015, revised and accepted for publication 23 June 2015

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Valnoctamide (VCD) is a constitutional isomer of valpromide (VPD), the corresponding amide of valproic acid (VPA) (1). It was originally marketed as an anxiolytic drug in Europe in the 1960s (2). As VCD is an amide derivative of VPA, one of the most commonly used antiepileptic drugs (AEDs), it has recently been considered as a possible therapeutic agent for the treatment of seizure disorders. Analysis of the anticonvulsant properties of VCD in animal models demonstrated that this compound has a wide spectrum of anticonvulsant activity in various animal models, and is 3–10 times more potent than VPA (3–5). In addition to being used as an AED, VPA has also been used to treat several other neurologic disorders, such as migraine, bipolar disorder, neuropathic pain, anxiety, phobia, and depression. Given its broad range of therapeutic

applications, VPA poses a high risk for pregnant women owing to its well-established teratogenicity (6–8). The teratogenic mechanism of VPA is believed to involve histone deacetylase (HDAC) inhibition (9, 10). Unlike VPA, VCD does not inhibit the HDACs, and studies in mice, rats, and rabbits indicated that it was significantly less likely to induce birth defects as compared to VPA (11–13). On the other hand, VCD, like VPA, induces cellular inositol depletion via inhibition of myo-inositol-1-P synthase, which is proposed to be one of its antiepileptic and anti-bipolar modes of action (14, 15). More recently, it has been demonstrated that VCD also inhibits noncompetitively the acylation of arachidonic acid (AA) by acyl-CoA synthetase 4, although at a very high Ki value of 6.38 mM (>10 and >30 times VPA and VCD therapeutic plasma levels, respectively) (16). This mechanism of anti-bipolar action has been previously described in VPA studies (17). Chronic treatment of rats with VPA reduced AA turnover and downregulated markers of the AA cascade in the brain, as did the other mood stabilizers (18). As markers of this cascade were found to be upregulated in the postmortem bipolar disorder brain, inhibition of the brain AA cascade by VPA, VCD, and other mood stabilizers is hypothesized to have a therapeutic effect in bipolar disorder. Based on the above findings, VCD has been considered as an alternative for VPA treatment in women of childbearing age suffering from a bipolar disorder. Initial clinical trials measuring the efficacy of VCD as an add-on to risperidone (RIS) for bipolar disorder treatment showed promising results (19). The follow-up Phase IIb randomized, double-blind, placebo- and RIS-controlled parallel group study, which evaluated the efficacy of VCD monotherapy in the treatment of patients with bipolar disorder, was terminated early owing to the higher dropout rate of subjects treated with VCD as compared to RIS and placebo groups (20).

As most, if not all, commercially available AEDs are teratogenic, it is essential to develop new AEDs that are nonteratogenic and safe for use in women of reproductive age (21, 22). This is particularly crucial for VPA, as on October 24, 2014, the Coordination Group for Mutual Recognition and Decentralized Procedures–Human (CMDh), part of the European Medicines Agency (EMA), decided to strengthen the restrictions on the use of VPA owing to the risk of malformation and developmental problems in children exposed to this drug *in utero* that might be associated with autistic spectrum disorder and childhood autism (three to five times higher than in the general population) (23).

RIS and olanzapine (OLA), second-generation antipsychotic medications, are most commonly used for the treatment of patients with bipolar disorder (24). Both of these drugs have been classified by the Food and Drug Administration (FDA) as 'pregnancy category C pharmaceuticals', suggesting that in the animal reproduction studies they showed an adverse effect on the fetus, but there are no adequate human epidemiological studies upon which to determine teratogenicity. Based on this classification, potential therapeutic benefits may still warrant use of these drugs in women of childbearing age, in spite of the potential risk. The readily available literature concerning the reproductive toxicity of RIS and OLA is limited. The only data available for review were supplied by the manufacturers of these drugs (Janssen Pharmaceutical, Ltd., Eli Lilly & Co., Caduceus Pharma, Ltd.) as the prescribing information (25–27), not independent publications in the medical literature. As VCD has been considered for the treatment of bipolar disorder, and because of the dearth of existing information on the prenatal toxicity of RIS and OLA, the aim of the present study was directly to compare the teratogenic potency of these three drugs, using the same animal model. For this purpose, we used SWV mice that have proved to be sensitive to AED-induced teratogenicity, and have been used in our laboratory for several years to test the teratogenicity of VPA and numerous of its central nervous system-active newer analogs and derivatives (28–30).

