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

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Abstract

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 aforementioned three drugs at the dose levels calculated as an equal proportion of the drugs' respective LD₅₀ value. Main reproductive indices examined included: numbers of implantations and resorptions, viable and dead fetuses, as well as fetal gross, visceral and skeletal abnormalities were assessed.

Results—Outcomes of this 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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Keywords

comparative teratogenicity; mice; olanzapine; risperidone; valnoctamide

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). Since VCD is an amide derivative of VPA, one of the most commonly used antiepileptic drug; it has recently been considered as a possible therapeutic agent for the treatment of seizure disorders. Analysis of VCD's anticonvulsant properties 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 antiepileptic drug, 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 due 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 on 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 uncompetitively acylation of arachidonic acid (AA) by acyl-CoA synthetase 4, although at a very high K_i 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 down-regulated markers of AA cascade in the brain as did the other mood stabilizers (18). Since markers of this cascade were found upregulated in the postmortem bipolar disorder brain, inhibiting of the brain AA cascade by VPA, VCD and other mood stabilizers is hypothesized to have 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 for bipolar disorder treatment showed promising results (19). The following-up *Phase IIb* randomized, double-blind, placebo and risperidone controlled parallel group study, which evaluated the efficacy of VCD monotherapy in the treatment of patients with bipolar disorder was terminated early due to the higher drop-out rate of subjects treated with VCD as compared to RIS and placebo groups (20).

Since most, if not all commercially available antiepileptic drugs (AEDs) are teratogenic, it is essential to develop new AEDs that are non-teratogenic and safe for use in women of reproductive age (21, 22). This is particularly crucial for VPA, since on October 24th, 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 due to the risk of malformation and developmental problems in children exposed to VPA in the womb that might be associated with autistic spectrum

disorder and childhood autism (three to five times higher than in the general population) (23).

Risperidone (RIS) and olanzapine (OLA), second-generation antipsychotic medications, are most commonly used for treatment of patients with bipolar disorder (24). Both of these drugs have been classified by the 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 despite the potential risk. The readily available literature concerning reproductive toxicity of RIS and OLA is very limited. The only data available for review was supplied by the drugs' manufacturers (Janssen Pharmaceutical, Ltd., Eli Lilly & Co., Caduceus Pharma, Ltd.) as the prescribing information (25–27), not independent publications in the medical literature. Since VCD has been considered for treatment of bipolar disorder, and the dearth of existing information on prenatal toxicity of RIS and OLA, the aim of this study was to directly 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 AEDs-induced teratogenicity, and have been used in our laboratory for years to test the teratogenicity of VPA and numerous of its CNS-active newer analogs and derivatives (28–30).

Methods

In order to be able to directly compare the teratogenicity of the drugs of interest, 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 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 but not well-suited approach.
- Comparing the teratogenicity of different drugs by applying 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, e.g., maximal recommended human dose (MRHD). This is the most commonly used method when assessing teratogenicity of a new drug with the purpose of predicting its teratogenicity in humans. However, due to several obvious

metabolic and physiological differences between laboratory rodents and humans, this method serves only as a rough approximation in extrapolating the results from animal models to humans. Since this method does not allow for the accurate prediction of teratogenicity in humans, it is even less well-suited to be used 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 that is the same portion of the respective drug's LD₅₀ value. This method allows one to directly compare any specific toxic effect (e.g., teratogenic) of the different drugs, compensating for their different acute toxicities. This *Compound Teratogenic Index* estimates at what fraction of its LD₅₀ the compound exerts its teratogenicity. The smaller the fraction/portion of the LD₅₀, the greater the teratogenicity of the compound.

Since the drugs tested in this study belong to different classes (as chemical compounds and drugs), have significantly different molecular weights, and an even more dissimilar acute toxicity (RIS is 16 times more toxic than VCD based on their LD₅₀), 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 1/6.54 fraction of the VCD-LD₅₀ 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 LD₅₀ values ($63:6.54 = 9.6$ mg/kg for RIS; $210:6.54 = 32.1$ mg/kg for OLA). Since 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 chose the 1/4.36 fraction of LD₅₀, 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 LD₅₀ 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. Due 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 LD₅₀ value. Lastly, since 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 this 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 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 AM of the previous night, the midpoint of the dark cycle (32).

Test compounds and treatment regimen

The test compounds included:

- i. VCD: 2-ethyl-3-methylpentamide, 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 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 μ L/g body weight. The control dams were gavaged with 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 gestational day (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–GD 17), and on GD 18, when the dams were euthanized. In order to calculate the dam's net weight gain, 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, and

subsequently examined for abnormalities in skeletal development (33). The remaining fetuses were fixed in Bouin's solution and examined for visceral malformations utilizing the Wilson free hand razorblade sectioning technique (34). The type and incidence of external, visceral, and skeletal abnormalities were recorded.

Statistical analysis

ANOVA with Tukey–Kramer multiple comparison test was used to evaluate 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.

