UNIVERSITAT ROVIRA i VIRGILI

IMPLEMENTATION OF CELL-BASED ASSAYS FOR THE DETECTION OF MARINE NEUROTOXINS

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BIOCHEMISTRY AND MOLECULAR BIOLOGY

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INDEX

ABREVIATION INDEX

1. APC: automated patch clamp
2. APs: action potentials
3. BSA: bovine serum albumin
4. BTX: brevetoxin
5. CBA: cell-based assay
6. CM: complete medium
7. CTX: ciguatoxin
8. DMSO: dimethyl sulfoxide
9. EDTA: ethylenediamine tetraacetic acid
10. EFSA: European Food and Safety Assembly
11. FBS: fetal bovine serum
12. Fura-2 AM: fura-2-acetoxymethyl ester
13. LH: locke hepes
14. MBA: mouse bioassay
15. MTT: thiazolyl blue tetrazolium bromide
16. N2a: Neuro-2a
17. NaV: sodium-voltage
18. PBS: phosphate buffered saline
19. SBFI: sodium-binding benzofuran isophthalate
20. STX: saxitoxin
21. TTX: tetrodotoxin
22. VGICs: voltage gated ion channels
23. VGSC: voltage gated sodium channel

ABSTRACT

Marine toxins represent a threat for human health, damage environments, and affect the productivity of fisheries and aquaculture industry. The geographical occurrence of these toxins is widening, and the producing organisms are becoming more diverse, so new structures of these compounds are appearing. Because of this, different detection techniques exist. All of them pursue high sensitivity, specificity, rapidity, and practicality. However, none of them is considered the gold standard method for the determination of marine toxins activity. In this research, a classical cell-based assay (CBA) and an innovative automated method are presented, comparing their efficiency and applicability.

INTRODUCTION

1. EMERGING MARINE TOXINS. IMPORTANCE

Marine toxins are secondary metabolites produced by aquatic microorganisms that accumulate in shellfish or finfish through the food chain and reach human consumers. Several marine toxins have been identified, such as tetrodotoxin (TTX), domoic acid (DA), okadaic acid (OA), azaspiracids (AZA), saxitoxins (STX), ciguatoxins (CTX), brevetoxins (BTX), or palytoxins (PLTX) [1]. Many of them cause a serious threat to human health due to poisoning after consumption of contaminated seafood, skin contact with contaminated water or inhalation of toxic aerosols. Despite the high number of intoxications that occur every year, marine toxins do not only represent a risk for human health but also have negative effects on the economies of countries that are highly dependent on seafood consumption like Japan or the Caribbean area.

Concerning food safety, the European Union asked the European Food Safety Authority (EFSA) to assess which must be the EU limits for six different types of toxins in shellfish, known as marine biotoxins, as well as the testing methods to be established by EU legislation. The EFSA opinion brings together the conclusions of six earlier risk assessments on marine biotoxins. For each type of toxin, the Panel established the amount which can be consumed within a 24-hour period without any appreciable health risk (the acute reference dose). These were then compared with shellfish consumption and incidence data from different EU countries in order to establish the EU limits. Using available consumption data, the experts identified 400 g as a large portion of shellfish and used this in assessing current permitted levels of toxins [2]. However, an adequate, universal, simple method of detection has yet to be established.

The risk for health caused by these toxins has a significant impact on the economy of several countries, since it directly affects the aquaculture industry, for instance, the bivalve mollusks. The EU food legislation focuses on them and provides the maximum limits for marine biotoxins and establishes monitoring and sampling plans in the production areas of live bivalve mollusks. Such monitoring is generally applied weekly during the periods when harvesting is allowed, but the sampling frequency must be representative for the considered area. The monitoring frequency may be reduced if the

risk assessment on toxins or phytoplankton occurrence suggests a very low risk of intoxication. On the contrary, if the results of monitoring exceed the regulatory limits the production area must remain closed by the competent authority and can be re-opened when at least two consecutive results of biotoxin levels in mollusks meet legislation requirements [3].

At the same time, marine toxins also have an important impact on the tourism sector. Countries that are highly dependent on tourism may also suffer the consequences of the marine toxins. An example of this is that, since 2008, different cases of ciguatoxin poisoning have been detected in the Canary archipelago, forcing a specific regulation in this area. In this period, 16 cases have been detected on 4 different islands, affecting a total of 108 people [4].

