

Determination of toxicity equivalent factors for paralytic shellfish toxins by electrophysiological measurements in cultured neurons

Journal:	<i>Chemical Research in Toxicology</i>
Manuscript ID:	tx-2011-00173d.R1
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Perez, Sheila; USC, Pharmacology Vale, Carmen; USC, Pharmacology Botana, Ana; USC, Pharmacology Vieytes, Mercedes; Universidad de Santiago de Compostela, Departamento de Fisiologia Animal Botana, Luis; Universidad de Santiago de Compostela, Departamento de Farmacologia

SCHOLARONE™
Manuscripts

1
2
3
4
5
6
7
8 ***Determination of toxicity equivalent factors for paralytic shellfish toxins***
9
10 ***by electrophysiological measurements in cultured neurons***
11

12 Sheila Perez¹, Carmen Vale¹, Ana M. Botana², Mercedes R. Vieytes³, and Luis
13
14 M. Botana^{2*}
15

16
17 ¹Departamento de Farmacología, Facultad de Veterinaria, USC, Lugo, Spain. ²Departamento
18 de Química Analítica, ³Departamento de Fisiología, Facultad de Veterinaria, USC, Lugo.
19
20

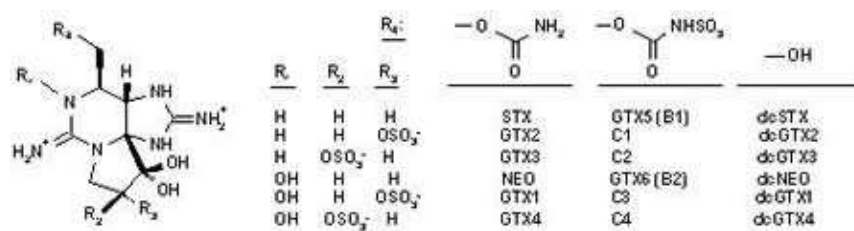
21
22
23
24 E-mail address: Luis.Botana@lugo.usc.es
25
26
27
28
29

30 Running title: Toxicity equivalent factors for PSP toxins.
31
32

33 *Corresponding author: Luis M. Botana. Departamento de Farmacología,
34 Facultad de Veterinaria, Universidad de Santiago de Compostela. Campus
35 Universitario s/n, 27002, Lugo, Spain.
36
37
38
39
40

41
42
43
44 Tel.: 34-982 252 242; FAX: 34-982 252 242. E-mail address:
45
46 Luis.Botana@lugo.usc.es
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table of Contents Graphic



Saxitoxin (STX) and PSP Toxins

Abstract

The establishment of toxicity equivalent factors to develop alternative methods to animal bioassays for marine-toxin detection is an urgent need in the field of phycotoxin research. Paralytic shellfish poisoning (PSP) is one of the most severe forms of food poisoning. The toxins responsible for this type of poisoning are highly toxic natural compounds produced by dinoflagellates, which bind to voltage-gated Na^+ channels causing the blockade of action potential propagation. In spite of the fact that several standards of PSP toxins are currently commercially available, there is scarcity of data on the biological activity of these toxins, a fact that limits the calculation of their toxicity equivalent factors. We have evaluated the potency of the commercial PSP toxin standards for their ability to inhibit voltage-dependent sodium currents in cultured neuronal cells by electrophysiological measurements. The *in vitro* potencies of the PSP toxin standards as indicated by their IC_{50} values were in the order Neosaxitoxin (NeoSTX) > decarbamoylsaxitoxin (dcSTX) > saxitoxin (STX) > gonyautoxin 1,4 (GTX1,4) > decarbamoylneosaxitoxin (dcNeoSTX) > gonyautoxin 2,3 (GTX2,3) > decarbamoylgonyautoxin 2,3 (dcGTX2,3) > gonyautoxin 5 (GTX5) > N-sulfocarbamoyl-gonyautoxin-2 and -3 (C1,2). The data obtained in this *in vitro* analysis correlated well with their toxicity values previously reported.

Abbreviations

PSP, paralytic shellfish poisoning; TEF, toxicity equivalent factor; CGC, cerebellar granule cells; DMEM; Dulbecco's Modified Eagle's medium; STX, saxitoxin; dcSTX, decarbamoylsaxitoxin; NeoSTX, Neosaxitoxin; dcNeoSTX, decarbamoylneosaxitoxin; GTX, gonyautoxin; dcGTX, decarbamoylgonyautoxin > C1,2, N-sulfocarbamoyl-gonyautoxin-2 and -3; MBA, mouse bioassay; MU, mouse unit; AOAC, Association of Official Analytical Chemists; VTD, veratridine.

Introduction

Paralytic shellfish poisoning (PSP) is one of the most severe forms of food poisoning. The toxins responsible for this type of poisoning are highly toxic natural compounds found in different phyla of both prokaryotic and eukaryotic organisms (1). PSP toxins comprise a series of heterocyclic guanidines with more than 57 known analogs of the parent compound saxitoxin (2, 3). The prevalent PSP toxins include saxitoxin and its related compounds neosaxitoxin, decarbamoylsaxitoxin, decarbamoylneosaxitoxin, gonyautoxins 1 to 6 (GTX) and their decarbamoyl analogs and four C toxins (4). Recently, several analogs of saxitoxin possessing a hydroxybenzoate moiety have been identified (5, 6). Paralytic shellfish poisoning is a public health concern worldwide (7) and it constitutes a serious illness in which neurological symptoms predominate. PSP toxins are neurotoxic and PSP poisoning exhibits symptoms including tingling sensation of the lips, numbness of extremities, gastrointestinal problems and difficulty breathing (8). The mechanism of action of PSP toxins is the blockade of sodium channels in excitable membranes that causes the arrest of action potential propagation (9, 10).