Non-parametric statistics including the Kruskal–Wallis test with the Dunn's post-test comparisons were used to evaluate the differences between treatment and control group 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 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 21.0 g weight gain in the control 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 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 statistically 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 very 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 due to the two litters with unusually high resorption rates (8 out of 13 and 11 out of 13). The third group, with a higher resorption rate 12.4% ($p > 0.05$), were the mice treated with the high dose of RIS 14.5 mg/kg. The average fetal weight compared to the control value was significantly lower in the three groups ($p < 0.05$); both OLA-treated groups and the group treated with the high RIS dose (14.5 mg/kg). 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 of fetuses 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 observed 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 of 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 number of fetuses with skeletal abnormalities in both OLA-treated groups ($p < 0.0001$), high-dose RIS group ($p < 0.005$), and the intermediate dose of VCD group ($p < 0.05$), were significantly higher than in the control group. The highest percentage of affected fetuses was found in both OLA-treated group (approximately 50% and 80%, respectively). The most commonly observed anomalies concerned the sternum, 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 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 (also one fetus in middle dose VCD group).

Comparison of teratogenic potency of OLA, RIS, and VCD

Based on the findings of this 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 in 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, resorption rates of over 70% of the embryos, 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 this 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 LD₅₀ the developmental toxicity of RIS also increased. The dams showed symptoms of toxicity (e.g., lower weight gain), the resorption rate as well as number of fetuses with structural malformations increased, and developmental delay was noted 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 was classified as developmentally delayed based on their incomplete skeletal ossification. OLA was not tested at a higher dose due to the toxicity observed at the initial dosage. The second dosage of OLA was actually the original starting dosage decreased by half, to 1/13.1 of its LD₅₀. Even with this lowered dosage, OLA treatment resulted in an increased percentage of resorbed embryos ($p > 0.05$), and significantly decreased average fetal weight ($p < 0.05$). Additionally, almost 50% of the fetuses in this OLA-treated group showed some skeletal abnormalities indicative of developmental delay. Since the VCD did not exert any teratogenic effect in the two tested doses, an additional (third), 50% higher dose (1/3.28 of LD₅₀) was tried. This highest VCD dosage 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. Due to its potent and wide spectrum of anticonvulsant activity in various animal models, including the kindled rats test, it has recently been investigated as a potential new antiepileptic drug, and more recently as a mood stabilizer in certain manic 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 antiepileptic drugs 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 strongly embryolethal (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. Additionally, that 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 or 1.8 mmol/kg of VCD or its stereoisomers indicated that VCD did not possess a teratogenic potential as measured by the number of fetuses with exencephaly. At the higher dosage (2.7 mmol/kg), VCD increased the number of resorptions compared to the control group (13). All of these aforementioned studies compared the teratogenic potential of VCD to VPA. For that reason, a simple

treatment protocol was used, that is: a single, parenteral administration of the test drugs to pregnant dams on GD 8. This standard treatment has been effectively utilized for decades in ours and several other laboratories to demonstrate VPA's teratogenicity in mice. This model system has repeatedly demonstrated that VPA induced congenital malformations (exencephaly), whereas VCD did 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).

Since 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 this study was to compare the teratogenic potential of this drug with the two other leading antipsychotic drugs (RIS and OLA) used for bipolar disorder treatment to determine their relative reproductive toxicity. Although clinical use of antipsychotic drugs has increased significantly, the available human epidemiological data concerning the effects of RIS and OLA on pregnancy are still very scarce and do not allow for adequate determination of these drugs' prenatal toxicity. 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). Despite this growing problem, the available treatment options for pregnant women with antipsychotic drugs 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). The meta-analysis indicated that exposure to OLA during the first trimester was not associated with increased risk of congenital malformations, whereas the results for RIS suggested that it was not associated with substantially increased risk. The malformation rates in this analysis were estimated 3.5% and 5.1% for OLA and RIS, respectively. It should be noted that the data analyzed in this meta-study had no confounders control and as a consequence of that a significance of concomitant medication (common in clinical practice) such as another antipsychotic, antidepressant, anticonvulsant or anxiolytic remained largely unaccounted for. The assessment of drug's teratogenicity based on human epidemiologic data is very difficult due to typically small number of cases available to study which results in an insufficient statistical power. This dilemma becomes even more complicated in case of polytherapy, due to possible unwanted interaction between used drugs. Unfortunately the desired synergistic pharmacologic effect of polytherapy can be accompanied by the detrimental toxicological side effect. This situation has been observed in pregnant women undergoing polytherapy with antiepileptic drugs. Generally polytherapy

regimens appear to exhibit increased risk of teratogenicity when compared to monotherapy, especially when valproic acid (VPA) is one of the compounds (41, 42). Since VPA and several other antiepileptic drugs (approved by FDA) have been most commonly prescribed for bipolar disorder treatment, an unpredicted interaction with antipsychotic drugs (even though there are no known reports of teratogenic effects unique to this combination) in case of polypharmacy is possible (43).