Considering the impact that emerging marine toxins have on different productivity sectors and ultimately, on our health and economy, the levels of toxicity in shellfish must be monitored accurately and the risk values for human health determined and regulated, since these toxins may cause substantial ecological damage and economic losses through frequent or prolonged contamination and closure of harvesting sites (aquaculture industry). But also because discoloration of coastal water and the contamination of water and fish may generate rejection in tourists, which would affect the economy as well.

2. ORIGIN AND CLASSIFICATION OF MARINE TOXINS

The different neurotoxins are classified according to their cellular target. Different molecular targets have been identified, and voltage-gated ion channels (VGICs) are one of the most relevant. These are transmembrane proteins that play important roles in the electrical signaling of cells. The activity of VGICs is regulated by the membrane potential of a cell. When the channel opens, it allows the passage of ions across cellular membranes along an electrochemical gradient. Depending on the ions that pass through the channel, VGICs can be classified as sodium, potassium, calcium, or chloride voltage-gated channels [5].

In this project, we are mainly focusing on neurotoxins that have voltage-gated sodium channels (VGSCs) as a target. These are known to transport Na^+ ions outside the cell into the cytoplasm in a voltage-dependent way.

In fact, the initiation and propagation of action potentials (APs) in excitable cells is due to the presence of VGSCs, which allow the essential Na^+ influx for neuronal functioning. Any substance that would interfere with VGSCs function would cause a potential neurological damage.

VGSCs are large proteins constituted of two or three subunits: α (260 kDa) pore-forming subunit, which is linked to one or two β1–4 auxiliary subunits (30–40 kDa). The α subunit can express the functionality of the channel itself. However, the auxiliary β subunits are needed for the localization of the channel as well as its interaction with cell adhesion molecules, intracellular cytoskeleton, and extracellular matrix. β subunits are also crucial for the modification of the kinetics and voltage dependency of the channel gating.

Alpha subunits contain four homologous, but not identical, domains (I–IV) in tandem. Each domain has six transmembrane helixes (S1–S6) and a pore-forming loop between the S5 and S6 segments (Figure 1). Every third position in the S4 segments of each domain contains positively charged amino acid residues. These residues operate as gating charges, moving across the membrane to activate channels in response to membrane depolarization [6].

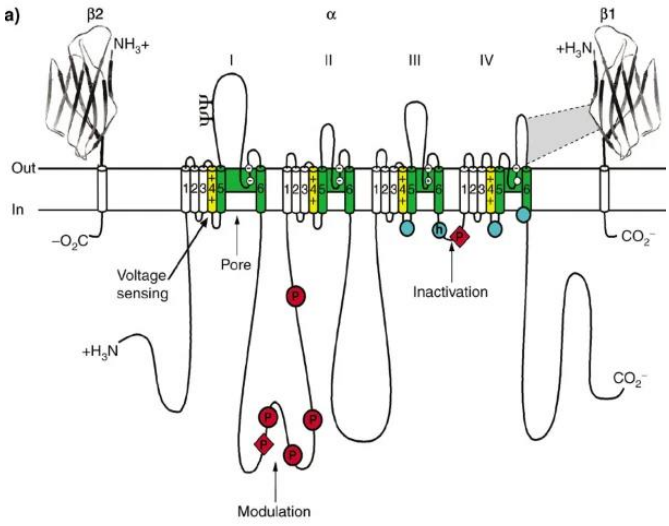


Figure 1. Schematic representation of the structure of a VGSC [7]

During the action potential and due to membrane depolarization, Na^+ ions move under the influence of the transmembrane electrochemical gradient. Regarding the inactivation gate, a small intracellular loop connecting the two homologous domains III and IV that folds into the channel structure is responsible for the blockage of the pore from the inside during sustained membrane depolarization [6].

Nine mammalian subunits have been discovered, which represent nine VGSC subtypes (NaV1.1–NaV1.9) encoded by different genes (Figure 2). Moreover, the α subunit provides a binding site for a wide range of drugs such as neurotoxins that target VGSC and can significantly modify the channel activity [6].