Methods

In order to be able to compare the teratogenicity of the drugs of interest directly, we modified the standard rationale for dosage selection. In a standard segment II (teratogenicity) study, a drug is usually tested at three dose levels. The highest tested dose should exert some maternal toxicity, the lowest dose should not produce any adverse effect to pregnant dams or to their embryos/fetuses (NOAEL), and the middle dose is used to demonstrate a possible dose-related response. However, this standard approach should not be used in situations where the teratogenicity of different drugs needs to be compared. For that purpose, a different methodology can and should be applied. The possible options include the following:

- Comparing the teratogenicity of different drugs by applying the same dose (as a mg dose/kg of body weight) of the comparison compounds. This is the most simplistic approach but not well-suited to the present study.

- Comparing the teratogenicity of different drugs by applying an equimolar dose (as a mole/kg of body weight) of the compared compounds. This is usually used to compare isomers, analogs, and compounds that belong to the same class of chemicals. This method compensates for the differences in the molecular weight of the test compounds.
- Comparing the teratogenicity of different drugs by applying doses equivalent to human doses – for example, the maximal recommended human dose (MRHD). This is the most commonly used method when assessing the teratogenicity of a new drug in humans. However, because of several obvious metabolic and physiological differences between laboratory rodents and humans, this method serves only as a rough approximation when extrapolating the results from animal models to humans. As this method does not allow for the accurate prediction of teratogenicity in humans, it is even less well suited for comparing the teratogenicity of different drugs.
- Comparing the teratogenicity of different drugs by administering a dose that is based on the acute toxicity of each compound. In situations where there is a significant difference in the toxicity of comparison compounds, the best approach is to use each compound at the dose representing the same proportion of its lethal dose, 50% (LD_{50}) value. This method allows one to compare directly any specific toxic effect (e.g., teratogenic) of the different drugs, compensating for their different acute toxicities. This *compound teratogenic index* estimates the fraction of its LD_{50} at which the compound exerts its teratogenicity. The smaller the fraction/proportion of the LD_{50}, the greater the teratogenicity of the compound.

As the drugs tested in the present study belong to different classes (as chemical compounds and as drugs), have significantly different molecular weights, and have an even more dissimilar acute toxicity (RIS is 16 times more toxic then VCD, based on their LD_{50}s), we decided to compare their teratogenicity using the approach based on their acute toxicity. More specifically, we followed this approach to establish the appropriate doses of each compound for testing.

Rationale for dosage selection

Based on the results of our previous study with VCD (13), as well as on the acute toxicity of VCD, RIS, and OLA, we chose the first dose of VCD for testing to be 152.8 mg/kg. This dose, after its conversion to human equivalent dose (HED) (12.4 mg/kg), based on the body surface area, is in the range of the recommended human dose (31). This selected dose corresponds to the fraction 1/6.54 of the VCD LD_{50} value (999:152.8 = 6.54). For this reason, the comparable doses of RIS and OLA were calculated as the same 1/6.54 fraction of their respective LD_{50} values (63:6.54 = 9.6 mg/kg for RIS; 210:6.54 = 32.1 mg/kg for OLA). As there were no symptoms of toxicity observed in the VCD- and RIS-treated mice, as a next step we decided to increase the dose for these two drugs. We choose the 1/4.36 fraction of $LD_{50,}$ which for VCD is equivalent to 229.2 mg/kg. This is also within the range of the lower dose (1.8 mmol/kg) tested in our previous study (13). For RIS, the 1/4.36 fraction of LD_{50} is equivalent to 14.5 mg/kg. On the other hand, the dosage of OLA used initially (32.1 mg/kg) induced significant maternal toxicity. Owing to this observed adverse effect, we could not increase the OLA dose to match the 1/4.36 fraction dose that we selected for RIS and VCD. Instead, we had to lower the OLA dose by 50%, to 16.05 mg/kg, which corresponds to 1/13.1 fraction of its LD_{50} value. Lastly, as we failed to observe any teratogenicity induced by VCD at the intermediate dosage, we decided to test VCD using an even higher dose of 304.6 mg/kg. All RIS, OLA, and VCD doses used in the present study are summarized in Table 1.

Animals and housing

SWV mice were housed in the Dell Pediatric Research Institute Vivarium, which is fully accredited by AAALAC. The animals were maintained in clear polycarbonate microisolator cages and were allowed free access to food and water (PicoLab Diet 20 no. 5053, LabDiet, Brentwood, MO, USA). The mice were maintained on a 12-hour light/dark cycle. Nulligravid females, 50–70 days

Table 1. Summary of the risperidone (RIS), olanzapine (OLA), and valnoctamide (VCD) doses used in this study

mg/kg	RIS	OLA	VCD
LD_{50} mouse, oral	63	210	999
1/6.54 of LD_{50}	9.6	32.1	152.8
1/4.36 of LD_{50}	14.5	Not tested[a]	229.2
1/13.1 of LD_{50}	Not tested	16.05	Not tested
1/3.28 of LD_{50}	Not tested	Not tested	304.6

LD_{50} = lethal dose, 50%.
[a]OLA could not be tested with the dose equal to 1/4.36 of its LD_{50} owing to significant maternal toxicity observed in mice treated with at the lower dose (32.1 mg/kg or 1/6.54 of LD_{50}).

of age, were mated overnight with males and examined for the presence of vaginal plugs the following morning. If a plug was observed, the onset of gestation ([gestation day (GD) 0:0; 0 days: 0 hours] was considered to be 1:00 a.m. on the previous night, the midpoint of the dark cycle (32).