The mouse bioassay (MBA) as standardized by the Association of Official Analytical Chemists (AOAC) (11) is commonly used to quantitatively determine the PSP toxicities of shellfish in many countries (7). In this reference method (11), shellfish extracts are administered by intraperitoneal injection and the assessment is based on symptomatology and time to death. However, the bioassay has been shown to have high variability and low sensitivity. In recent years, considerable progress has been made in developing alternatives to the MBA method for almost all of the common PSP analogs. Alternative testing

1
2
3 possibilities include antibody-based quantitation (12), assay of cytotoxicity
4 blockade in neuroblastoma cells in culture (13, 14), binding assays (15) and
5 assays using voltage-sensitive fluorescent dyes (16-19). Highly sensitive
6 analytical methods such as HPLC (20), liquid chromatography-mass
7 spectrometry (LCMS) or capillary electrophoresis (21, 22) have also been
8 developed. Of these, a postcolumn liquid chromatographic method described by
9 Oshima (4) is extensively used in research applications, while an alternative
10 procedure based on precolumn oxidation (23, 24) constitutes the only validated
11 and approved alternative method to MBA for the detection paralytic shellfish
12 toxins (20). However, the toxicological database for PSP toxins is limited and
13 comprises mostly studies on their acute toxicity following intraperitoneal
14 administration. Since PSP analogs display different toxicities depending on their
15 functional groups, and toxicity equivalent factors (TEFs) may differ depending
16 on the system used, for monitoring purposes using high performance liquid
17 chromatography (HPLC) techniques, TEFs have been applied to express the
18 detected analogs as STX equivalents. Currently, the Scientific Panel on
19 Contaminants in the Food Chain (CONTAM Panel) proposed the TEFs for PSP
20 toxins based on acute *i.p.* toxicity in mice (25, 26). The European Commission
21 considers the TEF provided by EFSA opinions as the reference values to apply
22 for all calculations (27). By using a fluorescent method we have previously
23 shown that PSP blockade of the changes in membrane potential in cerebellar
24 granule cells provides an estimation of PSP potency that strongly correlates
25 with the *in vivo* toxicity of paralytic toxins (19) for the vast majority of toxin
26 standards. Therefore, the aim of this work was to obtain the toxicity equivalent
27 factors of commercial PSP toxins by a functional method using
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 electrophysiological recordings in primary cultured neurons. With this approach
4
5 the unambiguous confirmation of TEF with different technologies would provide
6
7 a solid ground to use them in any future screening program.
8
9

10 11 12 **Experimental procedures**

13 *Chemicals and solutions*

14
15
16
17 Seven-day-old Swiss Mice were obtained from the animal care facilities of the
18
19 University of Santiago de Compostela. Animals were used according to current
20
21 European legal ethical regulations. Plastic tissue-culture dishes were purchased
22
23 from Falcon (Madrid, Spain). Fetal calf serum was obtained from Gibco
24
25 (Glasgow, UK) and Dulbecco's Modified Eagle's medium (DMEM) was from
26
27 Biochrom (Berlin, Germany). The certified PSP standards were STX-
28
29 dihydrochloride, dc-STX, GTX 2&3-b, GTX1&4b, NEO-b, GTX5-b, dc-GTX2&3-
30
31 b, and C1&2; all were obtained from the Institute for Marine Biosciences,
32
33 National Research Council of Canada. All other chemicals were reagent grade
34
35 and purchased from Sigma.
36
37
38
39
40
41

42 *Cell cultures*

43
44
45 Primary cultures of CGC were obtained from cerebella of 7-day-old mice
46
47 following previously described methods (28, 29). In brief, cells were dissociated
48
49 by mild trypsinization with trypsin from bovine pancreas (0.002% w/v) at 37 °C,
50
51 followed by trituration in a DNase solution (0.004% w/v) containing a soybean
52
53 trypsin inhibitor (0.05% w/v). The cells were suspended in DMEM containing 25
54
55 mM KCl, 31 mM Glucose, and 0.2 mM glutamine supplemented with p-amino
56
57 benzoate (0.1% w/v), insulin (0.04% w/v), penicillin (0.03% w/v) and 10% fetal
58
59
60

1
2
3 calf serum. The cell suspension was seeded in glass coverslips precoated with
4 poly-L-lysine and incubated in 6-multiwell plates for 7-11 days in a humidified
5
6 5% CO₂/95% air atmosphere at 37 °C. Cytosine arabinoside (20 μM) was
7
8 added before 48 h in culture to prevent glial proliferation.
9
10
11