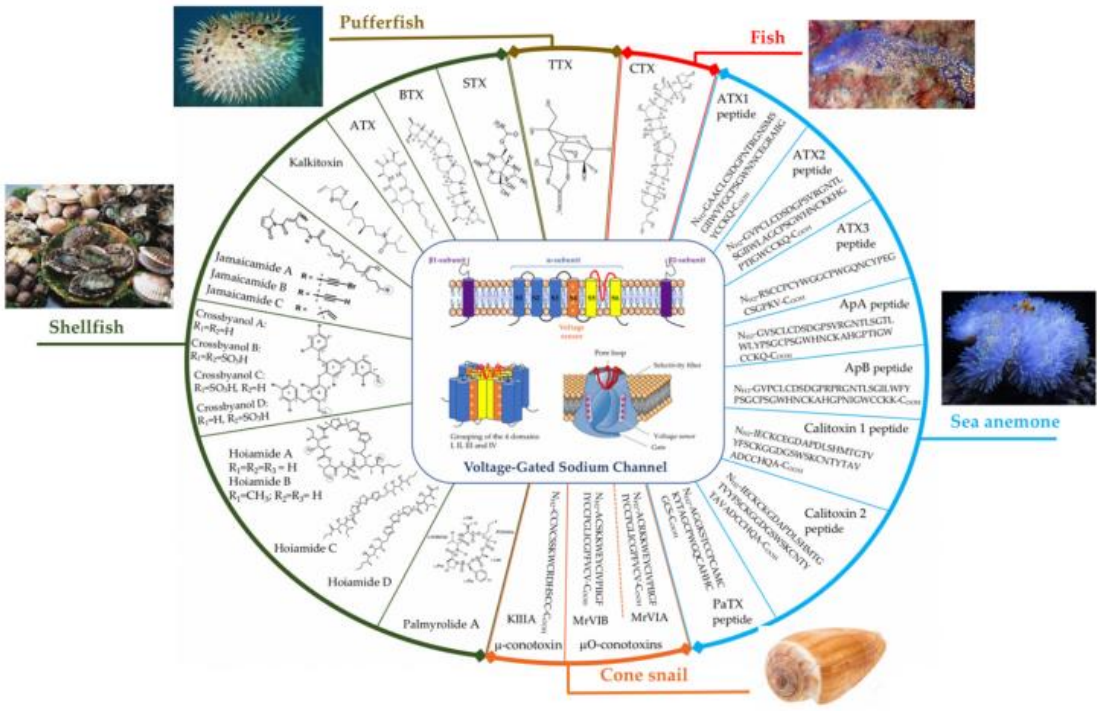


Figure 2. Toxins from marine origin that act on VGSCs and their structure [6]

Some toxins from marine origin that target these VGSCs are:

- TTX: tetrodotoxin, is particularly found in pufferfish. The onset of TTX in marine species is not due to the animal itself. It is synthesized by different bacteria genera such as *Pseudoalteromonas, Pseudomonas, Vibrio, Aeromonas, Actinomycetes, Microbacterium, Serratia*, etc. These bacteria are present in the animal microbiome or can bioaccumulate through the food chain. It presents a high toxicity for the animal physiology. It suppresses almost any function that is dependent on nerves and muscles,

as well as other cells excitability. TTX produces its blocking effect by binding to the toxin site 1 of the α-subunit of the channel at the neuronal membrane exterior surface (Figure 3). However, when applied intracellularly, even at concentrations as high as 1 µM, it has no effect on the channel [6].

- STX: Saxitoxin is a non-peptide neurotoxin produced by three species of marine dinoflagellates: *Alexandrium, Pyrodinium,* and *Gymnodinium*. It accumulates in shellfish and it is known as a "paralytic shellfish toxin" (PST) since it is responsible for a food intoxication named "paralytic shellfish poisoning" (PSP) syndrome. For this reason, it is considered one of the most lethal toxins for humans. The poisoning symptoms start after 30 minutes of the ingestion, beginning with a burning or tingling sensation on the lips and face and continuing with a total numbness that may expand to the fingers and toes and reach the extremities. Other symptoms may include perspiration, vomiting, diarrhea, and stomach cramps. An overdose (toxic dose in humans is 1–4 mg/person) of STX can cause death due to respiratory failure and cardiovascular shock.

