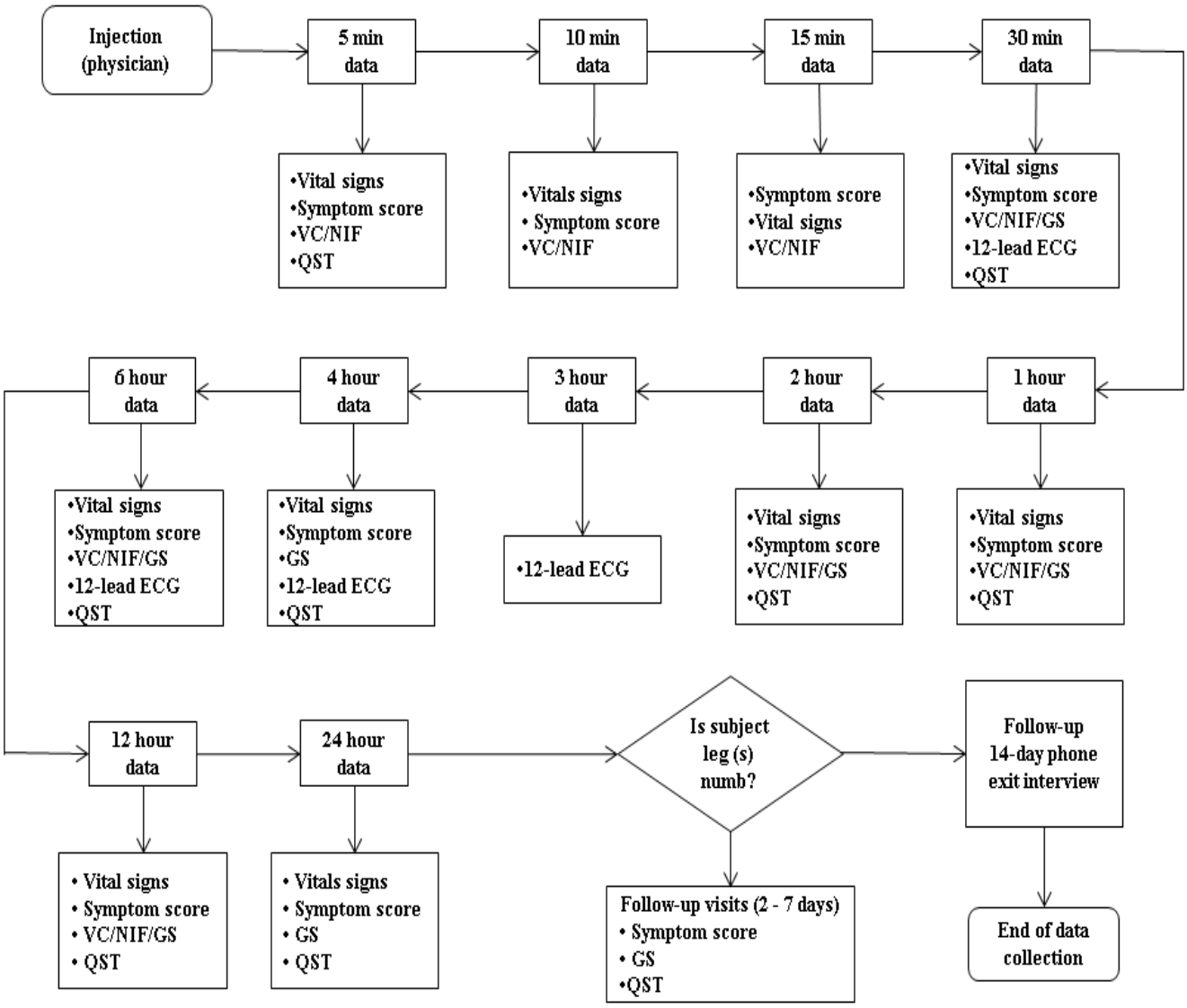
SUPPLEMENTAL DIGITAL CONTENT 1

A1. DRUG PRODUCTION AND PRE-CLINICAL TESTING

NeoSTX drug substance was produced at Proteus SA and drug product was packaged by Saval Laboratories (Santiago, Chile). The drug product was packaged at a concentration of 20mcg/mL in sodium chloride solution, 0.9mg/ml, at pH 4.5 in 1 ml sealed ampules. Drug assays for different manufacturing, toxicologic, and pharmacokinetic studies used multiple approaches, including HPLC followed by fluorescence detection and HPLC followed by tandem mass spectrometry. A series of studies confirmed sterility, stability, purity, and non-pyrogenicity. Absence of cyanobacterial DNA was confirmed by an rt-PCR method using positive and negative controls and absence of cyanobacterial protein or peptides was confirmed by a combined approach using Bradford protein assays, proteomics (mass spectrometry), and amino acid analysis following acid hydrolysis. NeoSTX did not appear mutagenic or carcinogenic in Ames and Chromosomal Aberration tests. All preparative methods, analytical methods, and Good Laboratory Practices toxicologic studies in rats and sheep were submitted to the U.S. FDA as part of the Investigational New Drug application.

A2. FIGURE 1. STUDY TEST AND TIME POINTS



VC: Vital Capacity; NIF: Negative Inspiratory Force; GS: Grip Strength; ECG; Electrocardiogram; QST: Quantitative Sensory Testing.

A3. PHARMACOKINETIC ANALYTICAL METHODOLOGY

Chromatography was carried out using an Atlantis HILIC Silica 3 μm (2.1 x 50 mm) analytical column (Waters Corporation, Milford, MA). The NeoSTX measurement procedure was formulated and produced at Boston Children's Hospital, Clinical Epidemiologic Research Laboratory. This method was validated in accordance with guidance from the U.S. Department of Health and Human Services Food and Drug Administration guidance for industry bioanalytical method validation and the European Medicines Agency.[1,2]