12 13 *Electrophysiology*

14
15 Membrane currents from single cells were studied at room temperature (22-25
16
17 °C) by electrophysiological recordings in voltage-clamp mode (30, 31) using a
18
19 computer-controlled current and voltage clamp amplifier (Multiclamp 700B,
20
21 Molecular Devices). Signals were recorded and analyzed using a Pentium
22
23 computer equipped with a Digidata 1440 data acquisition system and pClamp10
24
25 software (Molecular Devices, Sunnyvale, CA). pClamp10 was also used to
26
27 generate current and voltage-clamp commands and to record the resulting data.
28
29 Signals were prefiltered at 10 kHz and digitized at 20 μs intervals.
30
31
32
33
34

35
36 Recording electrodes were fabricated from borosilicate glass microcapillaries
37
38 (outer diameter, 1.5mm) and the tip resistance was 5-10 MΩ. The internal
39
40 pipette solution contained (in mM): 108 Cs gluconate, 1.7 NaCl, 0.9 EGTA, 9.0
41
42 HEPES, 1.8 MgCl₂, 4.0 Na₂ATP and 0.3 NaGTP, pH 7.2 (32). The extracellular
43
44 medium contained (in mM): 154 NaCl, 5.6 KCl, 3.6 NaHCO₃, 1.3 CaCl₂, 1.0
45
46 MgCl₂, 5.0 glucose and 10 HEPES (pH 7.4). Moreover, 20 mM TEA and 1.0
47
48 mM 4-AP were added in the extracellular recording solution in order to block
49
50 voltage-dependent potassium currents. Voltage dependent sodium currents
51
52 were elicited in CGCs by applying a series of 25 ms depolarizing pulses
53
54 (voltage steps), in 5 mV increments, from a holding potential of -100 mV (33,
55
56 34). Dose response curves for the inhibition of voltage-gated sodium currents
57
58
59
60

1
2
3 were obtained by plotting the percent inhibition of the peak sodium current
4
5 amplitude at each toxin concentration.
6
7

8 9 *Statistical analysis*

10
11 The percent inhibition of peak sodium current amplitude of all cells was
12 averaged. All the experiments were carried out at least three times. Results
13 were analyzed using the Student's *t*-test for unpaired data. A probability level of
14 0.05 or smaller was used for statistical significance. Results were expressed as
15 the means \pm SEM.
16
17
18
19
20
21
22
23
24

25 26 **Results**

27
28 Our objective was to determine the toxicity equivalent factor of commercial PSP
29 standards by electrophysiological measurement; to do so, we evaluated their
30 ability to inhibit voltage-dependent sodium currents in cultured cerebellar
31 neurons. This cellular preparation was chosen because PSP toxins are known
32 to block neuronal transmission through inhibition of voltage-gated Na⁺ channels
33 (9, 10). In addition, this same neuronal system has been previously used to
34 provide data on the relative potency of PSP toxins to inhibit veratridine-induced
35 depolarization, and show a good correlation with the data obtained by a
36 standard mouse bioassay (19).
37
38
39
40
41
42
43
44
45
46
47
48

49 Therefore, the ability of the different PSP standards to inhibit voltage-dependent
50 sodium currents was evaluated directly. Figure 1 shows the effect of different
51 saxitoxin related compounds on the amplitude of voltage-dependent sodium
52 currents. As shown in this figure, the parent compound saxitoxin inhibited
53 VGSCs with an IC₅₀ (95 % confidence intervals) of 3.59 nM (1.8 to 7.2 nM),
54 whereas dcSTX gave an IC₅₀ of 3.57 nM (95% confidence intervals: 1.5 to 8.2
55
56
57
58
59
60

1
2
3 nM). For Neosaxitoxin the IC_{50} to inhibit the peak amplitude of VGSCs was 3.53
4
5 nM (95% confidence intervals: 1.9 to 6.5 nM) and dcNeoSTX yielded an IC_{50} of
6
7 8.06 nM (95% confidence intervals from 2.2 to 20.9 nM). Thus, the *in vitro*
8
9 assay for the saxitoxin standards yielded the following order of potency:
10
11 NeoSTX > STX ~ dcSTX > dcNeoSTX.
12

13
14
15 In the next series of experiments the ability of the gonyautoxin standards to
16
17 block the amplitude of voltage-dependent sodium currents was evaluated. As
18
19 shown in Figure 2, the GTX 2,3 standard inhibited voltage-dependent sodium
20
21 currents with an IC_{50} of 12.79 nM (95% confidence intervals from 5.8 to 28.1
22
23 nM), whereas decarbamoyl GTX2,3 (dcGTX2,3) yielded an IC_{50} of 27.0 nM
24
25 (95% confidence intervals from 13.7 to 53.4 nM). The IC_{50} value for the GTX1,4
26
27 standard was 7.14 nM (95% confidence intervals from 2.1 to 23.7 nM) and for
28
29 the GTX5 standard was 39.6 nM (95% confidence intervals from 16.0 to 97.8
30
31 nM). Therefore, the potency of the GTX standards to inhibit voltage-gated
32
33 sodium channels was in the order GTX1,4 > GTX2,3 > dcGTX2,3 > GTX5.
34
35

36
37
38 In addition we also evaluated the effect of the C1&2 PSP standards on voltage-
39
40 gated sodium channels. As shown in Figure 3 this toxin inhibited sodium
41
42 channel amplitude with an IC_{50} of 151.3 nM (95% confidence intervals from 74.5
43
44 to 307.1 nM).
45
46