Just like TTX, STX is a powerful neurotoxin that preferentially targets VGSCs. STX acts on VGSCs via binding to the pore-forming region of the alpha-loop VGSC, located between S5 and S6 (Figure 3). This mechanism is similar to that of TTX, as they both belong to guanidinium toxins [6].

- BTX: Brevetoxins are lipid soluble neurotoxins found in shellfish and produced essentially by the dinoflagellate *Karenia brevis*. BTXs can also be produced by other species such as *K. selliformis, K. papilionacea, K. mikimotoi, K. brevisulcata, Chatonella antiqua, Fibrocapsa japonica,* and *Heterosigma akashiwo*.

The most common symptoms of severe poisoning with BTXs are headache, gastroenteritis, diarrhea, nausea, sensory problems, cranial nerve dysfunction, scorching sensation in the rectum, bradycardia, ataxia, paresthesia, and ataxia. BTXs can also lead to a condition known as respiratory irritation syndrome.

BTXs have two different types of backbone structures: Type 1: BTX-2, 3, 5, 6, 8, and 9 (Brevetoxin B backbone) and type 2: BTX-1, 7, and 10 (Brevetoxin A backbone). Notably, all BTXs can be considered as derivates of the BTX-1 and BTX-2 [6].

- CTX: ciguatoxins are lipophilic cyclic polyethers with more than 20 analogues that have been described so far. They are synthesized by benthic dinoflagellates of the genus *Gambierdiscus*. These toxins accumulate throughout the food chain and the fish carrying CTXs store them without being affected by their harmful effects. Moreover, CTXs are heat-resistant and do not alter the organoleptic properties of the contaminated fish.

In humans, CTXs are responsible for a complex human food syndrome called ciguatera, characterized by peripheral neurological symptoms (myalgias, cold allodynia, arthralgias, paresthesia, pruritus) and central symptoms (ataxia, headache). Cardiovascular signs (bradycardia and low blood pressure) and gastrointestinal signals (abdominal pain, vomiting, nausea) are also observed. All these symptoms persist for several days, and in most cases disappear spontaneously. There is no specific treatment for ciguatera, and the treatment is purely symptomatic. It is a disease present in the Pacific Ocean, the Indian Ocean, and the Caribbean area.

CTXs act like Brevetoxins (BTXs) by binding to site 5 of NaV channels at the intracellular segments 6 and 5 of domains I and IV, respectively (Figure 3). Their mode of action is therefore similar: they slow down the inactivation of the Na^+ current, which facilitates the persistent entry of Na^+ into the cells. They also shift the activation of the Na^+ current towards more negative potentials. These toxins are activators of NaV channels, resulting in the generation of repetitive action potentials, characteristic of the exacerbated excitability, which is at the origin of the symptoms observed in humans [6].

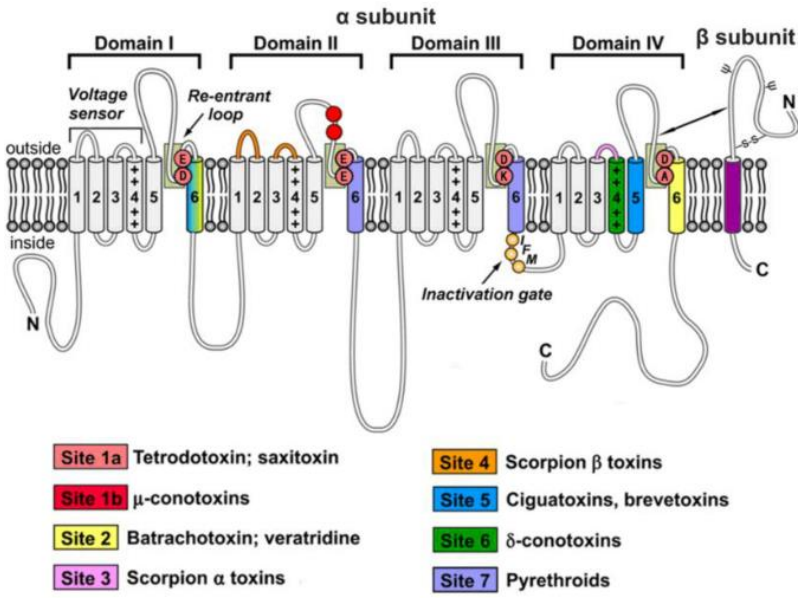


Figure 3. Binding sites of different toxins [8]