Test compounds and treatment regimen

The test compounds included:

(i) VCD: 2-ethyl-3-methylpentanamide (Zyfine, Ahmedabad, Gujarat, India, Lot # 2).
(ii) RIS: 3-(2-(4-(6-fluoro-1,2-benzisoxazol-3-yl) piperidino)ethyl)-6,7,8,9-tetrahydro-2-methyl-4H-pyrido(1,2-a)pyrimidin-4-one (Sigma-Aldrich Co., St. Louis, MN, USA, Lot # 041M4706V).
(iii) OLA: 2-methyl-4-(4-methyl-1-piperazinyl)-10H-thieno(2,3-b)(1,5)benzodiazepine (Sigma-Aldrich Co., St. Louis, MN, USA, Lot # 072M4720V).

All compounds were dissolved/suspended in a 25% water solution of Cremophor EL (Sigma-Aldrich, Fluka, St. Louis, MO, USA). Treatments were administered by oral gavage (p.o.) at a volume of 10 μL/g body weight. The control dams were gavaged with a 25% water solution of Cremophor EL (vehicle). Twenty pregnant females were randomly assigned to each of the eight treatment groups:

- Control (25% Cremophor EL)
- RIS 9.6 mg/kg
- RIS 14.5 mg/kg
- OLA 16.05 mg/kg
- OLA 32.1 mg/kg
- VCD 152.8 mg/kg
- VCD 229.2 mg/kg
- VCD 304.6 mg/kg

The dams were weighed and treated daily from GD 4 to GD 17. On GD 18, all dams were euthanized.

Observations and measurements

All animals were observed at least once/day during the study period for health monitoring. Gross maternal body weights were measured on the plug day (GD 0), the days of treatment (GD 4 to GD 17), and on GD 18, when the dams were euthanized. In order to calculate the net weight gain of the dams, the weight of the gravid uterus was recorded. Litters were assessed by counting the number of implants, resorptions/dead, normal, and abnormal fetuses. A detailed external examination of each viable fetus was conducted. For each litter, about half of the fetuses were processed for double staining with Alizarin Red S/Alcian Blue (Sigma-Aldrich, St. Louis, MO, USA), and subsequently examined for abnormalities in skeletal development (33). The remaining fetuses were fixed in Bouin's solution (Sigma-Aldrich) and examined for visceral malformations using the Wilson free-hand razor-blade sectioning technique (34). The type and incidence of external, visceral, and skeletal abnormalities were recorded.

Statistical analysis

Analysis of variance using the Tukey–Kramer multiple comparison test was used to evaluate the differences between treatment groups, for the mean values of continuously distributed variables (i.e., maternal body weight, gross and net weight gain, and fetal weight). In cases where the group failed the normality test, the Mann–Whitney test was applied.

Nonparametric statistics, including the Kruskal–Wallis test with Dunn's post-test comparisons, were used to evaluate the differences between the treatment and control groups in the distribution of the number of implantations, resorptions, and dead and live fetuses, as well as fetuses with congenital abnormalities. For comparisons, each litter was used as a statistical unit. All statistical analyses were conducted using Graph-Pad InStat (version 3.06; GraphPad Software, San Diego, CA, USA). The differences between the comparison groups were considered to be statistically significant when the p-value was ≤ 0.05.

Results

Maternal body weight

The initial (GD 0) mean body weight of dams taken at the recognition of pregnancy in all treatment and control groups did not differ significantly from each other ($p > 0.05$) (Table 2). The terminal (GD 18) mean body weight of dams treated with OLA 32.1 mg/kg was significantly lower than the control dams (34.9 g versus 49.1 g, respectively) ($p < 0.05$). The OLA-treated dams gained significantly less weight than did the controls (less than 50% of the average weight gain in control group) ($p < 0.05$). The only other treatment that resulted in a lower weight gain was the high dose of RIS (14.5 mg/kg). Dams in this group gained, on average, 18.1 g during the pregnancy, which was barely enough to show the significant statistical difference compared with the 21.0 g weight gain in the con-