47
48 Therefore, the results presented here indicate that the toxicity of the GTX
49
50 standards as evaluated with electrophysiological recordings strongly correlated
51
52 with the *in vitro* and *in vivo* potencies of the GTX standards previously reported
53
54 by our laboratory (19) as shown in Table 1 and with the recently reported EFSA
55
56 equivalent toxicity factors as shown in Figure 4.
57
58
59
60

Discussion

1
2
3
4
5
6 Paralytic shellfish poisoning testing methods alternative to the mouse bioassay
7
8 are currently being pursued. In this sense, the knowledge of toxicity equivalent
9
10 factors for each toxin or toxin combination is very important as this will allow
11
12 national monitoring systems to use analytical methods for toxin detection. To
13
14 date only an HPLC method for paralytic toxins has been interlaboratory
15
16 validated (20, 24), although single laboratory validation of additional method is
17
18 currently pursued (35). However, the toxicological database for PSP toxins is
19
20 limited and comprises mostly studies on their acute toxicity after intraperitoneal
21
22 administration (26). For monitoring purposes using HPLC techniques the
23
24 European Food Safety Authority has applied TEF factors based on the acute
25
26 intraperitoneal toxicity of these toxins in mice (36). In this work we examined the
27
28 potency of commercial PSPs standards to inhibit voltage-gated sodium
29
30 channels by electrophysiological recordings in cerebellar granule cells. This
31
32 cellular model was chosen because we have previously shown that PSP
33
34 blockade of the changes in membrane potential in cerebellar granule cells
35
36 provides an estimation of PSP potency that strongly correlates with *in vivo*
37
38 toxicity of paralytic toxins (19).
39
40
41
42
43
44

45
46 Assays detecting the direct effect of PSP toxins on voltage-gated sodium
47
48 channels are attractive because they more closely follow the underlying
49
50 mechanism of *in vivo* toxicity. Although electrophysiological measurements
51
52 applying patch-clamp techniques to single nerve cells have been used for
53
54 screening shellfish (37), the relative potency of PSP standards has not yet been
55
56 evaluated by this approach. The results presented here indicate that
57
58 electrophysiological recordings in neuronal cells may provide a valuable tool to
59
60

1
2
3 develop functional assays for PSP toxin screening. In this regard, it is
4
5 noteworthy to indicate that the IC₅₀ for STX inhibition of neuronal sodium
6
7 channels was very close to that reported previously using recombinant sodium
8
9 channels yielding IC₅₀ values of 3.6 nM in this work and 1.17 nM in recombinant
10
11 sodium channels (37). This point is important in order to develop automatic
12
13 patch clamp functional assays for screening of PSP toxins. Since granule cells
14
15 in the cerebellum predominantly express Nav1.2 sodium channels in the axon,
16
17 and Nav1.6, Naβ1 and Naβ2 sodium channels in the soma (38), the
18
19 development of automated patchclamp techniques using this channel should be
20
21 considered for PSP screening. In this sense, it is interesting that besides
22
23 providing a lower IC₅₀ value for PSP standards than our previous *in vitro*
24
25 method in cultured cerebellar neurons (19), a fact probably due to the
26
27 determination of isolated sodium currents in this work, the saxitoxin and
28
29 gonyautoxins standards employed in this work also differed in their ability to
30
31 inhibit voltage-dependent sodium currents at the largest concentrations
32
33 evaluated, a fact that may be useful to identify the PSP profile in a toxic sample.
34
35 The relative potencies of PSP toxin standards as indicated by their IC₅₀ values
36
37 to inhibit voltage-gated sodium currents were in the order NeoSTX > STX ≈
38
39 dcSTX > dcNeoSTX > GTX1,4 > GTX2,3 > dcGTX2,3 > GTX5 □ C1,2,
40
41 which closely correlates with their inhibitory potencies on the veratridine-
42
43 induced depolarization and mouse bioassay (19). In general, the IC₅₀ values for
44
45 PSP standards reported in this work are consistently lower than previously
46
47 reported in the same neuronal model by the veratrine-induced depolarization, a
48
49 fact that is probably due that the effect was measured using isolated sodium
50
51 currents in this work, thus eliminating potassium current interference.
52
53
54
55
56
57
58
59
60

1
2
3 Noteworthy, the results reported here corroborate the proposed toxicity
4
5 equivalent factors for PSP toxins reported by EFSA (36). This fact is especially
6
7 important in the case of GTX1,4 reference materials since the toxicity of this
8
9 standard could have been overestimated previously (GTX1,4: 0.9 (36) and 0.5
10
11 in this work).
12
13

14
15 Currently the mouse bioassay and the Association of Official Chemists HPLC
16
17 method (Lawrence method) are official methods for PSP detection in the
18
19 European Union. Although not yet formally validated, other methods that have
20
21 potential to determine STX-group toxins are receptor-based assays, antibody-
22
23 based methods and liquid-chromatography- tandem mass spectrometry (LC-
24
25 MS/MS). However, most of these analytical methods do not provide information
26
27 on the toxicity of the sample and require the use of toxicity equivalent factors.
28
29 Here we evaluated the potency of commercial PSP toxins by a simple,
30
31 economical and ethically acceptable functional assay and demonstrated that
32
33 electrophysiological recordings in cultured neurons provide a sufficient tool to
34
35 detect and quantify PSP toxins. While this method is also useful for ciguatoxins
36
37 (34), the method may discriminate between both types of toxins because PSP
38
39 toxins do not change sodium channel activation. Therefore, this method could
40
41 be suitable for high throughput screening of PSP toxins by automated patch-
42
43 clamp in commercially available ready-to use cell lines expressing voltage-
44
45 gated sodium channels.
46
47
48
49
50
51
52
53
54

