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Comparative Efficacy of Valnoctamide and *sec*-Butylpropylacetamide (SPD) in Terminating Nerve Agents-Induced Seizures in Pediatric Rats

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Summary

Objectives: Children and adults are likely to be among the casualties in a civilian nerve agent exposure. This study evaluated the efficacy of valnoctamide (racemic-VCD), *sec*-butylpropylacetamide (racemic-SPD) and phenobarbital to stop nerve agent seizures in both immature and adult rats.

Methods: Female and male post-natal day (PND) 21, 28 and PND70 (adult) rats, previously implanted with EEG electrodes were exposed to seizure-inducing doses of the nerve agents sarin or VX and EEG was recorded continuously. Five minutes after seizure onset, animals were treated with SPD, VCD or phenobarbital. The up-down-method was used over successive animals to determine the drugs' anticonvulsant-ED_{50}.

Results: SPD-ED_{50} values in the VX-model were: PND21, 53mg/kg (male) and 48mg/kg (female); PND28, 108mg/kg (male) and 43mg/kg (female); PND70, 101mg/kg (male), and 40mg/kg (female). SPD-ED_{50} values in the sarin-model were: PND21, 44mg/kg (male) and 28mg/kg (female); PND28, 7**9**mg/kg (male) and 34mg/kg (female); PND70, 53mg/kg (male) and 53mg/kg (female). VCD-ED_{50} values in the VX-model were: PND21, **34**mg/kg (male) and 43mg/kg (female); PND28, 165mg/kg (male) and 59mg/kg (female); PND 70, 87mg/kg (male)

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Conflict of interest disclosure

Kari M. Haines, Liana M. Matson, Emily N. Dunn, Cherish E. Ardinger, Robyn Lee-Stubbs, David Bibi, and John H. McDonough have no conflict of interest to disclose. Meir Bialer has received in the last three years speakers or consultancy fees from Alkaloid, Arkin Holdings, Bial, Boehringer Inglheim, Teva, Upsher-Smith and Vidac Pharma. He received research support from the Israel Ministry of Defense and Rafa Pharmaceuticals. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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and 91mg/kg (female). VCD-ED_{50} values in the sarin-model were: PND21, 45mg/kg (male), 48mg/kg (female), PND28, 152mg/kg (male) 79mg/kg (female); PND70, 97mg/kg (male), 79mg/kg (female). Phenobarbital-ED_{50} values in the VX-model were: PND21, 43mg/kg (male) and 18mg/kg (female); PND28, 48mg/kg (male) and **97**mg/kg (female). Phenobarbital-ED_{50} values in the sarin-model were: PND21, 3**2**mg/kg (male) and 32mg/kg (female); PND28, 58mg/kg (male) and **97**mg/kg (female): PND70, 65mg/kg (female).

Significance: SPD and VCD demonstrated anticonvulsant activity in both immature and adult rats in the sarin- and VX-induced SE models. Phenobarbital was effective in immature rats, while in adult rats higher doses were required that were accompanied by toxicity. Overall, significantly less drug was required to stop seizures in PND21 animals than in the older animals and overall males required higher amounts of drug than females.

Keywords

status epilepticus (SE); valproic acid CNS-active amide derivatives; *sec*-butylpropylacetamide (SPD); valnoctamide (VCD); nerve agents; sarin; VX; pediatric rats

Introduction

One of the most severe conditions of epilepsy is status epilepticus (SE). This is a medical emergency condition that may lead to a permanent brain damage or even death.[1,2] Nerve agents elicit prolonged seizures that have all the behavioral and electrographic characteristics of SE.[3] The most widely used therapy for early treatment of SE are the benzodiazepines.[4] More common antiepileptic drugs (AEDs), such as valproic acid (VPA), phenytoin or levetiracetam, are considered second-line therapy for established SE, but are all ineffective in stopping soman-induced SE.[1,5] However, in various animal models for SE as well as clinically, it has been shown that the longer a seizure progresses before benzodiazepine treatment, the less responsive seizures become to benzodiazepines and other antiepileptic drugs.[3,6] Racemic-valnoctamide (VCD) and racemic-*sec*-butylpropylacetamide (SPD) have proven in pilocarpine- and soman-induced SE animal models to be active in benzodiazepine-resistant SE models when given 30 and even 60 m after seizure onset.[7–16,] For more severe cases, anesthetic agents like propofol and pentobarbital are used as a third-line therapy. First- and second-line therapies often do not suppress electrographic SE, and both second- and third-line therapies must be administered in a medical treatment facility.[2,5] Therefore, there is currently a substantial need to develop novel therapies to treat refractory SE.