A4. QUANTITATIVE SENSORY TESTING (QST) PROCEDURES

To test the duration and density of sensory blockade, a series of QSTs evaluating sensitivity to mechanical and thermal stimuli were conducted. The QST procedure started with the evaluation of mechanical thresholds for detection and pain followed by documentation of thermal thresholds. Data on the thenar eminence, as a control remote site was also collected from a subset of subjects (n=72). The testing procedures were performed in the following order per previously published recommendations from Grone et. al.[3]:

Mechanical testing

Mechanical detection thresholds (MDT) and mechanical pain thresholds (MPT), measured using a standardized set of 20 von Frey filaments (hairs) that exert a fixed force between 0.008g and 300g upon bending (North Coast Medical, Gilroy, CA). To establish MDT, filaments of incrementally increasing force were applied to the subjects' skin and subjects were asked to report when they first felt it. To establish MPT, subjects were asked when the sensation first felt "sharp" and uncomfortable. If a subject did not feel the maximum experimental force applied (300g), a value of 300g was recorded. We used the

up-down method where the appearance and disappearance of thresholds were established until 2 values were obtained. If the difference in values recorded were >2 hairs for MDT, and >1 hair for MPT, a third value was obtained.

Thermal testing

Thermal testing was conducted using a Peltier-based computerized thermode with a 1.5cm x 1.5cm contact probe (TSA II, Medoc Inc., Ramat Yishai, Israel). First, thermal detection thresholds for the cool detection (CDT) and warm detection (WDT) stimuli were assessed, followed by cold pain threshold (CPT) and heat pain threshold (HPT). Baseline thermode temperature was 32°C, cutoff values were 0°C and 50°C. A third value was collected if the difference in the recorded threshold to thermal stimuli were >2°C for CDT and WDT; >5°C for CPT; and >3°C for HPT.

