InterVivo Solutions Final Study Report

Evaluation of the antiepileptic properties of LX9211 in the mouse 6Hz (32mA) seizure test

Study Number: VRI181-19145-RE
Sponsor Study Number: LX9211-N87

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Testing facility name and address:	InterVivo Solutions 8224 Sideroad 15, R.R. #3 Fergus, Ontario, N1M 2W4, Canada
Scientific Director name and address:	Leo Silenieks, InterVivo Solutions 8224 Sideroad 15, R.R. #3 Fergus, Ontario, N1M 2W4, Canada
Study initiation date:	2019-09-04
Study completion date:	Date report is signed by Scientific Director
Experimental initiation date:	2019-12-04
Experimental completion date:	2019-12-06

Scientific Director signature and date:

_____ 2020-03-18

Leo Silenieks Date

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APPENDICES

A. STUDY TITLE

Examination of the antiepileptic properties of LX9211 in the mouse 6Hz (32mA) seizure test

B. STUDY NUMBER

VRI181-19145-RE

C. SPONSOR REPRESENTATIVE

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D. OBJECTIVE

The primary objective of this study was to evaluate changes in the susceptibility to seizures in the mouse 6Hz (32mA) seizure test following the administration of a novel chemical entity (NCE), LX9211. The antiseizure property was assessed using the 6Hz seizure test, and motor functioning was assessed using the rotarod performance test. This was a non-GLP study that adhered to CCAC guidelines on animal care.

E. SCIENTIFIC DIRECTOR

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F. STUDY COORDINATOR

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G. LOCATION OF FACILITIES

Test facility:
InterVivo Solutions
8224 Sideroad 15, R.R. #3
Fergus, Ontario N1M 2W4, Canada

H. INTRODUCTION

The Electro Convulsive Therapy unit (Ugo Basile) contains a pulse train generator designed to deliver a series of 0.2 ms rectangular electrical pulses. This pulse train, when applied to mice using a set of corneal electrodes, causes partial seizures; making it an effective method for screening potential anti-epileptic compounds. The present study was designed to evaluate the effect of multiple doses of LX9211 against tonic seizures induced by the 6Hz (32mA) seizure test in mice. In addition to antiseizure efficacy, side effect measures were evaluated using the rotarod treadmill.

I. STUDY SCHEDULE

Table 1. Schedule of Operations

Study Day	Key Event	Procedure
-7	Animal Arrival	- Acclimation
-1	Rotarod Training	- Rotarod training (n=56)
0	Drug Testing	- Rotarod testing (n=56) - 6Hz test (n=56)

J. STUDY DESIGN

Fifty-six (56) male CD-1 mice were included in the study. There were 7 treatment groups as outlined in Table 2. Each group contained 8 animals and was tested by technicians blinded to treatment allocation. Locomotor activity (LMA) testing was conducted 10 minutes prior to 6Hz testing, which was performed approximately 6 hours post-dose for all NCE treated groups, and 30 minutes post-dose for positive control treated animals.

Table 2. Treatment Groups Summary

Group	Treatment	Dose (mg/kg)	Dosing Solution Conc. (mg/mL)	Frequency	Group Size	Route	Pre-Treatment Time (h)
A	Vehicle	-	-	Single Dose	8	P.O.	6
B	LX9211	5	10	Single Dose	8	P.O.	6
C	LX9211	10	10	Single Dose	8	P.O.	6
D	LX9211	30	10	Single Dose	8	P.O.	6
E	LX9211	100	10	Single Dose	8	P.O.	6
F	LX9211	200	10	Single Dose	8	P.O.	6
G	Sodium Valproate	600	10	Single Dose	8	I.P.	0.5

K. EXPERIMENTAL MATERIALS (NON-GLP)

1 Investigational Product

i *Chemical name*
LX9211

ii *Doses tested*
5, 10, 30, 100, 200 mg/kg

iii *Dosage form*
Oral solution; 10 mL/kg

iv *Vehicle*
10% ethanol, 40% PEG-400, 15% Tween 80

v *Drug storage during study*
Room temperature, in desiccator

2 Positive control

i *Chemical name*
Sodium Valproate

ii *Doses tested*
300 mg/kg

iii *Dosage form*
Intraperitoneal solution; 10 mL/kg

iv *Vehicle*
0.9% Saline

v *Drug storage during study*
Room temperature, in desiccator

L. MATERIALS AND METHODS

1 Test System

Fifty-six (56) male CD-1 mice, purchased from Charles River Laboratories, were included in the study. Subjects weighed approximately 20-24 g upon arrival to the test facility.