Table 2. Weight gain in mice treated orally with RIS, OLA, and VCD on GDs 4–17

Compound	Control	RIS	RIS	OLA	OLA	VCD	VCD	VCD
Dose, mg/kg	0	9.6	14.5	16.05	32.1	152.8	229.2	304.6
Pregnant dams, n	20	20	20	21	20	20	20	20
Dams weight GD 0	28.1 ± 3.4	27.5 ± 2.3	28.9 ± 3.7	27.8 ± 2.7	26.9 ± 2.0	28.2 ± 2.4	27.2 ± 2.6	27.7 ± 2.8
Dams weight GD 18	49.1 ± 4.0	46.9 ± 4.8	47.1 ± 3.2	46.5 ± 3.6	34.9 ± 4.0[a]	48.5 ± 3.4	47.3 ± 4.2	48.2 ± 2.9
Dams weight gain	21.0 ± 2.9	19.4 ± 4.2	18.1 ± 2.5[a]	18.7 ± 3.8	8.0 ± 3.5[a]	20.2 ± 3.0	20.1 ± 2.9	20.5 ± 3.5
Dams net weight gain	9.1 ± 2.5	7.5 ± 2.0	7.4 ± 2.3[a]	8.8 ± 1.8	5.6 ± 2.0[a]	7.9 ± 2.1	7.8 ± 2.1	8.6 ± 1.8

Data presented in grams as average per group (mean ± standard deviation). The highlighted columns represent the matching doses for different drugs:
1/6.54 of LD_{50}.
1/4.36 of LD_{50}.
GD = gestational day; LD_{50} = lethal dose, 50%; OLA = olanzapine; RIS = risperidone; VCD = valnoctamide.
[a]Significantly different from the control group.

trol group ($p < 0.05$). In order to exclude the influence of litter size on weight gain, the net weight gain was also calculated. This lower net weight gain observation was statistically confirmed in both groups (OLA 32.1 mg/kg and RIS 14.5 mg/kg), suggesting the presence of a slight maternal toxicity.

Litters

The litter reproductive parameters are summarized in Table 3. The number of implantations was not significantly different between the groups ($p > 0.05$); however, the number of live fetuses was significantly lower in the high dose of OLA (32.1 mg/kg) treatment group, compared to controls ($p < 0.05$). This decrease in the number of live fetuses was striking (mean = 13.1 in controls versus 3.4 in the OLA group), which was further confirmed by the significantly increased number of resorptions in the same experimental group ($p < 0.001$). Almost 74% of the conceptuses were resorbed in mice treated with 32.1 mg/kg OLA, compared to 7% resorbed in the control group ($p < 0.001$). In the OLA low-dose treatment group, 15% of the embryos were resorbed. This relatively high percentage was skewed owing to the two litters with unusually high resorption rates (eight out of 13 and 11 out of 13, respectively). The group treated with the high dose of RIS (14.5 mg/kg) also had a higher than control resorption rate of 12.4% ($p > 0.05$). The average fetal weight in both of the OLA-treated groups and the group treated with the high RIS dose (14.5 mg/kg) was significantly lower than the control value ($p > 0.05$). Consistent with the other recorded parameters, the lowest fetal weight was documented in mice treated with the high dose of OLA (32.1 mg/kg).

With respect to the fetal gross morphological evaluation, there was only one fetus found with exencephaly in the RIS 14.5 mg/kg treatment group. Detailed examination revealed that there were significantly more fetuses with visceral abnormalities in both RIS-treated groups [7% ($p < 0.05$) and 10.8% ($p < 0.005$)], and in the group treated with OLA at 32.1 mg/kg [21.4% ($p < 0.01$)], compared to the control group (0.6%) (Table 4).

The vast majority of the detected visceral abnormalities consisted of cleft palates, which were found in both RIS and OLA-treated mice (Table 4). Significant numbers of fetuses with clefts were found at both RIS dosages ($p < 0.05$), but not in the control group. Less frequently, cleft palate was observed in both groups treated with OLA. None of the fetuses in the VCD treatment groups presented with cleft palate. Another frequently observed abnormality was dilated renal pelvis. This mild dilation was observed in single fetuses in almost all treatment groups, including controls (Table 4). Dilated brain ventricles were noted in four fetuses, two in the high-dose RIS group and two in the high-dose OLA group. One fetus in the highest-dose VCD group was found with a right aortic arch and a stomach malposition (Table 5).