When three tests were performed for each QST parameter, the mean of the closest two values was used. For QST missing data, the mean value for the dose cohort was calculated at that time point and used as an estimation for that participant; where less than 3 values were available the Last Observation Carried Forward approach was used. To present a more accurate representation of time to partial and time to near-complete recovery, we extrapolated the exact time at which a participant crossed the cut-off by taking the time points before and after they crossed the cut-off, assuming a linear pattern of recovery.

A5. SAFETY RESULTS

Adverse events

Table 1. Percentage of subjects who experienced any nausea, vomiting, dizziness, and peri-oral tingling and numbness following NeoSTX injection

NeoSTX dose (mcg)	Total	Nausea		Vomiting		Dizziness		Tingling		Numbness	
	n	N	%	n	%	N	%	n	%	n	%
0	8	1	12.5	0	0.0	0	0.0	0	0.0	0	0.0
Part 1: Dose Escalation											
5	6	0	0.0	0	0.0	1	16.7	0	0.0	1	16.7
10	14	0	0.0	0	0.0	0	0.0	4	28.6	6	42.9
15	10	0	0.0	0	0.0	3	30.0	6	60.0	8	80.0
20	10	0	0.0	0	0.0	0	0.0	8	80.0	9	90.0
30	10	0	0.0	0	0.0	1	10.0	10	100.0	9	90.0
40	10	8	80.0	3	30.0	5	50.0	9	90.0	10	100.0
Part 2: Three way combination											
10	8	0	0.0	0	0.0	0	0.0	1	12.5	1	12.5
30	8	1	12.5	0	0.0	0	0.0	2	25.0	0	0.0

NeoSTX = Neosaxitoxin

Neuromuscular and respiratory function

Two subjects exhibited a > 30% decrease in Grip Strength (GS); the decrease was not dose-dependent. Of these, one subject in the 40mcg *NeoSTX-Saline* group exhibited a decrease of 33.3% at 30min post-injection. The second subject in the 30mcg *NeoSTX-Bup* group exhibited an overall decrease in GS at all post-injection time points with a maximum change of 45.5% observed at 6hrs; at 24hrs his mean GS was 30.4% lower than baseline. None of the changes in GS were associated with changes in the respiratory or vital sign parameters evaluated. All subjects had Negative Inspiratory Force (NIF) and Vital Capacity (VC) values within normal ranges. No subjects in the two *NeoSTX-Bup-Epi* groups showed any clinically significant reduction in GS, VC, and NIF at any time point.

Vital signs

One subject at the 30mcg dose experienced relative bradycardia (HR=53 bpm) and hypotension (BP= 79/48mmHg) at the 15min time point post-injection following an apparent vasovagal episode. Vital signs were stable 5mins after the vasovagal episode (HR=68bpm; BP=96/67mmHg). One subject in the 15mcg *NeoSTX-Bup* group exhibited a HR of 44bpm 1hr post-injection (HR ranged between 44 and 55bpm). Five subjects presented with HR>100bpm (up to a maximum of 108bpm); this increase was not dose-dependent. Four of these five subjects had higher heart rate at the 10min post-injection time point and one, at the 15min time point. Other outliers for systolic blood pressure (SBP) and diastolic blood pressure (DBP) readings noted in Table 2 of the manuscript were isolated values not having any clinical impact on the subject with the highest overall SBP value being 155mmHg and the lowest DBP value being 45mmHg over the 24 hour post-injection period. The mean oxygen saturation rates remained stable across all doses and were maintained at 98% or higher. No subject required any form of respiratory, hemodynamic, or any other type of medical intervention throughout the evaluation period. No subject in the *NeoSTX-Bup-Epi* groups showed any clinically significant changes in vital signs throughout the evaluation period.

A6. EFFICACY RESULTS

Table 2. NeoSTX 10mcg Block Onset: Percentage of subjects with dense, moderate and mild block at 5 and 30min post-injection.