VCD is a CNS-active chiral constitutional isomer of VPD, the corresponding amide of VPA, and SPD is a one-carbon homologue of VCD with an additional methyl group. In contrast to VPD which is a prodrug of VPA, both VCD and SPD act as drugs on their own with minimal biotransformation to their corresponding acids. Both racemic-SPD and racemic-VCD (Figure 1) possess unique and broad-spectrum antiepileptic profiles in a wide array of anticonvulsant models; these profiles are superior to that of VPA with ED_{50} values 3–20 times smaller than those of VPA. [7,8,12] SPD and VCD possess two stereogenic carbons in their chemical structure and exhibit stereoselective pharmacokinetics in humans and dogs (VCD) and rats (VCD and SPD). [7,8,13–15]

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Some AEDs exhibit qualitative or quantitative changes of their anticonvulsant activity during postnatal development.[17–22] In addition, children are likely to be among the casualties in a civilian nerve agent exposure as recent events in Syria have demonstrated (http://www.who.int/mediacentre/news/statements/2018/chemical-attacks-syria/en). Therefore, the efficacy of anticonvulsants used to treat nerve agent-induced SE needs to be studied in both immature and adult subjects.

Consequently, the aims of the current study were to determine if racemic-SPD, racemic-VCD and phenobarbital, three drugs that enhance GABAergic signaling,[11] were able to provide anticonvulsant protection against seizures elicited by the nerve agents sarin and VX in both immature and adult animal models, and to investigate if the efficacy of these compounds changes over the different developmental ages. Sarin and VX were chosen as the challenge nerve agents since they have been used most frequently in terrorist attacks (Japan nerve agent attacks by the Aum Shinrikyo ·cult; assassination of Kim Jong Namand and in the recent civil conflict in Syria). The recently developed model of nerve agent-induced SE in both immature and adult rats[22,23]was used to evaluate the anticonvulsant efficacy of these drugs.

Methods.

Chemicals and Reagents

All the solvents were of analytical grade or HPLC grade and were purchased from J.T. Baker (The Netherlands). SPD and VCD were synthesized according to previously described syntheses.[24] The chemical structures of the synthesized SPD and VCD and their purity were assessed by ^{1}H NMR, GC-MS and elemental analysis,[14,24]and the results obtained were as follows:

sec-Butylpropylacetamide. White powder. **Melting point** 120–121 °C. ^{1}H NMR (300 MHz, CDCl3, δ TMS): 0.8–0.96 (m, 9H), 1.06–1.3 (m, 2H), 1.3–1.64 (m, 5H), 0.9–2.0 (m, 1H), 5.4–5.54 (s, 1H), 5.7–5.9 (s, 1H). **Elemental Analysis Calculated** for $C_9H_{19}NO$: C, 68.74; H, 12.18; N, 8.91; O, 10.17. Found: C, 68.79; H, 13.10; N, 8.92; O, 10.19. **High Resolution Mass Spectrometry (**HRMS) (ESI-TOF) (*m/z*) **calculated** for $C_9H_{19}NO$ ($[M+H]^+$): 157.1466; found: 157.1452, **indicating 99% purity.**

Valnoctamide. White powder. **Melting point** 117–118 °C. ^{1}H NMR (300 MHz, CDCl3, δ TMS): 0.8–0.96 (m, 9H), 1.06–1.3 (br m, 1H), 1.3–1.64 (br m, 4H), 1.7–1.85 (br m, 1H), 5.4 (s, 1H), 5.6 (s, 1H). **Elemental Analysis Calculated** for $C_8H_{17}NO$: C, 67.07; H, 11.96; N, 9.78; O, 11.17. Found: C, 67.05; H, 11.93; N, 9.84; O, 11.12. **High Resolution Mass Spectrometry** HRMS (ESI-TOF) (*m/z*) **calculated** for $C_8H_{17}NO$ ($[M+H]^+$): 143.1310; found: 143.1322, **indicating 99% purity.**

Sarin and VX (>97% pure by phosphorus NMR analysis) came from US Army Medical Research Institute of Chemical Defense stocks; aliquots were kept at −80ºC, thawed on the day of use and maintained on ice during the study. **Pralidoxime Cl** (2PAM; **100% purity)** was purchased from Sigma-Aldrich, St. Louis, MO; **atropine sulfate** and **methyl atropine nitrate** (**pharmacopeal purity of 90–105%**) were purchased from Wedgewood Pharmacy

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(Swedesboro, NJ); **phenobarbital** sodium for injection (**pharmacopeal purity of 90–105%**) was purchased from West-Ward (Eatontown, NJ).