The skeletal abnormalities and anomalies were much more common, and were found in fetuses from groups treated with all three test compounds (Tables 4 and 6). The numbers of fetuses with skeletal abnormalities in both OLA-treated groups, the high-dose RIS group, and the intermediate-dose VCD group were significantly higher than in the control group ($p < 0.0001$, $p < 0.005$ and $p < 0.05$, respectively). The highest percentage of affected fetuses was found in both OLA-treated groups (approximately 50% and 80%, respectively). The most commonly observed anomalies concerned the sternum, and metacarpal and metatarsal bones. Less frequently, skull bones, ribs, or vertebra were affected. The anomalies most frequently noticed in the fetuses were incomplete ossification and absent ossification (Table 6), which

Table 3. Pregnancy outcomes in mice treated orally with RIS, OLA, and VCD on GD 4–17

Compound	Control	RIS	RIS	OLA	OLA	VCD	VCD	VCD
Dose, mg/kg	0	9.6	14.5	16.05	32.1	152.8	229.2	304.6
Pregnant dams, n	20	20	20	21	20	20	20	20
Implantations/litter	14.3 ± 2.0	14.5 ± 2.2	15.0 ± 1.6	14.3 ± 1.2	13.1 ± 2.7	14.9 ± 1.7	14.8 ± 1.6	14.5 ± 1.7
Live fetuses/litter	13.2 ± 1.9	13.2 ± 3.2	12.9 ± 1.9	12.0 ± 3.3	3.4 ± 3.2[a]	13.8 ± 2.1	13.7 ± 2.0	13.2 ± 2.8
Dead fetuses/litter	0.2 ± 0.5	0.2 ± 0.7	0.2 ± 0.4	0.1 ± 0.5	0	0.2 ± 0.4	0.1 ± 0.3	0.1 ± 0.2
Resorptions/litter	1.0 ± 0.8	1.1 ± 1.1	1.8 ± 1.2	2.1 ± 2.8	9.6 ± 3.9[a]	1.0 ± 0.9	0.9 ± 0.8	1.2 ± 1.4
Fetus weight, g	0.90 ± 0.08	0.89 ± 0.06	0.83 ± 0.05[a]	0.83 ± 0.10[a]	0.66 ± 0.09[a]	0.91 ± 0.07	0.90 ± 0.07	0.91 ± 0.06
Fetus gender ratio F/M	67/73	66/69	66/63	65/61	18/18	71/73	58/77	57/73

Data presented as average per litter (mean ± standard deviation). The highlighted columns represent the matching doses for different drugs:
1/6.54 of LD_{50}.
1/4.36 of LD_{50}.
F = female; GD = gestational day; LD_{50} = lethal dose, 50%; M = male; OLA = olanzapine; RIS = risperidone; VCD = valnoctamide.
[a]Significantly different from the control group.

are signals of developmental delay rather than overt structural teratogenicity. The only true defects noted were fusion of the cervical vertebra (one fetus in the high-dose VCD group) and a bent fibula (one fetus in the intermediate-dose VCD group).

Comparison of teratogenic potency of OLA, RIS, and VCD

Based on the findings of the present study and the applied direct dose comparison based on the acute toxicity of the test compounds, it is possible to rank these drugs according to their teratogenic potential. When the drugs were administered to pregnant mice at a dose that was the same fraction (1/6.54) of their respective LD_{50} values, the most teratogenic compound was OLA. It induced maternal toxicity, embryo resorption rates of over 70%, significantly reduced fetal weight, structural malformations (cleft palate and dilated brain ventricles), and delayed fetal development. The effect of RIS at a comparable dosage was much less toxic than OLA as it induced only a limited number of structural defects (cleft palates) in fetuses. VCD at the corresponding treatment dose was the least developmentally toxic drug in the present study, with no statistically significant teratogenicity and only mild developmental delay (presented as skeletal anomalies) in 10% of treated fetuses. When the dose was increased to 1/4.36 of the LD_{50}, the developmental toxicity of RIS also increased. These dams showed symptoms of toxicity (e.g., lower weight gain), an increase in the resorption rate and in the number of fetuses with structural malformations, and a developmental delay, as evidenced by lower fetal weight and delayed skeletal ossification. VCD treatment at the higher dose did not induce any obvious developmental toxicity; about the same fraction (11%) of fetuses like at the lower dose was classified as developmentally delayed based on their incomplete skeletal ossification. OLA was not tested at a higher dose owing to the toxicity observed at the initial dosage. The second dose of OLA was actually the original starting dose decreased by half, to 1/13.1 of its LD_{50}. Even with this lowered dose, OLA treatment resulted in an increased percentage of resorbed embryos ($p > 0.05$) and a significantly decreased average fetal weight ($p < 0.05$) relative to controls. Additionally, almost 50% of the fetuses in this OLA-treated group showed some skeletal abnormalities indicative of developmental delay. As VCD did not exert any teratogenic effect at either of the two tested doses, an additional, 50% higher dose (1/3.28 of LD_{50}) was tried. This highest VCD dose

Table 4. Abnormalities in fetuses from mice treated orally with RIS, OLA, and VCD on GD 4–17

Compound	Control	RIS	RIS	OLA	OLA	VCD	VCD	VCD
Dose, mg/kg	0	9.6	14.5	16.05	32.1	152.8	229.2	304.6
Fetuses with external abnormalities, n (%)	0	0	1 (0.4)	0	0	0	0	0
Fetuses with visceral abnormalities, n (%)	1 (0.6)	9 (7.0)[a]	13 (10.8)[a]	2 (1.9)	6 (21.4)[a]	2 (1.3)	2 (1.8)	1 (0.6)
Fetuses with skeletal abnormalities, n (%)	3 (3.1)	9 (10.8)	25 (20.1)[a]	61 (48.4)[a]	23 (79.2)[a]	14 (10.4)	16 (11.1)[a]	6 (8.6)

The highlighted columns represent the matching doses for different drugs:
1/6.54 of LD_{50}.
1/4.36 of LD_{50}.
GD = gestational day; LD_{50} = lethal dose, 50%; OLA = olanzapine; RIS = risperidone; VCD = valnoctamide.
[a]Significantly different from the control group.