2 Selection and Allocation of Animals

Animals in good health that were responsive, alert and maintaining their coats were selected for the study. Allocation to treatment groups was balanced with respect to body weight to the best extent possible.

3 Acclimation

Animals received a minimum 3 days of acclimation to the test facility prior to beginning the testing phase of the study.

4 Administration of Test Articles

LX9211 was dissolved in vehicle and administered by oral gavage at a dose volume of 10 mL/kg. Dosing was achieved using a ball-tipped gavage needle attached to a syringe containing the dosing formulation.

5 Housing and Management of Test System

The study was conducted at InterVivo Solutions, located at 8224 Sideroad 15, R.R. #3, Fergus, Ontario, Canada. Animals were housed in transparent polycarbonate cages in groups of 5. Subjects were provided with standard corn cob bedding and PVC tubing as enrichment. Cages were changed once per week or more often as needed.

Animals were housed under an automated 12-hour light/12-hour dark cycle. All manipulations were performed during the animals' light cycle. Heating and cooling were electronically controlled and were set to maintain the animal room in a temperature range from 19-22 °C and with a relative humidity of approximately 50%.

All animals had access to food and water *ad libitum.* Standard rodent chow was provided in stainless steel feeders. Tap water was provided in glass water bottles with rubber stoppers and stainless-steel sipper tubes.

6 Health Observations

General health observations were performed on the mice daily prior to drug treatment and at regular intervals on drug test days. Checked signs included, but were not limited to, mortality, loss of righting reflex, general coat appearance, and general activity/reflexes. Only abnormal health findings were recorded.

7 Body Weights

Animals were weighed prior to dosing on their designated treatment day for group allocation purposes and calculation of accurate dose volumes. The scales were operated and maintained according to standard operating procedures.

8 6Hz Psychomotor Seizure Test

Following an acclimation period to the test facility, 56 CD-1 mice were administered LX9211 (5, 10, 30, 100, 200 mg/kg), sodium valproate (300 mg/kg), or vehicle by oral gavage with a pre-treatment time of 6 h (LX9211) or 0.5 h (sodium valproate) pre-6Hz test according to Table 2.

Following the appropriate pretreatment time (see Table 2) all animals were tested in the 6Hz procedure (6Hz, 0.2ms pulse width, 3s duration, 32mA) via corneal electrodes moistened with saline (ECT unit 57800; Ugo Basile) for the presence or absence (i.e. protection) of a tonic seizure.

Immediately following this endpoint, the animals were euthanized via instantaneous stun and cervical dislocation following standard operating procedures.

The total seizure score was determined by observing for the presence of the following behaviours after the electrical stimulus had been administered: stun/immobility, forelimb clonus, straub tail, and lateral head movement. For each behaviour present, a score of 1 was assigned. The sum total of all scores was considered to be the total seizure score, from which the % seizure was calculated and analyzed using one and two-way ANOVA tables (Statistica Version 11, StatSoft, Tulsa OK).

9 Rotarod Testing

Prior to the day of testing (refer to Table 1), subjects were trained on rotarod procedures to ensure competence of the task. Ten minutes prior to conducting the 6Hz test, mice were tested on the accelerating rotarod. All subjects were tested three times at an accelerating speed of 4 to 40 RPM (Med Associated ENV-575M). Potential motor impairment resulting from test article administration was measured by latency (in seconds) to fall from the rotarod apparatus. Each session was a maximum of five minutes.

10 Statistical Analyses

All statistical analyses were performed using Statistica Version 11 (StatSoft, Tulsa OK). Analysis of variance was completed for both total seizure score and rotarod performance data with the independent grouping variable of treatment (i.e. test article dose and controls). In the event of a significant main effect, post-hoc Dunnett's test was conducted to compare vehicle with drug pretreatment.