Treatment combination	N	Mechanical Detection Threshold					Mechanical Pain Threshold					Cold Detection Threshold				
		Dense block (%)	Moderate block (%)	Mild block (%)	Minimal block (%)	*P*	Dense block (%)	Moderate block (%)	Mild block (%)	Minimal block (%)	*P*	Dense block (%)	Moderate block (%)	Mild block (%)	Minimal block (%)	*P*
At 5 min post –injection																
Bup	8	100.0	0.0	0.0	0.0		100.0	0.0	0.0	0.0						
NeoSTX-Saline	7	42.9	57.1	0.0	0.0		42.9	28.6	28.6	0.0				--		
NeoSTX-Bup	7	100.0	0.0	0.0	0.0	0.007	100.0	0.0	0.0	0.0	0.003					
NeoSTX-Bup-Epi	8	50.0	50.0	0.0	0.0		100.0	0.0	0.0	0.0						
At 30 min post- injection																
Bup	8	50.0	25.0	12.5	12.5		75.0	12.5	0.0	12.5		87.5	0.0	0.0	12.5	
NeoSTX-Saline	7	14.3	57.1	28.6	0.0		0.0	57.1	42.9	0.0		57.1	14.3	28.6	0.0	
NeoSTX-Bup	7	100.0	0.0	0.0	0.0	0.022	100.0	0.0	0.0	0.0	<0.001	85.7	0.0	14.3	0.0	0.339
NeoSTX-Bup-Epi	8	75.0	25.0	0.0	0.0		87.5	12.5	0.0	0.0		87.5	12.5	0.0	0.0	

Bup – 0.2% Bupivacaine; *NeoSTX-Saline* – Neosaxitoxin in saline; *NeoSTX-Bup* – Neosaxitoxin in Bup; *NeoSTX-Bup-Epi* – Neosaxitoxin in Bup with Epinephrine.
Fisher's exact test

Table 3 Time to Partial and Near-complete Recovery

NeoSTX dose (mcg)	Treatment combination	N	Time to partial recovery (hr)						Time to near-complete recovery (hr)					
			MDT		MPT		CDT		MDT		MPT		CDT	
			Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
0	Saline	8	0.5	0.5-0.5	0.5	0.5-0.5	0.5	0.5-0.5	0.5	0.5-0.7	0.5	0.5-1.0	0.5	0.5-0.5
	Bup	8	7.2	2.7-9.1	11.7	3.5-16.3	7.6	4.5-20.2	10.3	5.7-12.7	20.9	9.3-21.8	17.6	8.3-39.5
Part 1: Dose escalation														
5	NeoSTX- Saline	3	0.9	0.8-0.9	0.9	0.5-0.9	0.5	0.5-3.5	3.8	1.4-12.0	3.5	0.5-4.0	3.6	0.8-11.3
	NeoSTX-Bup	3	36.5*	34.5-67.0	37.7*	34.8-64.3	43.1	1.6-88.3	47.0	39.0-84.0	44.6*	36.0-74.4	52.5	2.0-96.1
10	NeoSTX- Saline	7	0.8	0.5-3.2	0.6	0.5-2.8	0.9	0.5-9.3	18.0	6.0-21.0	1.8	1.0-4.0	3.9	2.0-18.8
	NeoSTX-Bup	7	30.2*	18.6-40.4	21.0*	11.5-56.0	4.2	1.6-39.5	34.7	24.0-67.2	39.3*	30.0-60.0	41.6	3.8-93.6
15	NeoSTX- Saline	5	8.4	4.5-9.5	7.5	3.5-8.0	9.5	5.3-9.8	20.0	19.2-20.0	12.0	10.8-14.4	11.0	9.4-11.7
	NeoSTX-Bup	5	31.2*	26.8-47.0	33.2*	21.5-50.0	40.3	0.7-44.2	38.8	36.8-64.5	38.8	30.0-54.0	47.1	0.8-65.6
20	NeoSTX- Saline	5	0.8	0.5-9.0	0.5	0.5-0.5	0.7	0.5-3.6	12.0	8.0-19.5	0.5	0.5-3.0	6.6	0.9-9.2
	NeoSTX-Bup	5	43.5*	32.4-63.5	42.5*	31.5-59.0	43.2*	29.6-66.5	61.3*	43.2-81.6	66.8*	45.0-72.0	88.4	58.3-92.5
30	NeoSTX- Saline	5	2.0	1.7-2.7	0.8	0.5-1.5	1.5	0.8-5.1	12.0	12.0-21.6	2.5	2.0-3.0	1.8	1.0-5.6
	NeoSTX-Bup	5	36.0*	31.2-56.7	34.7*	21.0-48.0	0.7	0.5-1.9	68.0*	57.6-144.0	40	36.0-63.0	39.2	22.7-49.1
40	NeoSTX- Saline	5	4.0	1.8-4.3	1.6	1.5-3.0	3.8	2.7-9.2	12.0	9.6-38.4	4.7	3.0-5.0	10.8	5.8-11.2
	NeoSTX-Bup	5	37.6*	33.3-37.7	32.7	32.0-39.0	40.3	39.9-40.9	48.0	40.0-52.0	40.0	39.3-48.0	50.9	47.9-53.5
Part 2: Three way combination														
10	NeoSTX- Bup-Epi	8	38.2*	34.5-49.7	38.2*	36.6-49.2	46.8*	13.4-86.5	49.5*	43.8-64.3	48.5*	40.1-64.4	105.5*	80.6-136.1
30	NeoSTX- Bup-Epi	8	39.5*	34.7-58.3	46.6*	34.7-52.8	42.7	23.3-63.1	66.6*	51.0-78.0	62.4*	47.7-77.6	94.2*	67.1-110.9