Animal Studies

Female and male post-natal day (PND) 21, 28 and PND70 adult control rats were surgically implanted with electroencephalographic (EEG) electrodes **(Plastics One, Roanoke, VA)** and headpieces (**Epitel Inc**, **Salt Lake City, UT**) two days (PND 21/28 rats) or seven (PND70 rats) before study. These rats' PND ages represent 3–6 year old, 8–10 year old and 15–18 year old human equivalent ages, respectively, depending upon the method used to calculate equivalence.[25]

On the day of study, the animals were connected to the EEG apparatus (**Epitel Inc**, **Salt Lake City, UT**) while in separate cages. After ~30 min of baseline EEG recording the animals were pre-treated with 2PAM (25 mg/kg, subcutaneously (SC) in PND21 and 28, intramuscular (IM) in PND70) 20 minutes prior to agent exposure. The animals were **randomly** exposed SC to SE-inducing doses of the nerve agents sarin (PND21, 120 μg/kg; PND28 and PND70, 190 μg/kg) or VX (PND21 and PND28, 40 μg/kg; PND70, 36 μg/kg). **Injected volume was 0.5 ml/kg for all nerve agents and treatment drugs.** These challenge doses were taken from a previous study[22] and take into account the differences in toxicity of these agents across developmental ages of rats.[26]Animals administered sarin received an admixture of atropine sulfate ($ATSO_4$; 0.5 mg/kg) and methyl atropine nitrate (AMN; 2 mg/kg) directly after agent exposure, animals given VX received the admixture at first signs of toxicity. For PND21 and PND28 rats all injections (including agent) were SC),PND70 rats also received agent SC but received 2PAM, $ATSO_4$, AMN IM due to the larger muscle mass in the adult animals. These treatments significantly reduced the early lethal effects of the two nerve agents without preventing development of SE.

Five minutes after seizure onset as confirmed on the EEG, animals were treated IP with SPD, VCD or phenobarbital. SPD and VCD were prepared in a solution of propylene glycol, alcohol, and water for injection at a ratio of 6:2:2; phenobarbital was diluted from its parent commercial solution to 32.5 mg/ml with sterile saline. The up-down method was used over successive testing sessions to determine the anticonvulsant-median effective dose (ED_{50}) of each of the three drugs. In the up-down method,[27] an initial testing dose is chosen, usually based on previous work, and then a succession of doses in 0.20 – 0.25 log units above and below this starting dose are chosen as fixed steps between doses. The first animal is tested at the initial dose, and if this dose terminates the seizure, the next test animal is tested at the next lower dose, whereas if the initial test dose does not terminate the seizure the next test animal is tested at the next higher dose. The rule is: if the seizure is terminated - go down a dose for the next test animal; if the seizure is not terminated - go up a dose in the next test animal. Testing proceeded in this fashion until 4 reversals occurred. ED_{50} values were calculated using the formulas and tables in Dixon and Massey.[27]To be rated as having the seizure terminated, all spiking and/or rhythmic waves had to stop within 1 hr of drug treatment and the EEG had to remain free of epileptiform activity for a minimum of 1hr. EEG examples of successful seizure terminations are displayed in Figure 2.

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Statistics

An ad hoc analysis was conducted on the log(10) of the ED_{50}s to compare the ED_{50}s among drug groups, PND age groups, agents, and sexes. The log(10) of the ED_{50}s was used in the analysis to reduce the variability of the data. A four factor analysis of variance (ANOVA) was used with all two factor interactions. All two factor interactions were not significant, therefore they were removed from further analysis. A four factor ANOVA with only main effects was conducted, followed by a Tukey's multiple comparison on ages and drugs if the main effect was significant.

Seizure termination latencies were grouped according to challenge nerve agent and treatment drug and then compared within an age group with Kruskal-Wallis ANOVAs followed by Dunn post-hoc test. No attempt was made to further distinguish if there were differences between the sexes on this measure due to the low and variable numbers of female and male animals within the different age and drug groups. In all statistical tests, $p<0.05$ was considered significant.