M. RESULTS AND DISCUSSION

LX9211 was tested at 5, 10, 30, 100, and 200 mg/kg via the oral route. Sodium valproate (300mg/kg) was included as a positive control. A main effect of treatment ($F_{6,49}=8.36$; $P<0.01$) for the measure of total seizure score was shown. Post-hoc tests that revealed that treatment with sodium valproate (300 mg/kg) produced significant protection against 6Hz (32 mA) induced seizures compared to vehicle group (0.1 ± 0.1 sodium valproate vs. vehicle: 3.0 ± 0.3). LX9211 showed a trend to increased protection, although not significantly ($P=0.066$). Refer to Figure 1 below.

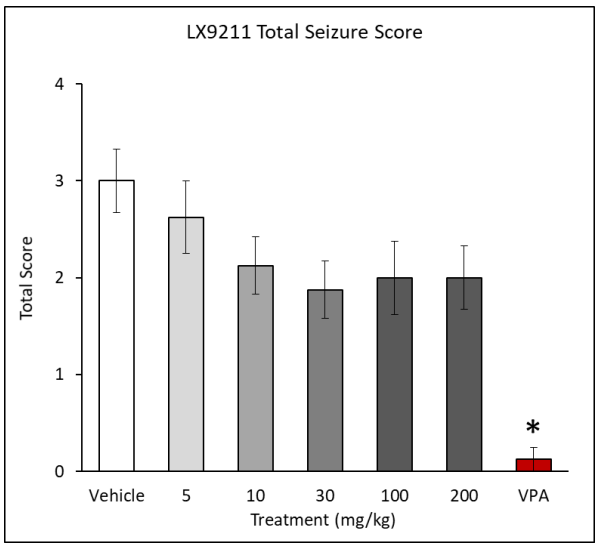


Figure 1: LX9211: total average seizure scores across treatment groups. Each bar represents the mean ± standard error of the mean (SEM) per treatment group (n=8). *P<0.01 vs Vehicle treatment group (Dunnett test following significant ANOVA).

Subjects were exposed to a 5-minute maximum rotarod treadmill challenge in order to determine potential motor impairment associated with test article. A main effect of treatment ($F_{6,49}=33.85$; $P<0.01$) for this measure reflected significant impairment compared to vehicle associated with both sodium valproate treatment (8.6 ± 2.7 s vs vehicle: 262.4 ± 20.3 s) and 200 mg/kg LX9211 treatment (183.4 ± 24.2 s vs Vehicle 262.4 ± 20.3 s). Refer to Figure 2 below.

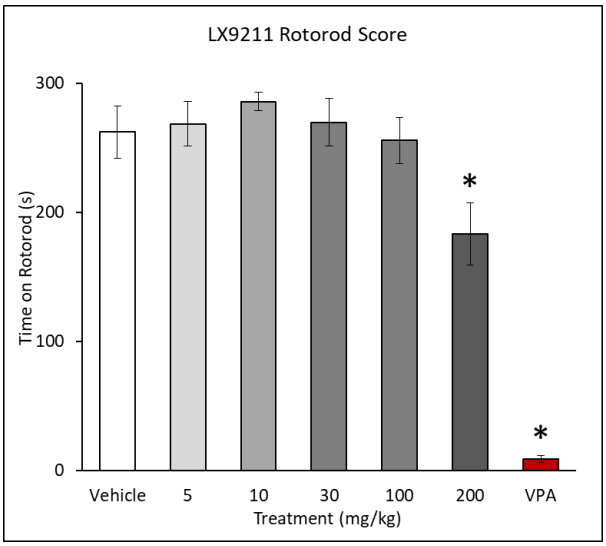


Figure 2: LX9211: total average rotarod scores across treatment groups. Each bar represents the mean ± standard error of the mean (SEM) per treatment group (n=8). *P<0.01 vs Vehicle treatment group (Dunnett test following significant ANOVA).

N. SCIENTIFIC DIRECTOR'S ADDITIONAL COMMENTS

Treatment with LX9211 did not protect mice against a 6Hz (32mA) induced seizure, however, a trend downward was seen in seizures scores, with the best reduction occurring at 30 mg/kg (oral), at which dose minimal adverse effects were captured in the rotarod test. The results of this study are consistent with the results from a previous study, VRI181-19145-RE, where the biggest reduction in seizure scores occurred at 30 mg/kg and no neurological impairments were identified at 30 mg/kg and 100 mg/kg.