Bup – 0.2% Bupivacaine; *NeoSTX-saline* – Neosaxitoxin in saline; *NeoSTX-Bup* – Neosaxitoxin in *Bup*; NeoSTX-Bup-Epi – Neosaxitoxin in *Bup* with Epinephrine; IQR – Interquartile ranges.

MDT – Mechanical Detection Threshold; MPT- Mechanical Pain Threshold; CDT – Cool Detection Threshold

* Significant difference (P <0.05) when compared with *Bup* group.

Exploratory Analysis

Exploratory analysis using generalized linear models of time to near-complete and partial recovery were performed to evaluate the influence and interaction of dose (5-40mcg) and treatment combinations (*NeoSTX-saline, NeoSTX-Bup*) for part 1 of the study. We designed individual models for either time to partial recovery or time to near-complete recovery (as dependent variables) in each one of the QST parameter (MDT, MPT and CDT), and included dose cohorts (5-40mcg) and treatment groups (*NeoSTX-saline, NeoSTX-Bup*) as independent variables. If the interaction term was not significant in the original model we removed this to allow for the proper interpretation of the main effect of dose and treatment.

For all models of time to partial and near-complete recovery, *treatment* had a significant effect as an individual predictor; however *dose* did not show a significant effect in any of the models. The interaction between treatment and dose was only significant for time to near-complete recovery for MDT (Table 4).

These analyses are only exploratory in nature due to the low number of subjects assigned to each one of the dose cohorts and treatments arms. We believe that at the doses studied, it is difficult to identify changes in the duration of block and most of the differences were expected by the combination treatment groups.

Table 4. Exploratory Generalized Linear Model

Variable	Model		Dose		Treatment		Interaction	
	F	p value	F	p value	F	p value	F	p value
Time to near-complete recovery								
MDT	6.59	<0.001	1.85	0.121	51.43	<0.001	2.60	0.037
MPT	19.23	<0.001	0.36	0.877	113.62	<0.001	0.81	0.547
CDT	4.41	0.001	0.37	0.868	24.61	<0.001	1.04	0.406
Time to partial recovery								
MDT	19.82	<0.0001	0.78	0.571	115.04	<0.001	0.83	0.533
MPT	15.94	<0.0001	0.32	0.900	94.04	<0.001	0.60	0.696
CDT	4.81	0.0006	1.26	0.297	22.59	<0.001	1.43	0.229