Veratridine, although not a marine toxin (it is produced by the plant *Veratrum album*) is a drug that is commonly used as an activator of VGSCs. Veratridine has been used commonly together with the Na^+/K^+ ATPase inhibitor, ouabain. The combined effect of these compounds results in elevated intracellular sodium levels leading to altered cell morphology and a subsequent decrease in cell viability. TTX, STX, and related toxins which block sodium channels, antagonize this effect (Figure 4), and other toxins, as ciguatoxins potentiate their toxicity. This phenomenon has been used in CBA to detect marine toxins (Manger *et al*., 1993).

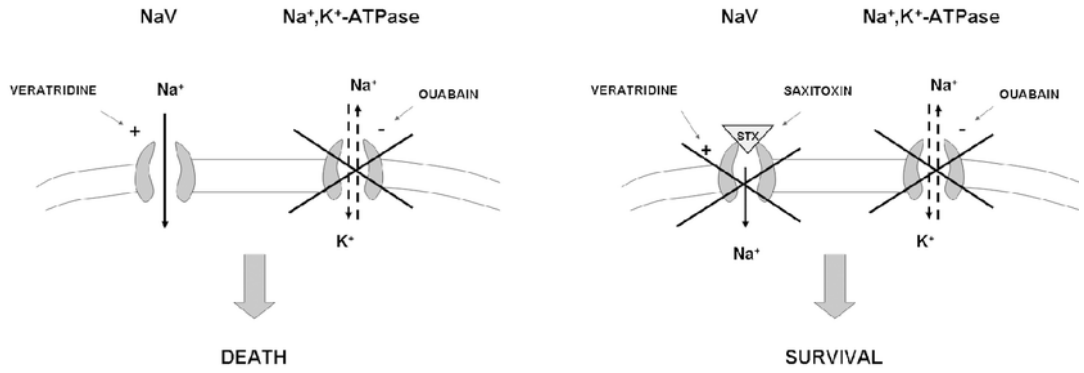


Figure 4. Cell response to STX binding to the VGSC in presence of veratridine and ouabain [10]

3. DETECTION METHODS FOR MARINE TOXINS

Because of the major implications in public health and economic activity mentioned above, producing countries need to monitor the presence of marine toxins in seafood. For this purpose, several detection methods have been developed. However, detection and quantification of these groups of marine toxins remains highly challenging due to the wide range of congeners present in contaminated biological samples. In addition, an official reference method is still missing for most of these toxins due to the lack of reference standards (figure 5).

Although the mouse bioassay (MBA) has been recommended by the European Food Safety Authority (EFSA) for years until 2015, the European Union now strongly supports the use of analytical techniques based on liquid chromatography coupled with mass spectrometry in tandem (LC-MS/MS) and high-performance liquid chromatography with

fluorescence detection (HPLC-FLD) for the detection of lipophilic and hydrophilic toxins, respectively. However, the need for alternative high-throughput methods for toxin screening and quantification purposes remains. Among the available and most widely used *in vitro* methods, the functional assay known as CBA that uses a neuroblastoma cell line appears as the most promising one [12].