55 **Funding Support**

56
57 This work was funded with the following FEDER cofunded-grants:
58
59
60

1
2
3 From Ministerio de Ciencia y Tecnología, Spain: AGL2007-60946/ALI,
4 SAF2009-12581 (subprograma NEF), AGL2009-13581-CO2-01, TRA2009-
5
6
7 0189, AGL2010-17875. From Xunta de Galicia, Spain: GRC 2010/10, and
8
9 PGIDT07CSA012261PR, PGDIT 07MMA006261PR, PGIDIT (INCITE)
10
11 09MMA003261PR, 2009/XA044, 2009/053 (Consell. Educación), 2008/CP389
12
13 (EPITOX, Consell. Innovación e Industria, programa IN.CI.TE.), 10PXIB261254
14
15 PR. From EU VIIth Frame Program: 211326 - CP (CONFIDENCE), 265896
16
17 BAMMBO, 265409 μ AQUA, and 262649 BEADS. From the Atlantic Area
18
19 Programme (Interreg IVB Trans-national): 2008-1/003 (Atlantox) and 2009-
20
21 1/117 Pharmatlantic.
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

References

- (1) Shimizu, Y. (1996) Microalgal metabolites: a new perspective. *Annu. Rev. Microbiol.*, *50*, 431-465.
- (2) Llewellyn, L. E. (2006) Saxitoxin, a toxic marine natural product that targets a multitude of receptors. *Nat. Prod. Rep.*, *23*, 200-222.
- (3) Wiese, M., D'Agostino, P. M., Mihali, T. K., Moffitt, M. C., and Neilan, B. A. (2010) Neurotoxic alkaloids: saxitoxin and its analogs. *Mar. Drugs.*, *8*, 2185-2211.
- (4) Oshima, Y. (1995) Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. *J. AOAC Int.*, *78*, 528-532.
- (5) Negri, A., Stirling, D., Quilliam, M., Blackburn, S., Bolch, C., Burton, I., Eaglesham, G., Thomas, K., Walter, J., and Willis, R. (2003) Three novel hydroxybenzoate saxitoxin analogues isolated from the dinoflagellate *Gymnodinium catenatum*. *Chem. Res. Toxicol.*, *16*, 1029-1033.
- (6) Vale, P. (2008) Complex profiles of hydrophobic paralytic shellfish poisoning compounds in *Gymnodinium catenatum* identified by liquid chromatography with fluorescence detection and mass spectrometry. *J. Chromatogr. A*, *1195*, 85-93.
- (7) Lehane, L. (2001) Paralytic shellfish poisoning: a potential public health problem. *Med. J. Aust.*, *175*, 29-31.
- (8) Shimizu, Y. (2000) Chemistry and mechanism of action, In *Seafood and Freshwater toxins* (Botana, L. M., Ed.) pp 151-172, Marcel Dekker Inc., New York, NY.
- (9) Hille, B. (1975) The receptor for tetrodotoxin and saxitoxin. A structural hypothesis. *Biophys. J.*, *15*, 615-619.

- 1
2
3 (10) Narahashi, T. (1974) Chemicals as tools in the study of excitable
4 membranes. *Physiol. Rev.*, *54*, 813-889.
5
6
7
8 (11) AOAC Official Method. (2000) Paralytic shellfish poison: biological
9 method. First action, 1959. Final action, Sec. 49.10.01. In *Official*
10 *Methods of Analysis of AOAC International, 17th ed. AOAC International*
11 (Horwitz, W., Ed.), Gaithersburg, MD.
12
13
14
15
16
17 (12) Carlson, R. E., Lever, M. L., Lee, B. E., and Guire, P. E. (1984)
18 Development of immunoassays for the PSA: a radioimmunoassay for
19 saxitoxin. In *Seafood toxins* (Ragelis, E. P., Ed.) pp 181-192,
20 Washington, DC.
21
22
23
24
25
26
27 (13) Jellett, J. F., Marks, L. J., Stewart, J. E., Dorey, M. L., Watson-Wright,
28 W., and Lawrence, J. F. (1992) Paralytic shellfish poison (saxitoxin
29 family) bioassays: automated endpoint determination and standardization
30 of the in vitro tissue culture bioassay, and comparison with the standard
31 mouse bioassay. *Toxicon*, *30*, 1143-1156.
32
33
34
35
36
37
38 (14) Truman, P., and Lake, R. J. (1996) Comparison of mouse bioassay and
39 sodium channel cytotoxicity assay for detecting paralytic shellfish
40 poisoning toxins in shellfish extracts. *J. AOAC Int.*, *79*, 1130-1133.
41
42
43
44
45 (15) Vieytes, M. R., Cabado, A. G., Alfonso, A., Louzao, M. C., Botana, A. M.,
46 and Botana, L. M. (1993) Solid-phase radioreceptor assay for paralytic
47 shellfish toxins. *Anal. Biochem.*, *211*, 87-93.
48
49
50
51
52 (16) David, L. S., and Nicholson, R. A. (2004) Quantitation of paralytic
53 shellfish toxins using mouse brain synaptoneurosomes. *Chemosphere*,
54 *55*, 1315-1321.
55
56
57
58
59
60