O. ANIMAL DISPOSITION

Carcasses and tissues from euthanized or deceased animals were disposed of according to standard operating procedures.

P. DATA INTEGRITY STATEMENT

There were no unforeseen circumstances that affected the quality or integrity of the data.

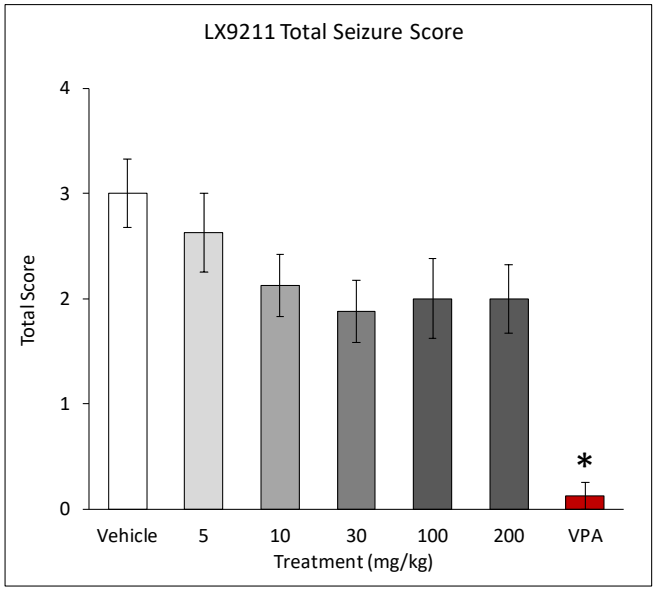
Q. ACCURACY OF REPORT STATEMENT

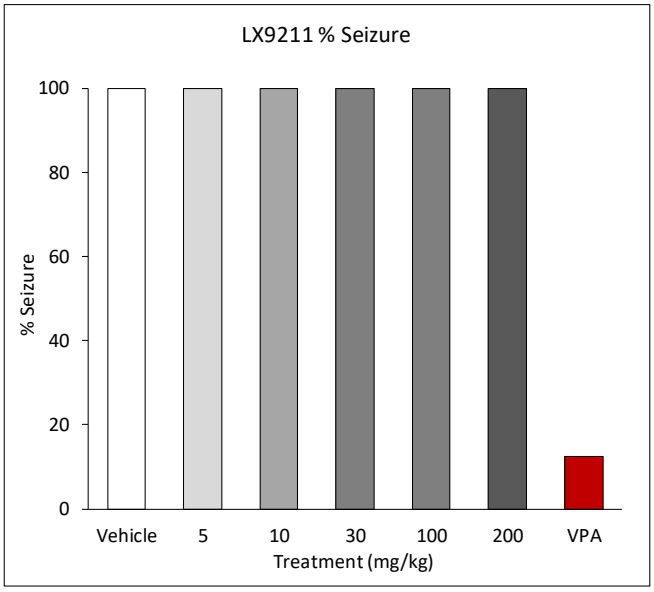
I certify that this report is a complete and accurate representation of all study observations.

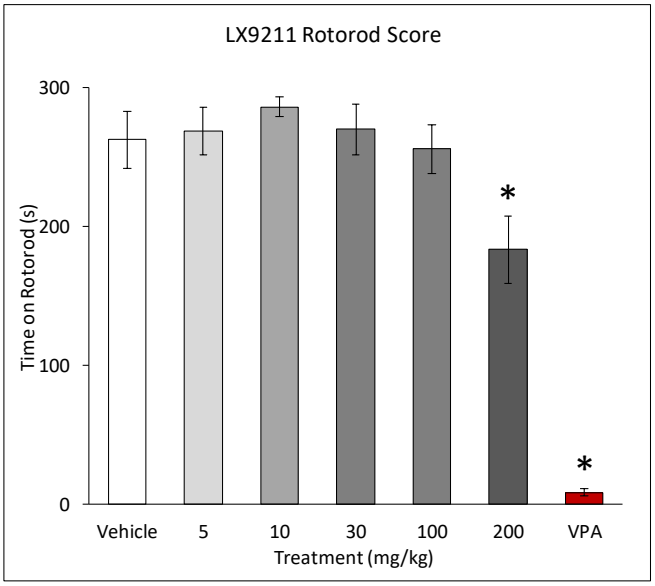
_____ 2020-03-18

Leo Silenieks, Scientific Director Date

APPENDIX A. SUMMARY FIGURES AND STATISTICAL ANALYSIS







*P<0.01 vs Vehicle treatment group

APPENDIX B. STATISTICAL ANALYSIS

Total Seizure Score

Kruskal-Wallis ANOVA by Ranks; Seizure (Revelstoke)
Independent (grouping) variable: Treatment
Kruskal-Wallis test: H (6, N= 56) =25.52224 p =.0003