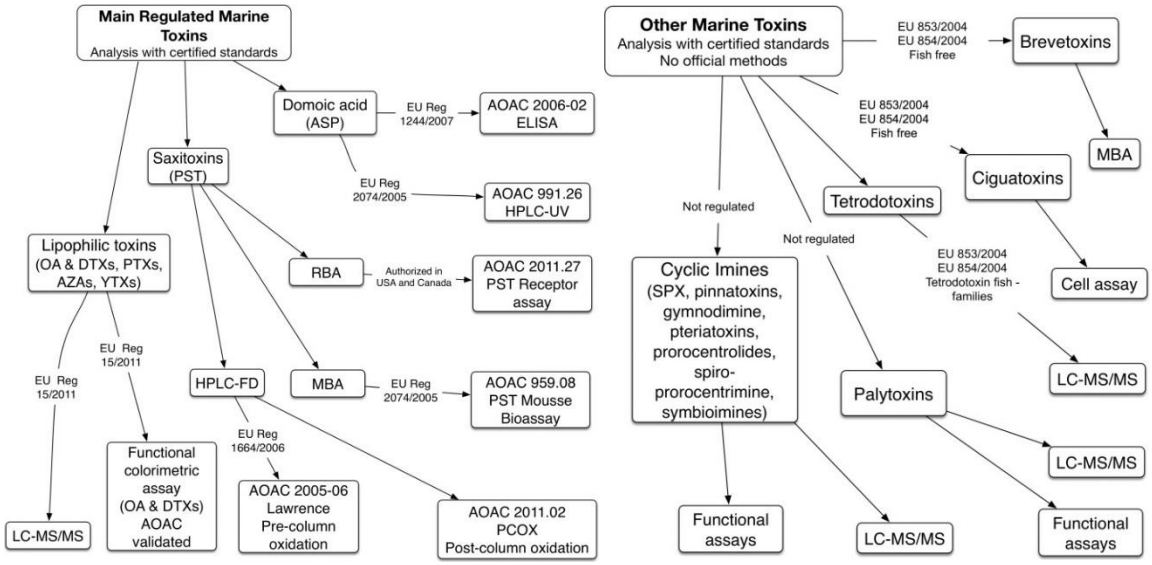


Figure 5. Main detection methods used depending on the toxin [11]

The Mouse Bioassay uses animal models such as rats and/or mice. They are not very sensitive and can be subject to interferences. Moreover, they do not give information about the concentration of the different toxins in target tissues, and it is difficult to identify which toxin causes the death of the mice. They are also time consuming, expensive and require expertise manipulators to work with animals, apart from ethical issues.

Because of the major drawbacks this method had, alternative methods such as biochemical immunological assays and chemical analytical methods have been developed.

Immunoassays are biochemical assays based on the immunological affinity between an antibody and its antigen. They have been successfully used in both the screening and precise quantification of some marine toxins, because of their high selectivity and sensitivity. It is necessary to keep in mind that immunoassays are based on a structural

recognition, thus they do not provide toxicological information. Moreover, antibodies may have the ability to detect different toxin analogues or derivatives if they share a structurally similar fragment. This cross-reactivity may be advantageous or not, depending on whether the purpose is to detect the whole family of toxins (not all of them necessarily having the same toxicological potency) or just a specific one. The most common immunoassay format is the enzyme-linked immunosorbent assay (ELISA), which relies on the use of specific Abs against the target analyte, and enzymes as labels. ELISAs can be direct or indirect. The indirect format involves the use of a labelled secondary Ab against the primary Ab, and the direct one implies the labelling of the primary Ab [13].

Chemical analytical methods are also powerful methods for toxin detection. For example, Amnesic Shellfish Poisoning (ASP) toxins present in seafood are quantified using high-performance liquid chromatography (HPLC-UV). In the EU, a liquid chromatography-coupled to tandem mass spectrometry (LC-MS/MS) method is currently the reference method for the quantification of lipophilic marine toxins, replacing completely the MBA by 2015. Instrumental analysis approaches have also been contemplated for emerging marine toxins. For example, cyclic imines such as gymnodimines, pinnatoxins and spirolides, can be identified and quantified by LC-MS/MS methods. Ciguatoxins can also be identified by LC-MS/MS, but the application of the method in routine is difficult due to the lack of certified material, the structural complexity of the toxins group, and the low sensitivity of the current instrumentation, since these toxins are extremely powerful and may be hazardous at concentrations in fish that are difficult to detect [13].

Although the instrumental analysis approach is certainly a good strategy to evaluate emerging marine toxins in food, alternative methods that can provide a higher sensitivity or that may have other advantages such as shorter analysis times or lower equipment complexity (figure 6) and cost, may be used as screening or quantification tools to facilitate the evaluation of multiple samples.