- 1
2
3 (17) Louzao, M. C., Rodriguez Vieytes, M., Garcia Cabado, A., Vieites
4
5 Baptista De Sousa, J. M., and Botana, L. M. (2003) A fluorimetric
6
7 microplate assay for detection and quantitation of toxins causing paralytic
8
9 shellfish poisoning. *Chem. Res. Toxicol.*, 16, 433-438.
10
11
12 (18) Manger, R., Woodle, D., Berger, A., and Hungerford, J. (2007) Flow
13
14 cytometric detection of saxitoxins using fluorescent voltage-sensitive
15
16 dyes. *Anal. Biochem.*, 366, 149-155.
17
18
19 (19) Vale, C., Alfonso, A., Vieytes, M. R., Romaris, X. M., Arevalo, F., Botana,
20
21 A. M., and Botana, L. M. (2008) In vitro and in vivo evaluation of paralytic
22
23 shellfish poisoning toxin potency and the influence of the pH of
24
25 extraction. *Anal. Chem.*, 80, 1770-1776.
26
27
28 (20) AOAC Official Method 2005.06. (2006) Quantitative determination of
29
30 paralytic shellfish poisoning toxins in shellfish using precromatographic
31
32 oxidation and liquid chromatography with fluorescence detection., In
33
34 *Official Methods of Analysis of AOAC international, 18th ed. AOAC*
35
36 *International.*
37
38
39 (21) Pineiro, N., Leao, J. M., Gago Martinez, A., and Rodriguez Vazquez, J.
40
41 A. (1999) Capillary electrophoresis with diode array detection as an
42
43 alternative analytical method for paralytic and amnesic shellfish toxins. *J.*
44
45 *Chromatogr. A*, 847, 223-232.
46
47
48 (22) Pleasance, S., Thibault, P., and Kelly, J. (1992) Comparison of liquid-
49
50 junction and coaxial interfaces for capillary electrophoresis-mass
51
52 spectrometry with application to compounds of concern to the
53
54 aquaculture industry. *J. Chromatogr.*, 591, 325-339.
55
56
57
58
59
60

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- (23) Lawrence, J. F., Niedzwiadek, B., and Menard, C. (2004) Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: interlaboratory study. *J. AOAC Int.*, *87*, 83-100.
- (24) Lawrence, J. F., Niedzwiadek, B., and Menard, C. (2005) Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. *J. AOAC Int.*, *88*, 1714-1732.
- (25) Botana, L. M., Vilariño, N., Alfonso, A., Vale, C., Louzao, C., Elliott, C. T., Campbell, K., and Botana, A. M. (2010) The problem of toxicity equivalent factors in developing alternative methods to animal bioassays for marine-toxin detection. *TrAC Trends in Analytical Chemistry*, *29*, 1316-1325.
- (26) EFSA. (2009) Marine biotoxins in shellfish – Saxitoxin group. Scientific opinion of the Panel on Contaminants in the Food Chain. (Question No EFSA-Q-2006-065E).
- (27) Regulation, C. (2011) COMMISSION REGULATION (EU) No 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs pp 3-4, Official Journal of The European Union.
- (28) Schousboe, A., Meier, E., Drejer, J., and Hertz, L. (1989) Preparation of primary cultures of mouse (rat) cerebellar granule cells, In *A dissection and tissue culture manual of the nervous system* (Shahar A, d. V. J., Vernadakis A, Haber BPP, Ed.) pp 203-206, Alan R Liss, New York.

- 1
2
3 (29) Vale, P. (2008) Fate of benzoate paralytic shellfish poisoning toxins from
4
5 Gymnodinium catenatum in shellfish and fish detected by pre-column
6
7 oxidation and liquid chromatography with fluorescence detection. *J.*
8
9 *Chromatogr. A*, 1190, 191-197.
10
11
12 (30) Vale, C., and Sanes, D. H. (2000) Afferent regulation of inhibitory
13
14 synaptic transmission in the developing auditory midbrain. *J. Neurosci.*,
15
16 *20*, 1912-1921.
17
18
19 (31) Vale, C., and Sanes, D. H. (2002) The effect of bilateral deafness on
20
21 excitatory and inhibitory synaptic strength in the inferior colliculus. *Eur. J.*
22
23 *Neurosci.*, 16, 2394-2404.
24
25
26 (32) Afshari, F. S., Ptak, K., Khaliq, Z. M., Grieco, T. M., Slater, N. T.,
27
28 McCrimmon, D. R., and Raman, I. M. (2004) Resurgent Na currents in
29
30 four classes of neurons of the cerebellum. *J. Neurophysiol.*, 92, 2831-
31
32 2843.
33
34
35 (33) Osorio, N., Alcaraz, G., Padilla, F., Couraud, F., Delmas, P., and Crest,
36
37 M. (2005) Differential targeting and functional specialization of sodium
38
39 channels in cultured cerebellar granule cells. *J. Physiol.*, 569, 801-816.
40
41
42 (34) Perez, S., Vale, C., Alonso, E., Alfonso, C., Rodriguez, P., Otero, P.,
43
44 Alfonso, A., Vale, P., Hiram, M., Vieytes, M. R., and Botana, L. M.
45
46 (2011) A Comparative Study of the Effect of Ciguatoxins on Voltage-
47
48 Dependent Na(+) and K(+) Channels in Cerebellar Neurons. *Chem. Res.*
49
50 *Toxicol.*, 24, 587-596.
51
52
53 (35) van den Top, H. J., Elliot, C. T., Haughey, S. J., Vilariño, N., van
54
55 Egmond, H. P., Botana, L. M., and Campbell, K. (2011) Surface plasmon
56
57 resonance biosensor screening method for paralytic shellfish poisoning
58
59
60