Results

The results of these experiments are shown Table 1 which presents the anticonvulsant-ED_{50} values and their 95% confidence intervals for each drug as a function of sex, age group and challenge nerve agent. The statistical analysis showed a significant effect of age ($p<.0001$), a small but significant effect of sex ($p=.0425$), and a slight, but not significant effect of drug ($p=.0627$). There was no significant effect of agents. The ED_{50}s for PND21s was significantly lower than those for both the PND28 and PND70 age groups, $p<0.05$. Overall the ED_{50} for males was slightly but significantly greater than the ED_{50} for females, $p=.0425$. While not statistically significant, treatments showed a minor difference in ED_{50}s with higher amounts of VCD being required for an anticonvulsant effect, followed by SPD and phenobarbital.

Anticonvulsant dose estimates could not be reliably determined for phenobarbital in PND70 animals in the VX model or sarin model (males only) because the test doses of phenobarbital resulted in rapid toxicity.

Figure 3 displays seizure termination latencies for each age group, challenge agent and treatment drug. There were no significant differences between treatments in the PND21 age group in the time to terminate seizures. However, PND28 animals that were challenged with sarin and treated with phenobarbital had significantly longer seizure termination latencies than VX challenged animals treated with phenobarbital which, in turn, had significantly longer latencies than VX challenged animals treated with VCD. In the PND70 age group, VX-challenged animals treated with phenobarbital had significantly longer seizure termination latencies than VX challenged animals treated with VCD.

In both the immature and adult animals treated successfully with SPD or VCD, a small percentage (8 of 90; 9%) developed sporadic episodes of seizure activity 2–3h after successful initial treatment. These developed as short (5–15s) episodes of spikes separated by periods of normal EEG. As time went on the duration of these episodes increased and

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became more frequent. The incidence of seizure reoccurrence was more frequent in SPD-treated animals (6 of 50; 12%) than VCD-treated subjects (2 of 40; 5%) and was distributed randomly across age groups, sexes and challenge agent (SPD: 2 PND21 males challenged with sarin, 1 PND21 female challenged with VX; 1 PND28 male challenged with VX; 2 PND70 males challenged with sarin; VCD: 1 PND21 male challenged with sarin; 1 PND28 female challenged with VX). This mostly occurred in animals treated with lower doses of these two compounds. A reoccurrence of seizure activity was not seen with phenobarbital.

Discussion

The results show that SPD, VCD and phenobarbital are capable of terminating SE seizures induced by the nerve agents VX or sarin in both immature and adult rats of both sexes, but with a number of important caveats. Overall, there was a trend for ED_{50} doses of all three treatments to increase with age with the most notable differences being between the PND21 age group and the older animals. Also, overall male animals required higher ED_{50} doses than females. While not statistically significant, there was a trend that animals treated with VCD required higher drug doses to achieve an anticonvulsant effect compared to SPD or phenobarbital. Also, there was no differential effect of these drugs to treat SE initiated by the two different nerve agents indicating a common neuropharmacological mechanism by which these toxic compounds initiate and maintain these seizures.[21,23]

The reason for the marked differences in the effectiveness of these drugs across developmental ages are most probably linked to body weight changes **coupled with developmental differences in the drugs' pharmacokinetics.** There was an approximate 90% increase in body weight over one week between PND21 (female = 47.1 g; male = 52.8 g) and PND28 (female = 88.3 g; male = 98.1 g) animals, the two developmental ages that differed the most. In contrast, the change in weights between PND28 and PND70 (female = 266.4 g; male = 437.4 g) aged animals is 302% and 446%, respectively, over six weeks. The greater proportional weight gain of males over this period could also contribute to the finding that overall males required higher drug amounts than females.

Previous work had shown that both VCD and SPD were capable of controlling SE induced by the nerve agent soman in adult male rats and guinea pigs.[7, 12–14] The present results extend those findings to SE seizures induced by two other nerve agents, VX and sarin, in both immature and adult male and female rats. In those previous studies, VCD and SPD were given at substantially longer delay times (20 m, 40 m after seizure onset) and were given in conjunction with the benzodiazepine diazepam at a dose (2.2 mg/kg, IM) that was incapable of stopping SE on its own. Under those conditions, the ED_{50}s for seizure control ranged from 67–92mg/kg (SPD) and 62–80mg/kg (VCD) which are very comparable to the ED_{50}s for adult rats obtained in the present study where these drugs were used at a shorter time following seizure onset and without the benefit of the co-administration of a benzodiazepine.