Depend.: Seizure	Code	Valid N	Sum of Ranks	Mean Rank
Veh	101	8	335.5000	41.93750
LX5mg/kg	102	8	296.0000	37.00000
LX10mg/kg	103	8	247.0000	30.87500
LX30mg/kg	104	8	217.0000	27.12500
LX100mg/kg	105	8	226.5000	28.31250
LX200mg/kg	106	8	232.0000	29.00000
VPA	107	8	42.0000	5.25000

Median Test, Overall Median = 2.00000; Seizure (Revelstoke)
Independent (grouping) variable: Treatment
Chi-Square = 8.603129 df = 6 p = .1972

Dependent: Seizure	Veh	LX5mg/kg	LX10mg/kg	LX30mg/kg	LX100mg/kg	LX200mg/kg	VPA	Total
<= Median: observed	3.00000	4.00000	5.000000	6.000000	6.000000	5.000000	8.00000	37.00000
expected	5.28571	5.28571	5.285714	5.285714	5.285714	5.285714	5.28571	
obs.-exp.	-2.28571	-1.28571	-0.285714	0.714286	0.714286	-0.285714	2.71429	
> Median: observed	5.00000	4.00000	3.000000	2.000000	2.000000	3.000000	0.00000	19.00000
expected	2.71429	2.71429	2.714286	2.714286	2.714286	2.714286	2.71429	
obs.-exp.	2.28571	1.28571	0.285714	-0.714286	-0.714286	0.285714	-2.71429	
Total: observed	8.00000	8.00000	8.000000	8.000000	8.000000	8.000000	8.00000	56.00000

Multiple Comparisons p values (2-tailed); Seizure (Revelstoke)
Independent (grouping) variable: Treatment
Kruskal-Wallis test: H (6, N= 56) =25.52224 p =.0003

Depend.: Seizure	Veh R:41.938	LX5mg/kg R:37.000	LX10mg/kg R:30.875	LX30mg/kg R:27.125	LX100mg/kg R:28.313	LX200mg/kg R:29.000	VPA R:5.2500
Veh		1.000000	1.000000	1.000000	1.000000	1.000000	0.000143
LX5mg/kg	1.000000		1.000000	1.000000	1.000000	1.000000	0.002076
LX10mg/kg	1.000000	1.000000		1.000000	1.000000	1.000000	0.035197
LX30mg/kg	1.000000	1.000000	1.000000		1.000000	1.000000	0.153463
LX100mg/kg	1.000000	1.000000	1.000000	1.000000		1.000000	0.098331
LX200mg/kg	1.000000	1.000000	1.000000	1.000000	1.000000		0.075316
VPA	0.000143	0.002076	0.035197	0.153463	0.098331	0.075316	

One way ANOVAs

Total Seizure Score

Univariate Tests of Significance for Seizure (Revelstoke)
Sigma-restricted parameterization
Effective hypothesis decomposition; Std. Error of Estimate: .8864052

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	216.0714	1	216.0714	275.0000	0.000000
Treatment	39.4286	6	6.5714	8.3636	0.000003
Error	38.5000	49	0.7857		

Dunnett test; variable Seizure (Revelstoke)
Probabilities for Post Hoc Tests (2-sided)
Error: Between MSE = .78571, df = 49.000

Cell No.	Treatment	{1} 3.0000
1	Veh	
2	LX5mg/kg	0.902474
3	LX10mg/kg	0.217295
4	LX30mg/kg	0.066455
5	LX100mg/kg	0.124010
6	LX200mg/kg	0.124010
7	VPA	0.000010

*P<0.05 vs. Vehicle (n=10 per group)
Values highlighted in red indicate statistical significance.