1
2
3 toxins: a pilot interlaboratory study. *Anal. Chem. Just accepted*
4
5
6 *Manuscript, DOI:10.1021/ac2005235.*

- 7
8 (36) (EFSA), E. F. S. A. (2009) Scientific opinion of the Panel on
9
10 Contaminants in the Food Chain on a request from the European
11
12 Commission on Marine biotoxins in shellfish – Saxitoxin group. (Question
13
14 No EFSA-Q-2006-065E), pp 1-76, EFSA J.
15
16
17 (37) Velez, P., Sierralta, J., Alcayaga, C., Fonseca, M., Loyola, H., Johns, D.
18
19 C., Tomaselli, G. F., Marban, E., and Suarez-Isla, B. A. (2001) A
20
21 functional assay for paralytic shellfish toxins that uses recombinant
22
23 sodium channels. *Toxicon*, 39, 929-935.
24
25
26
27 (38) Schaller, K. L., and Caldwell, J. H. (2003) Expression and distribution of
28
29 voltage-gated sodium channels in the cerebellum. *Cerebellum*, 2, 2-9.
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10 **Figure Legends**

11
12 **Figure 1.** Concentration-response curves for the inhibitory effect of the PSP
13 standards for STX, dcSTX, NeoSTX and dcNeoSTX on voltage-dependent
14 sodium currents in primary cultures of mouse cerebellar neurons. Values are
15 Means \pm SEM of three to thirteen independent experiments.
16
17
18
19
20
21
22
23

24 **Figure 2.** Concentration-response curves for the inhibitory effect of the PSP
25 standards for GTX2,3, dcGTX2,3, GTX1,4 and GTX5 on voltage-dependent
26 sodium currents in primary cultures of mouse cerebellar neurons. Values are
27 Means \pm SEM of three to eleven independent experiments.
28
29
30
31
32
33
34
35

36 **Figure 3.** Concentration-response curves for the inhibitory effect of the PSP
37 standards for C1,2 on voltage-dependent sodium currents in primary cultures of
38 mouse cerebellar neurons. Values are Means \pm SEM of three to five
39 independent experiments.
40
41
42
43
44
45
46
47

48 **Figure 4.** Linear relationship between the *in vitro* potency of PSP toxins to
49 inhibit the peak sodium current amplitude in cerebellar neurons and the PSP
50 toxicity equivalent factors reported by EFSA
51
52
53
54
55
56
57
58
59
60

Table 1. Comparative results of the relative potency and IC₅₀ of PSP standards to inhibit Nav current amplitude in electrophysiological measurements and to inhibit the veratridine (VTD)-induced depolarization in cerebellar neurons (from (19)). nd: not determined

	Relative potency Nav currents	IC ₅₀ (μM) Nav currents	Relative potency (19) VTD depolarization	IC ₅₀ (μM) (19) VTD depolarization
STX	1	0.00359	1	0.0051
dcSTX	1.00	0.00357	0.84	0.0063
NeoSTX	1.02	0.00353	0.82	0.0062
dcNeoSTX	0.44	0.00806	0.48	0.0105
GTX1.4	0.50	0.00714	0.53	0.0095
GTX2.3	0.28	0.01279	0.375	0.0136
GTX5	0.09	0.0396	0.09	0.052
dcGTX2.3	0.13	0.027	0.22	0.0228
C1&2	0.02	0.151	nd	nd