In a previous study utilizing the pentylenetetrazol-induced seizures in immature rats, VCD, two of its stereoisomers (2S,3S)-VCD and (2R,3S)-VCD and VCD-constitutional isomer propylisopropylacetamide (PID) exhibited anticonvulsant activity in all four age groups

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studied.[28] VCD (racemate) was more potent than its constitutional isomer PID against the generalized tonic-clonic seizures (GTCS) and generalized seizures without tonic phase (GCS) in the PND18- and PND25 age groups. However no differences were found between these two constitutional isomers in the PND7 and PND12 age groups. The two tested VCD stereoisomers had similar ED50 values that were not significantly different from that of racemic-VCD. All four tested drugs were more potent than VPA. VPA doses of 50 to 400 mg/kg (i.p.) were used in a previous study and only high doses of 150 mg/kg and higher VPA suppressed GTCS in all age groups of immature rats.[29] All of these studies confirm previous reports showing that VPA has the weakest ED_{50} values among the antiepileptics in various anticonvulsant animal (rodent) models.[30]

In the present study SPD, VCD and phenobarbital demonstrated good anticonvulsant activity in two new nerve agent models of SE in both adult (PND70) and immature (PND21, PND28) rats of both sexes. To date, SPD and VCD have shown anticonvulsant activity in multiple other rat-SE models (pilocarpine, soman, paraoxon, TETS) demonstrating the broad anti-SE spectrums of these two homologous CNS-active compounds.[7–16] There is an ongoing need to identify new drugs to treat SE, whether induced by nerve agents or of other etiologies. The present data show that SPD, VCD and to a lesser extent phenobarbital are effective in both sexes across all age groups.

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Key Points

- Anticonvulsant-ED_{50} values varied with age and sex
- SPD, VCD and phenobarbital are more effective in PND21 rats, therefore requiring that younger animals receive lower doses of the drugs
- Phenobarbital has been effective in pediatric rats but its ED_{50} values could not be determined in adult rats due to higher doses required that were accompanied by toxicity
- SPD and VCD have now displayed excellent anticonvulsant activity **in multiple** rat models of SE

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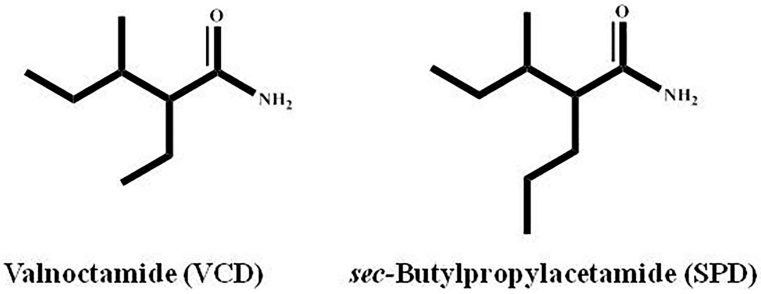

Valnoctamide (VCD) *sec*-Butylpropylacetamide (SPD)

Figure 1.
Chemical structures of sec-butylpropylacetamide (SPD) and valnoctamide (VCD).

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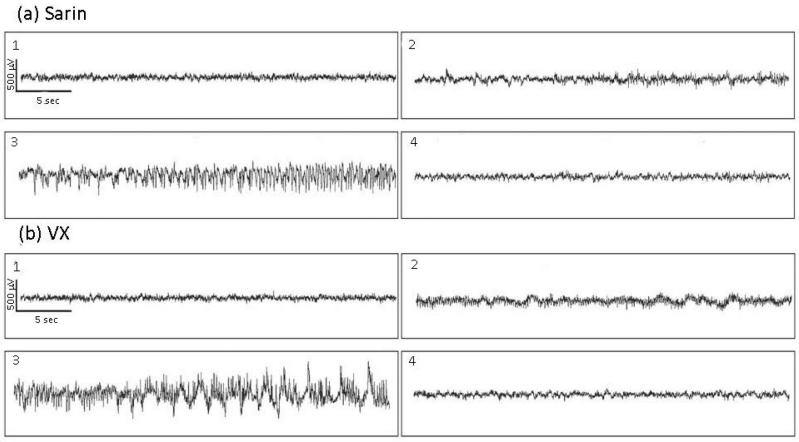

Figure 2.