Table 5. External and visceral abnormalities in mouse fetuses from dams treated orally with RIS, OLA, and VCD on GD 4–17

Compound	Control	RIS	RIS	OLA	OLA	VCD	VCD	VCD
Dose, mg/kg	0	9.6	14.5	16.05	32.1	152.8	229.2	304.6
Total examined fetuses/litters	140/20	135/20	129/20	126/21	36/20	144/20	135/20	130/20
Exencephaly	0	0	1/1	0	0	0	0	0
Cleft palate	0	8[a]/6	8[a]/7	2/2	2/1	0	0	0
Dilated brain ventricles	0	0	2/2	0	2/2	0	0	0
Right aortic arch	0	0	0	0	0	0	0	1/1[b]
Stomach malposition	0	0	0	0	0	0	0	1/1
Dilated renal pelvis	1/1	2/2	3/2	0	2/2	2/2	2/2	0

The highlighted columns represent the matching doses for different drugs:
1/6.54 of LD_{50}.
1/4.36 of LD_{50}.
Data presented as number of affected fetuses/litters. GD = gestational day; LD_{50} = lethal dose, 50%; OLA = olanzapine; RIS = risperidone; VCD = valnoctamide.
[a]Significantly different from the control group.
[b]The right aortic arch was accompanied by an enlarged right ventricle.

proved to be safe for the dams as well as for the developing embryos/fetuses. There were no signs of developmental toxicity observed in this treatment group.

Discussion and conclusions

VCD has been marketed in Europe for more than 40 years as an anxiolytic drug, although, because of the low sales, the marketing was stopped in 2005. Owing to its wide spectrum of potent anticonvulsant activity in various animal models, including the kindled rats test, it has recently been investigated as a potential new AED, and more recently as a mood stabilizer in certain maniac depressive diseases (e.g., bipolar disorder) (5, 19, 35). In both cases, VCD is considered as an alternative for carbamazepine and VPA, two of the most effective and commonly prescribed AEDs that, unfortunately, have a well-established teratogenic side effect. The initial comparative toxicological studies demonstrated that VCD was significantly safer than VPA when administered to pregnant mice on midgestational Day 8. In a study using NMRI mice that were injected on GD 8 with 3 mmol/kg of VPA or its derivatives (VPD, valnoctic acid, or VCD), VCD did not induce any embryotoxicity, whereas VPA was highly embryo lethal (53% resorption rate) and teratogenic, inducing exencephaly in 52% of exposed fetuses (11). In another study using NMRI mice that were injected subcutaneously on GD 8 with 800 mg/kg of VPA or VCD, similar results were obtained with respect to resorption and exencephaly rates (36). Additionally, the latter study also showed that VPA induced a significant number of skeletal defects in mouse fetuses, whereas the VCD treatment was not teratogenic (36). Our preliminary study on SWV mice injected intraperitoneally on GD 8 with either 2.7 mmol/kg or 1.8 mmol/kg of VCD or its stereoisomers indicated that VCD had no teratogenic potential, as measured by the number of fetuses with exencephaly. At the higher dose (2.7 mmol/kg), VCD increased the number of resorptions compared to the control group (13). All of the aforementioned studies compared the teratogenic potential of VCD to that of VPA. For that reason, a simple treatment protocol was used;

Table 6. Skeletal abnormalities in mouse fetuses from dams treated orally with RIS, OLA, and VCD on GD 4–17