Due to significant differences in acute toxicity and a lack of prenatal toxicity data on mice (RIS and OLA), we decided that the standard equimolar approach that we used in several previous studies for comparisons of the antiepileptic drug depakote (VPA) to VCD or its other derivatives, was not an adequate approach for this experiment. Instead, we applied a method that allows direct comparison of 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 so called *agent's teratogenic hazard*, which represents the relationship between the teratogenic dose to the adult toxic dose, arguing that from 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 dosage 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, or comparing dosage equivalents by weight, or even considering multiples of human dosages, all fail to consider the factor of specific compound toxicity. 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 very useful for comparative studies as well as for evaluation of risk assessment. In our study, we also utilized the relationship of teratogenic and adult toxic dose. More specifically, we have calculated the doses for teratogenicity testing based on the LD₅₀ of the drugs. Further, by using the same fraction of their respective LD₅₀ value, we were able to directly 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: significant decrease of dam's weight gain, increase of resorptions, visceral and skeletal abnormalities as well as decreased fetal weight. RIS was also maternal-, embryo-, and fetotoxic, although its adverse effect was less pronounced compared to that exerted by OLA. On the other hand valnoctamide did not show any teratogenic effect even when used in comparatively higher dose than OLA and RIS. It did, induced increased skeletal abnormalities but only when compared to the control group. These skeletal abnormalities signal rather a slight developmental delay than true teratogenicity.

Results of clinical studies concerning VCD used as monotherapy or polytherapy for treatment of patients with bipolar disorder are equivocal (19, 20). The *Phase IIa* clinical trial with racemic-VCD as 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 three to five (19). On the other hand, the

Phase IIb study with VCD used as a monotherapy in a three-week, double-blind, randomized, placebo- and RIS-controlled parallel group trial, was terminated due to lack of efficacy in the treatment of patients with bipolar disorder in a manic or mixed episode (20). In this latter study, two cohort 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 (due to lack of clinical efficacy and not side effects) was higher as 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 as compared to placebo ($p = 0.0446$) while treatment with VCD showed similar reduction in Young Mania Rating Scale (YMRS) score as compared to placebo ($p = 0.9169$). In contrast, the second cohort analysis demonstrated the superiority of both RIS ($p = 0.0188$) and VCD ($p = 0.0159$) over placebo treatment in YMRS change from baseline at Week 3. The difference in the VCD effect between the first and second cohorts is unclear. The subsequent futility analysis demonstrated that in the best case scenario there is 57.1% conditional probability of study success in achieving the primary outcome measure for the VCD *versus* placebo comparison if the study will continue to enroll the originally preplanned population ($N = 313$). Due to the results of this analysis the study was terminated. Concluding, this *Phase IIb* study showed that patients can tolerate VCD daily doses of 1500 mg with minimal side effects and that patients who stayed on VCD may have benefited from it. Nevertheless VCD has a great potential in epilepsy particularly in light of the recent EMA restriction on the use of valproic acid in women and girls (23).

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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

^aOLA could not be tested with the dose equal to 1/4.36 of its LD₅₀ due to significant maternal toxicity observed in mice treated with at the lower dose (32.1 mg/kg or 1/6.54 of LD₅₀).

Table 2

Weight gain 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
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 LD50.

1/4.36 of LD50.

RIS = risperidone; OLA = olanzapine; VCD = valnoctamide; GD = gestational day.

^aSignificantly different from the control group.

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
Fetuses sex 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 LD₅₀.

1/4.36 of LD₅₀.

RIS = risperidone; OLA = olanzapine; VCD = valnoctamide; GD = gestational day; F = female; M = male.

^aSignificantly different from the control group.

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 LD50.

1/4.36 of LD50.

RIS = risperidone; OLA = olanzapine; VCD = valnoctamide; GD = gestational day.

^aSignificantly 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
Malposition stomach	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

Data presented as number of affected fetuses/litters. RIS = risperidone; OLA = olanzapine; VCD = valnoctamide; GD = gestational day.

The highlighted columns represent the matching doses for different drugs:

1/6.54 of LD50.

1/4.36 of LD50.

^a Significantly different from the control group.

^b Right aortic arch was accompanied by enlarged right ventricle.

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
Missshapen	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 centrum	0	0	0	0	0	0	0	1/1
Ribs								
Absent 13 th	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								

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Compound	Control	RIS	RIS	OLA	OLA	VCD	VCD	VCD	VCD
Incomplete ossification	0	1/1	0	20/8 ^b	4/3	0	0	0	0
Absent ossification	0	0	6/1	8/8 ^b	14/9 ^b	0	0	0	1/1

Data presented as number of affected fetuses/litters. RIS = risperidone; OLA = olanzapine; VCD = valnoctamide; GD = gestational day.
The highlighted columns represent the matching doses for different drugs:

1/6.54 of LD50.

1/4.36 of LD50.

^aIncomplete ossification.

^bSignificantly different from the control group.