2a Example of anticonvulsant EEG response of a male PND21 rat exposed to sarin and treated 5 m after seizure onset with 42 mg/kg SPD (IP). 1. Baseline EEG prior to sarin exposure; 2. Seizure onset ~7 m after sarin challenge; note repetitive spiking that progressively increases in amplitude; 3. Seizure activity at time of SPD treatment; 4. EEG 15 m after SPD treatment; EEG remained seizure free throughout the remainder of the recording session ~4 h.

b. Example of anticonvulsant response of a female PND28 rat exposed to VX and treated 5 m after seizure onset with 56 mg/kg VCD (IP). 1. Baseline EEG prior to VX; 2. Seizure onset ~19 m after VX challenge; note repetitive spiking that progressively increases in amplitude; VX-induced seizure were always substantially delayed compared to sarin;[23,31] 3. EEG activity at time of VCD treatment; 4. EEG 22 m after VCD treatment; EEG remained seizure free throughout the remainder of the recording session ~4 h.

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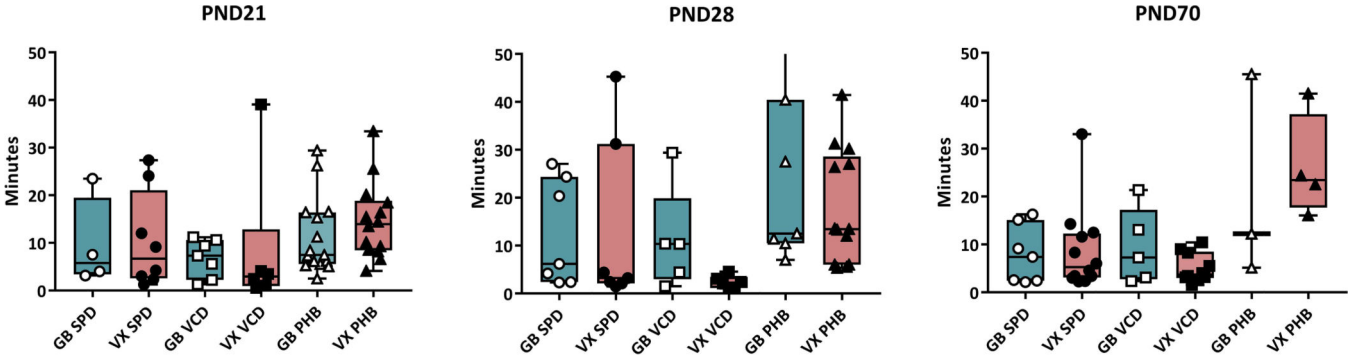

Figure 3.

Seizure termination latencies for each treatment drug as a function of age group and nerve agent challenge. Data are presented as box-and-whiskers plots; bar within the box represents medians, edges of the box are 95% confidence limits, bars outside the box are the minimum and maximum responders. Each symbol represents a successful anticonvulsant response; both male and female animals are represented in each condition.

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Table 1.

Anticonvulsant ED_{50} (mg/kg) values (and their 95%CI)[c] of SPD, VCD and phenobarbital, given IP, in protecting against seizures induced by the nerve agents sarin and VX in female (F) and male (M) rats of different developmental ages

		Sarin			VX		
Age	**Sex**	**SPD**	**VCD**	**Phenobarbital**	**SPD**	**VCD**	**Phenobarbital**
[a]PND21	F	28 (21 –36)[b] N = 10	48 (35–67) N = 11	32 (24–43) N = 8	48 (37–64) N = 8	43 (30–62) N = 10	18 (14–24) N = 10
	M	44(32–61) N = 9	45 (35–59) N = 9	32 (24–43) N = 11	53 (39–74) N = 9	34 (24–49) N = 8	43 (30–62) N = 9
[a]PND28	F	34 (24–49) N = 8	79 (56–109) N = 12	97 (68–139) N = 9	43 (30–62) N = 9	59(42–81) N = 9	97 (68–139) N = 9
	M	79 (57–109) N = 8	152 (112–205) N = 10	58 (45–74) N = 11	108 (81–143) N = 11	165(116–236) N = 11	48 (37–63) N = 9
[a] PND70	F	53(39–74) N = 9	79 (57–109) N = 10	65 (48–88) N = 9	40 (32–50) N = 12	91(73–113) N = 13	ND[d] (240) N = 9
	M	53 (39–74) N = 12	97 (68–139) N = 13	ND* (320) N = 10	101(78–132) N = 13	87(65–115) N = 8	ND[d] (240) N = 10

[a]Rats were assessed at post-natal day (PND) 21, 28 and 70.

[b]N-Number of animals tested per group.

[c]The 95% confidence interval (95%CI) around the ED_{50} value.

[d]ND – ED_{50} could not be determined due to early toxicity (maximum dose tested)