Rotorod

Kruskal-Wallis ANOVA by Ranks; Rotorod (Revelstoke)
Independent (grouping) variable: Treatment
Kruskal-Wallis test: H (6, N= 56) =29.20707 p =.0001

Depend.: Rotorod	Code	Valid N	Sum of Ranks	Mean Rank
Veh	101	8	281.0000	35.12500
LX5mg/kg	102	8	277.5000	34.68750
LX10mg/kg	103	8	307.0000	38.37500
LX30mg/kg	104	8	277.5000	34.68750
LX100mg/kg	105	8	264.5000	33.06250
LX200mg/kg	106	8	152.5000	19.06250
VPA	107	8	36.0000	4.50000

Median Test, Overall Median = 269.500; Rotorod (Revelstoke)
Independent (grouping) variable: Treatment
Chi-Square = 15.00000 df = 6 p = .0203

Dependent: Rotorod	Veh	LX5mg/kg	LX10mg/kg	LX30mg/kg	LX100mg/kg	LX200mg/kg	VPA	Total
<= Median: observed	3.00000	3.00000	2.00000	2.00000	4.000000	6.00000	8.00000	28.00000
expected	4.00000	4.00000	4.00000	4.00000	4.000000	4.00000	4.00000	
obs.-exp.	-1.00000	-1.00000	-2.00000	-2.00000	0.000000	2.00000	4.00000	
> Median: observed	5.00000	5.00000	6.00000	6.00000	4.000000	2.00000	0.00000	28.00000
expected	4.00000	4.00000	4.00000	4.00000	4.000000	4.00000	4.00000	
obs.-exp.	1.00000	1.00000	2.00000	2.00000	0.000000	-2.00000	-4.00000	
Total: observed	8.00000	8.00000	8.00000	8.00000	8.000000	8.00000	8.00000	56.00000

Multiple Comparisons p values (2-tailed); Rotorod (Revelstoke)
Independent (grouping) variable: Treatment
Kruskal-Wallis test: H (6, N= 56) =29.20707 p =.0001

Depend.: Rotorod	Veh R:35.125	LX5mg/kg R:34.688	LX10mg/kg R:38.375	LX30mg/kg R:34.688	LX100mg/kg R:33.063	LX200mg/kg R:19.063	VPA R:4.5000
Veh		1.000000	1.000000	1.000000	1.000000	1.000000	0.003633
LX5mg/kg	1.000000		1.000000	1.000000	1.000000	1.000000	0.004495
LX10mg/kg	1.000000	1.000000		1.000000	1.000000	0.375321	0.000686
LX30mg/kg	1.000000	1.000000	1.000000		1.000000	1.000000	0.004495
LX100mg/kg	1.000000	1.000000	1.000000	1.000000		1.000000	0.009677
LX200mg/kg	1.000000	1.000000	0.375321	1.000000	1.000000		1.000000
VPA	0.003633	0.004495	0.000686	0.004495	0.009677	1.000000	

Rotorod

Univariate Tests of Significance for Rotorod (Revelstoke)
Sigma-restricted parameterization
Effective hypothesis decomposition; Std. Error of Estimate: 47.92543

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	2691952	1	2691952	1172.020	0.000000
Treatment	466485	6	77748	33.850	0.000000
Error	112546	49	2297		

Dunnett test; variable Rotorod (Revelstoke)
Probabilities for Post Hoc Tests (2-sided)
Error: Between MSE = 2296.8, df = 49.000

Cell No.	Treatment	{1} 262.38
1	Veh	
2	LX5mg/kg	0.999809
3	LX10mg/kg	0.828834
4	LX30mg/kg	0.999326
5	LX100mg/kg	0.999667
6	LX200mg/kg	0.009486
7	VPA	0.000009

*P<0.05 vs. Vehicle (n=10 per group)
Values highlighted in red indicate statistical significance.