Compound	Control	RIS	RIS	OLA	OLA	VCD	VCD	VCD
Dose, mg/kg	0	9.6	14.5	16.05	32.1	152.8	229.2	304.6
Total examined fetuses/litters	121/20	130/20	129/20	125/21	33/20	130/20	140/20	134/20
Whole skeletal system								
Incomplete ossification	0	1/1	2/1	0	7/3[b]	0	0	0
Skull								
Incomplete ossification	0	3/2	6/1	0	1/1	6/2	1/1	0
Parietal[a]	0	0	1/1	3/2	0	0	0	0
Interparietal[a]	0	0	0	4/3	1/1	1/1	0	1/1
Supraoccipital[a]	0	0	0	1/1	1/1	1/1	0	0
Sternebra								
Incomplete ossification	3/3	5/4	13/9[b]	25/11[b]	8/7	0	3/2	1/1
Absent ossification	0	0	7/2[b]	4/4	8/5[b]	2/2	0	1/1
Misaligned	0	0	0	0	0	1/1	0	0
Misshapen	0	3/3	1/1	0	0	0	6/4	3/2
All vertebra								
Incomplete ossification	0	0	0	0	3/1	0	0	0
Cervical vertebra								
Ossified fusion of centrums	0	0	0	0	0	0	0	1/1
Ribs								
Absent 13th	0	0	0	0	1/1	3/1	3/3	0
Branched	0	0	0	0	0	1/1	1/1	0
Fibula								
Bent	0	0	0	0	0	0	1/1	0
Metacarpal								
Incomplete ossification	0	1/1	0	47/12[b]	6/3	0	0	0
Absent ossification	0	0	6/1	1/1	12/8[b]	0	0	1/1
Metatarsal								
Incomplete ossification	0	1/1	0	20/8[b]	4/3	0	0	0
Absent ossification	0	0	6/1	8/8[b]	14/9[b]	0	0	1/1

The highlighted columns represent the matching doses for different drugs:
1/6.54 of LD_{50}.
1/4.36 of LD_{50}.
Data presented as number of affected fetuses/litters. GD = gestational day; LD_{50} = lethal dose, 50%; OLA = olanzapine; RIS = risperidone; VCD = valnoctamide.
[a]Incomplete ossification.
[b]Significantly different from the control group.

that is: a single, parenteral administration of the test drugs to pregnant dams on GD 8. This standard treatment has been utilized effectively for several decades in ours and several other laboratories to demonstrate the teratogenicity of VPA in mice. This model system has repeatedly demonstrated that VPA induces congenital malformations (exencephaly), whereas VCD does not.

Following a successful Phase IIa study in patients with mania, Covance Laboratories conducted teratogenicity studies comparing VCD to VPA (head to head) in mice, rats, and rabbits. In these additional reproductive toxicity studies, VCD, in contrast to VPA, failed to demonstrate a teratogenic potential in mice and rabbits at therapeutic plasma concentrations. In rats, a modest teratogenic signal was observed at plasma concentrations 15 times higher than the suggested VCD therapeutic plasma levels (12).

As VCD is currently being considered as a therapeutic agent for the treatment of epilepsy and other neurological disorders such as bipolar disorder, the aim of the present study was to compare the teratogenic potential of this drug with the two other leading antipsychotic drugs (RIS and OLA) used for the treatment of bipolar disorder, to determine their relative reproductive toxicity. Although the clinical use of antipsychotic drugs has increased significantly, the available human epidemiological data concerning the effects of RIS and OLA on pregnancy are still scarce and do not allow for adequate determination of the prenatal toxicity of these drugs. Verdoux and colleagues (37) demonstrated that the actual number of patients with psychiatric disorders has increased considerably over the last decade, and they are often treated with second-generation antipsychotic drugs. A recent large population-based study demonstrated that depression and bipolar disease were the most prevalent indication for antipsychotic treatment in pregnant women (38). In spite of this growing problem, the available antipsychotic drug treatment options for pregnant

women remain controversial and, unfortunately, limited. This stems largely from insufficient reliable epidemiological data and animal studies, which complicates risk estimations concerning the teratogenicity of these compounds (39). The most up-to-date systematic review of published epidemiological studies concerning the risk of congenital malformation after exposure to selected antipsychotic drugs was recently published and included, among others, both OLA and RIS (40). This meta-analysis indicated that exposure to OLA during the first trimester was not associated with an increased risk of congenital malformations, and that exposure to RIS was not associated with a substantially increased risk. The malformation rates in this analysis were estimated at 3.5% and 5.1% for OLA and RIS, respectively (40). It should be noted that the data analyzed in this meta-study had no control for confounders, as a consequence of which the significance of concomitant medication (common in clinical practice), such as another antipsychotic, antidepressant, anticonvulsant, or anxiolytic agent, remained largely unaccounted for. It is difficult to assess a drug's teratogenicity based on human epidemiologic data owing to the typically small number of cases available to study, resulting in insufficient statistical power. This dilemma becomes even more complicated in the case of polytherapy, owing to possible unwanted interactions between the drugs used. Unfortunately, the desired synergistic pharmacologic effect of polytherapy can be accompanied by adverse side effects. This situation has been observed in pregnant women undergoing polytherapy with AEDs. Generally, polytherapy regimens appear to exhibit an increased risk of teratogenicity when compared to monotherapy, especially when VPA is one of the compounds (41, 42). As VPA and several other AEDs (approved by the FDA) have been most commonly prescribed for the treatment of bipolar disorder, an unpredicted interaction with antipsychotic drugs (even though there are no known reports of teratogenic effects unique to this combination) in the case of polypharmacy is possible (43).