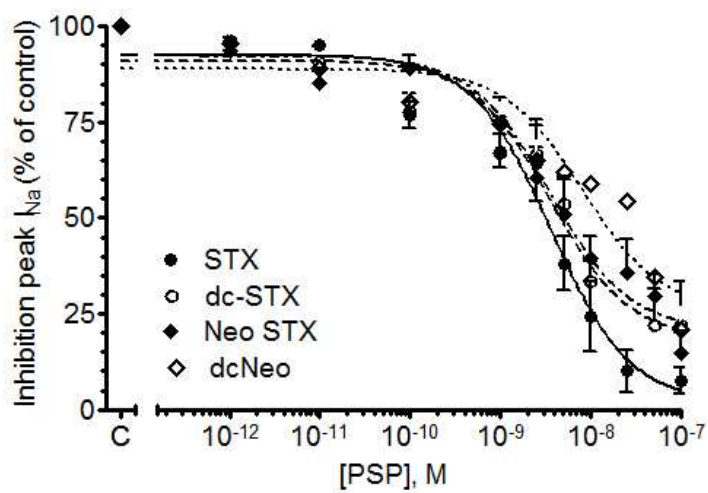


Figure 1

Figure 1

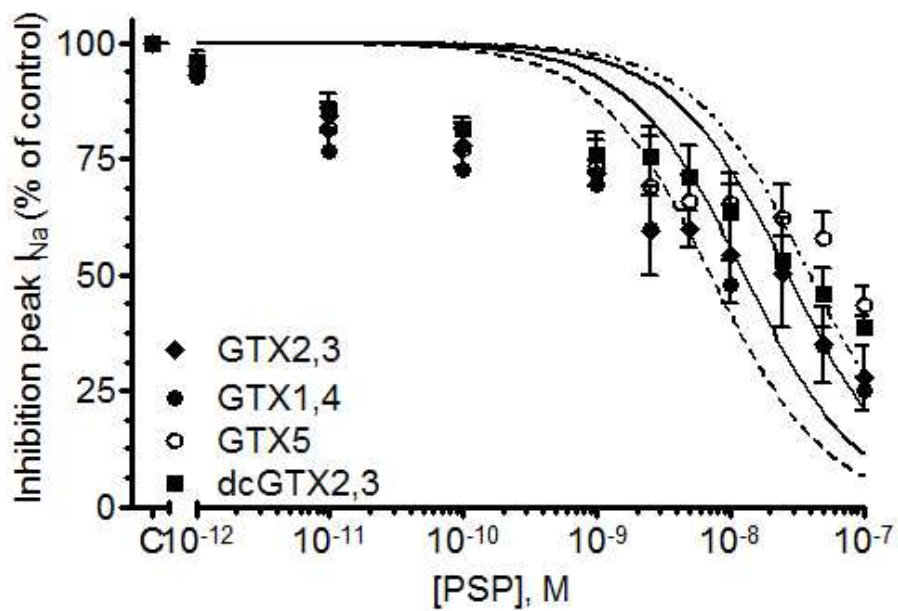


Figure 2

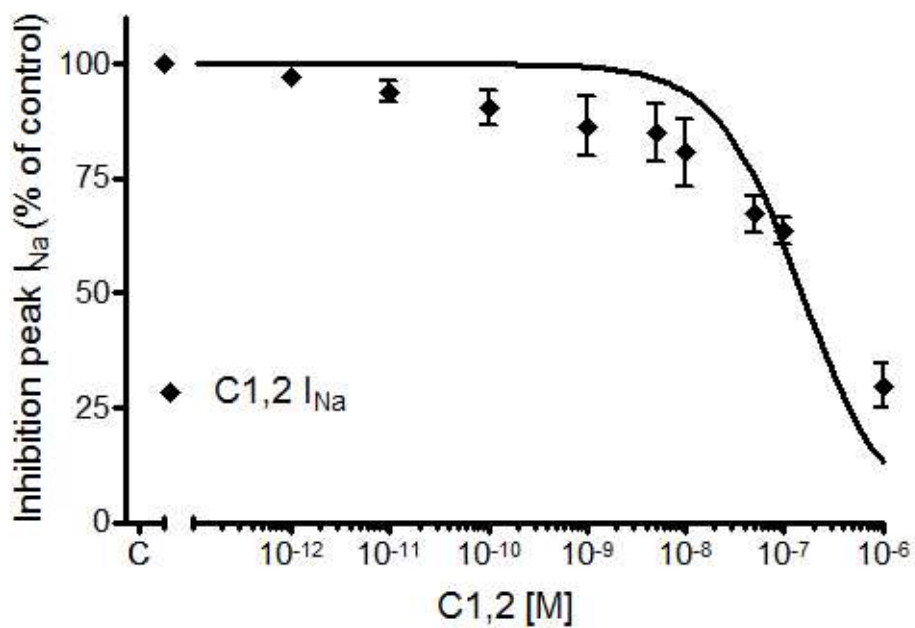


Figure 3

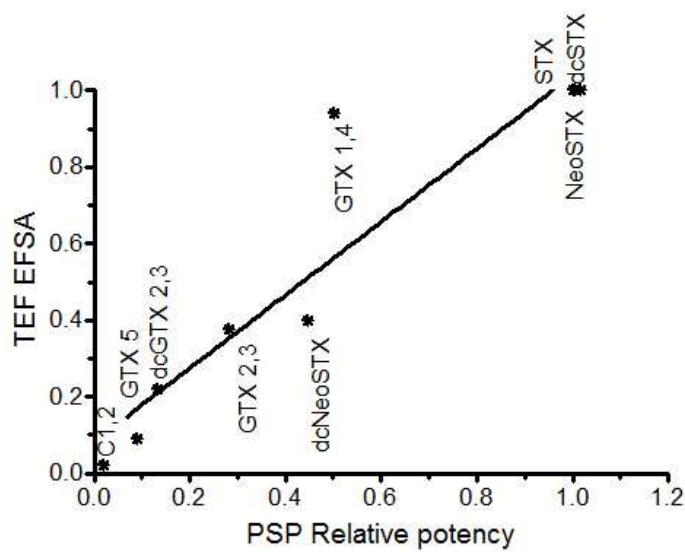


Figure 4