MDT – Mechanical Detection Threshold; MPT- Mechanical Pain Threshold; CDT – Cool Detection Threshold

Possible systemic effects of NeoSTX

Given the possible systemic effect of NeoSTX, we performed secondary analysis of the *Bup* control group who received saline in the active treatment leg vs. those receiving NeoSTX (any combination or dose) in the active treatment leg (Table 5). This analysis showed some contradictory results. We observed an increase in time to near-complete recovery of bupivacaine when *NeoSTX-Bup* or *NeoSTX-Bup-Epi* combinations (but not with *NeoSTX-Saline*) were injected in the contralateral (active treatment) side, for MDT. Although this result could suggest possible mild systemic effect of NeoSTX, these differences are not consistently seen across all NeoSTX groups, and that data on the thenar eminence (naïve area remote from the site of injection) did not show any hypoesthesia post-injection (data not shown). One possible explanation could be related to the expectation of the subjects and the experimenters. The prolonged block provided by NeoSTX combinations (*NeoSTX-Bup* and *NeoSTX-Bup-Epi*) could influence the participant responses (increase in the thresholds for MDT and CDT) for bupivacaine in the contralateral side.

Table 5. Time to Partial and Near-complete recovery in the Bupivacaine control leg. Groups separated according with the active-treatment combination injected in the contralateral leg

		Time to Partial recovery (hr)					
		MDT		MPT		CDT	
Bupivacaine (contralateral treatment)	**n**	**Median**	**IQR**	**median**	**IQR**	**Median**	**IQR**
Bup (Saline)	8	6.3	2.6-8.0	4.7	1.8-9.8	5.0	3.8-9.0
Bup (NeoSTX-Saline)	30	7.3	3.0-10.5	5.2	1.3-9.4	3.3	0.8-28.9
Bup (NeoSTX-Bup)	30	8.6	3.5-14.5	5.0	0.8-16.4	14.7	1.7-34.6
Bup (NeoSTX-Bup-Epi)	16	7.0	5.4-13.2	5.3	3.7-9.5	32.8	8.2-66.6
		Time to near-complete recovery (hr)					
		MDT		MPT		CDT	
Bupivacaine (contralateral treatment)	**n**	**median**	**IQR**	**median**	**IQR**	**median**	**IQR**
Bup (Saline)	8	10.0	3.3-12.2	13.7	4.3-20.3	9.9	6.2-23.3
Bup (NeoSTX-Saline)	30	15.0	9.3-20.0	9.9	6.0-18.0	20.1	2.8-41.5
Bup (NeoSTX-Bup)	30	19.0*	10.3-32.5	17.9	10.0-33.0	32.0	5.8-46.9
Bup (NeoSTX-Bup-Epi)	16	20.4*	12.0-24.0	17.4	9.2-28.5	42.0	13.6-85.4

Bup – 0.2% Bupivacaine; NeoSTX-Saline – Neosaxitoxin in saline; NeoSTX-Bup – Neosaxitoxin in BUP; NeoSTX-Bup-Epi – Neosaxitoxin in BUP with Epinephrine.
MDT – Mechanical Detection Threshold; MPT – Mechanical Pain Threshold; CDT – Cool Detection Threshold.
* Significant difference (p-value <0.05) when compared with BUP (Saline) group using Kurskal-Wallis for multiples comparisons.

References

1. US Department of Health and Human Services, Food and Drug Administration: Bioanalytical Method Validation (Guidance for Industry); May 2001
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3. Gröne E, Crispin A, Fleckenstein J, Irnich D, Treede RD, Lang PM: Test order of quantitative sensory testing facilitates mechanical hyperalgesia in healthy volunteers. J Pain Off J Am Pain Soc 2012; 13: 73–8013.