Owing to significant differences in acute toxicity and a lack of prenatal toxicity data in mice (RIS and OLA), we decided that the standard equimolar approach that we had used in several previous studies for comparisons of VPA to VCD or its other derivatives was not appropriate for the current study. Instead, we applied a method that allows direct comparison of the teratogenic effect of different drugs, compensating for their differing acute toxicities. A similar approach was advocated by Fabro and coworkers (44) in their proposed method of developmental toxicity risk assessment. They introduced the so-called *agent's teratogenic hazard*, which represents the relationship between the teratogenic dose and the adult toxic dose, arguing that, from a regulatory point of view, the most important factor is not whether an agent is teratogenic but whether an agent can exert developmental toxicity at a dose level significantly lower than the adult toxic dose. Additionally, for comparative investigations, a quantitative estimation of teratogenicity is valuable. The more traditional methods, such as comparison of a compound's potency using the effects of compounds at equimolar doses, comparing dose equivalents by weight, or even considering multiples of human dosages, all fail to consider the specific toxicity of the compound. Fabro and coworkers, in their study on mice, using groups of anticonvulsant drugs, demonstrated that incorporation of teratogenic hazard, measured as a relative teratogenic index, could be useful for comparative studies as well as for evaluating risk assessment. In the present study, we also utilized the relationship between the teratogenic and adult toxic dose. More specifically, we calculated the doses for teratogenicity testing based on the LD_{50} of the drugs. Further, by using the same fraction of their respective LD_{50} values, we were able directly to compare the embryo–fetal developmental toxicity of these drugs.

Under our experimental conditions, OLA turned out to be the most teratogenic of the three test drugs. It induced maternal, embryo-, and fetotoxicity as evidenced by: a significant decrease in the weight gain of the dams, an increase in the number of resorptions, visceral and skeletal abnormalities, as well as decreased fetal weight. RIS also induced maternal, embryo-, and fetotoxicity, although its adverse effect was less pronounced than that exerted by OLA. By contrast, VCD showed no teratogenic effect, even when used at a comparatively higher dose than used for OLA and RIS. It induced increased skeletal abnormalities but only when compared to the control group. These skeletal abnormalities signal a slight developmental delay, rather than true teratogenicity.

The results of clinical studies of VCD use, as monotherapy or polytherapy, for the treatment of patients with bipolar disorder are equivocal (19, 20). The Phase IIa clinical trial with racemic-VCD as an add-on to RIS in patients with acute mania showed that, in all efficacy measures, VCD was significantly more effective than placebo, with differences between the two groups being significant from treatment Weeks 3–5 (19). However, the Phase IIb study of VCD use, as a monotherapy, in a three-week, double-blind, ran-

domized, placebo- and RIS-controlled parallel group trial was terminated owing to a lack of efficacy in the treatment of patients with bipolar disorder in a manic or mixed episode (20). In this latter study, two cohorts of patients (n = 151 and n = 114) were analyzed, with a total dropout rate of 24.5%. The dropout rate of subjects treated with VCD (owing to a lack of clinical efficacy, rather than side effects) was higher compared to groups treated with RIS or with placebo, and resulted in the shorter follow-up duration. Analysis of the primary study endpoint for the first cohort demonstrated the superiority of the RIS treatment compared to placebo ($p = 0.0446$), while treatment with VCD showed a similar reduction in Young Mania Rating Scale (YMRS) score compared to placebo ($p = 0.9169$). By contrast, the second cohort analysis demonstrated the superiority of both RIS ($p = 0.0188$) and VCD ($p = 0.0159$) over placebo treatment in the change in the YMRS score from baseline at Week 3. The difference in the VCD effect between the first and second cohorts was unclear. The subsequent futility analysis demonstrated that, in the best-case scenario, there is a 57.1% conditional probability of study success in achieving the primary outcome measure for the VCD versus placebo comparison if the study continues to enroll the originally preplanned population (N = 313). Owing to the results of this analysis, the study was terminated. In conclusion, this Phase IIb study showed that patients can tolerate daily VCD doses of 1,500 mg with minimal side effects and that patients who stayed on VCD may have benefited from it. Nevertheless, VCD has a great potential in the treatment of epilepsy, particularly in light of the recent EMA restriction on the use of VPA in women and girls (23).

Acknowledgements

We would like to thank Kelly Huang and Rigoberto Gutierrez for their technical assistance with fetal preparation and assessment. This study was supported by a Stanley Medical Research Institute (SMRI) grant 12R-0001-A, as well as partial support from NIH grant HD072251.

Disclosures

The authors of this paper do not have any commercial associations that might pose a conflict of interest in connection with this manuscript.

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