APPENDIX C. SUMMARY DATA TABLE

VRI181-19145-RE (Revelstoke) Mouse 6Hz

Treatment: LX9211 (5, 10, 30, 100 and 200 mg/kg) 10mL/kg *p.o.* , VPA (600 mg/kg) 10mL/kg *i.p.*
Pretreatment Time: 6hrs (LX9211), 30 min (VPA)
Date: 2019-12-05
Experimenters: NC, LBS, JES, CS

Group	ID	Treatment	Stun	F. Clonus	Straub T.	Lat H. Movement	Total Seizure Score	% Seizure	Rotorod (s)
A	A1	Vehicle	0	1	0	1	2	1	300
	A2		1	0	0	1	2	1	300
	A3		1	1	1	1	4	1	169
	A4		1	1	1	1	4	1	178
	A5		1	1	0	0	2	1	252
	A6		1	0	1	1	3	1	300
	A7		1	1	1	1	4	1	300
	A8		1	0	1	1	3	1	300
		AVG					**3.0**	**100.0**	**262.4**
		SEM					**0.3**		**20.3**
B	B1	LX9211 (5mg/kg)	1	1	1	1	4	1	300
	B2		1	1	0	0	2	1	300
	B3		1	1	0	1	3	1	300
	B4		1	1	1	1	4	1	298
	B5		0	0	1	0	1	1	300
	B6		1	1	0	0	2	1	206
	B7		1	0	1	0	2	1	177
	B8		1	1	0	1	3	1	269
		AVG					**2.6**	**100.0**	**268.8**
		SEM					**0.4**		**17.5**
C	C1	LX9211 (10mg/kg)	1	1	0	1	3	1	300
	C2		1	1	0	0	2	1	270
	C3		1	0	1	0	2	1	255
	C4		1	0	0	0	1	1	300
	C5		1	1	1	0	3	1	300
	C6		1	1	0	0	2	1	300
	C7		1	0	1	1	3	1	300
	C8		1	0	0	0	1	1	263
		AVG					**2.1**	**100.0**	**286.0**
		SEM					**0.3**		**7.0**

VRI181-19145-RE (Revelstoke) Mouse 6Hz

Treatment: LX9211 (5, 10, 30, 100 and 200 mg/kg) 10mL/kg *p.o.* , VPA (600 mg/kg) 10mL/kg *i.p.*
Pretreatment Time: 6hrs (LX9211), 30 min (VPA)
Date: 2019-12-05
Experimenters: NC, LBS, JES, CS

Group	ID	Treatment	Stun	F. Clonus	Straub T.	Lat H. Movement	Total Seizure Score	% Seizure	Rotorod (s)
D	D1	LX9211 (30mg/kg)	1	1	1	0	3	1	155
	D2		1	0	0	1	2	1	234
	D3		1	1	0	0	2	1	300
	D4		0	1	0	0	1	1	272
	D5		1	0	0	0	1	1	300
	D6		1	1	1	0	3	1	300
	D7		0	1	1	0	2	1	298
	D8		1	0	0	0	1	1	300
		AVG					**1.9**	**100.0**	**269.9**
		SEM					**0.3**		**18.4**
E	E1	LX9211 (100mg/kg)	1	1	0	1	3	1	246
	E2		1	0	0	0	1	1	300
	E3		0	0	1	0	1	1	190
	E4		1	0	1	0	2	1	300
	E5		1	1	1	1	4	1	300
	E6		1	0	1	0	2	1	200
	E7		1	0	1	0	2	1	300
	E8		1	0	0	0	1	1	210
		AVG					**2.0**	**100.0**	**255.8**
		SEM					**0.4**		**17.7**
F	F1	LX9211 (200mg/kg)	1	0	1	0	2	1	300
	F2		0	0	1	0	1	1	157
	F3		1	1	1	0	3	1	190
	F4		1	1	1	0	3	1	124
	F5		1	0	0	0	1	1	274
	F6		0	0	0	1	1	1	132
	F7		1	1	0	0	2	1	120
	F8		1	1	1	0	3	1	170
		AVG					**2.0**	**100.0**	**183.4**
		SEM					**0.3**		**24.2**
G	G1	VPA (600mg/kg)	0	0	0	0	0	0	10
	G2		0	0	0	0	0	0	2
	G3		0	0	0	0	0	0	5
	G4		1	0	0	0	1	1	3
	G5		0	0	0	0	0	0	26
	G6		0	0	0	0	0	0	7
	G7		0	0	0	0	0	0	6
	G8		0	0	0	0	0	0	10
		AVG					**0.1**	**12.5**	**8.6**
		SEM					**0.1**		**2.7**