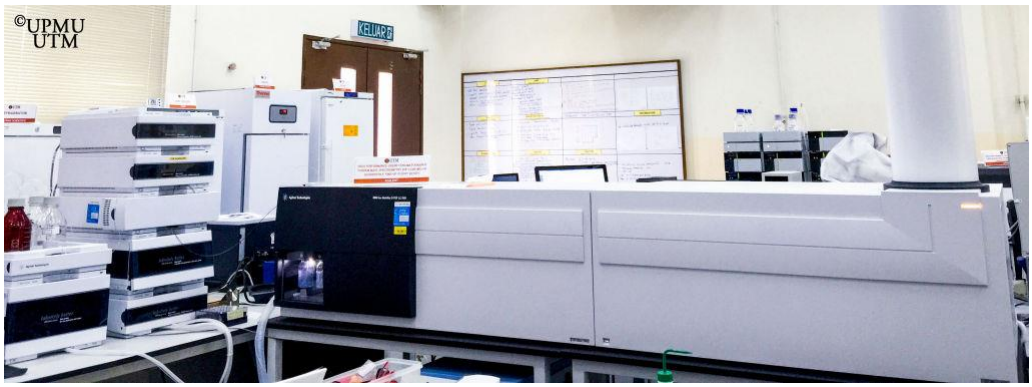


Figure 6. HPLC-MS/MS equipment in the laboratory [14]

Finally, functional methods such as the ones we use in this project, the functional CBA, also are being developed. These are based on the detection of the biochemical effect of toxins on cells. Marine toxins usually produce a change in the physiology, the morphology, or the viability of cells, which can be measured and quantified. Most CBAs require the presence of agonists or antagonists, e.g., the drugs veratridine and ouabain mentioned above, in order to counteract or emphasize the action of those toxins. Veratridine is a well-known activator of the voltage-gated sodium channels (VGSCs), which binds to these channels and blocks them in an open position. Ouabain binds to the Na^+/K^+-ATPase pump and blocks it in a closed position, thus inhibiting the flux of sodium from the inside of the cells and potentiating the toxic effects of CTX, for instance.

Currently, the most well-established CBA to detect marine toxins is the cell viability assay, which consists of determining the number of healthy cells exposed to a sample after an incubation period. Many different types can be employed, such as dye exclusion, colorimetric, luminescent or the one we use in great part of our research, the fluorescence assay. Among the different assays used, the Neuro2A CBA is able to detect both TTX or CTX toxins and is quite versatile.

CBA usually offer high sensitivities. Nevertheless, other factors such as cost, low speed of analysis, the need of trained professionals and equipment limits this method when choosing it as the most appropriate for the detection of marine toxins [15]. Also, the fact that CBA is used to study the effect that appears on the cells (e.g., depolarization of the membrane) without establishing the exact substance that is causing it, makes it unsuitable to determine which compound from the sample is the actual cause of the effect observed. There is a need of new assays that may detect neurotoxins of different

origins that can be reliable, high throughput and cost-effective, so it is a promising field to explore in the world of marine toxins detection.

4. NEW APPROACHES TO MARINE TOXIN DETECTION

Novel technologies are emerging, and so are detection methods for marine toxins. An innovative type of cell-based assay which may overcome many of the limitations mentioned above about this type of detection technique is based on the patch clamp technology.

The patch clamp technique is the gold standard for real-time investigation of ion channels, the target of many marine toxins.

Automated patch clamp increases throughput and efficiency compared to a conventional cell viability assay. This technology shows reliable and precise results, and it does not require highly qualified technicians.

Several companies are developing APC systems meeting different levels of throughput. Nanion is one of the leading companies in the field and in 2009 launched the Patchliner system (figure 7). It is an automated patch clamp robot for medium throughput. The downsized version of it is the Port-a-Patch, a semi-automated device with a small footprint for the analysis of ion channels in cells and lipid bilayers. Finally, the SyncroPatch 384 is a high-throughput patch clamp instrument [16].

Figure 7. Nanion's Patchliner [16]

The patch-clamp technique allows the research of a small set or even single ion channels. Therefore, it is interesting for the study of the conductance of these channels which are present in excitable cells such as Neuro-2a.

The principle of this technique explains how a glass pipette containing electrolyte solution is tightly sealed onto the cell membrane and thus isolates a membrane patch electrically. Currents fluxing through the channels in this patch hence flow into the pipette and can be recorded by an electrode that is connected to a highly sensitive differential amplifier. In order to force the formation of the high-resistance seal (giga-ohm range), suction is applied. The ions fluxing the membrane patch flow into the pipette and are recorded by a silver chloride electrode connected to a highly sensitive electronic amplifier.

To prevent alterations in the membrane potential, a compensating current that resembles the current that is flowing through the membrane is generated by the amplifier as a negative feedback mechanism (Figure 8) [17].

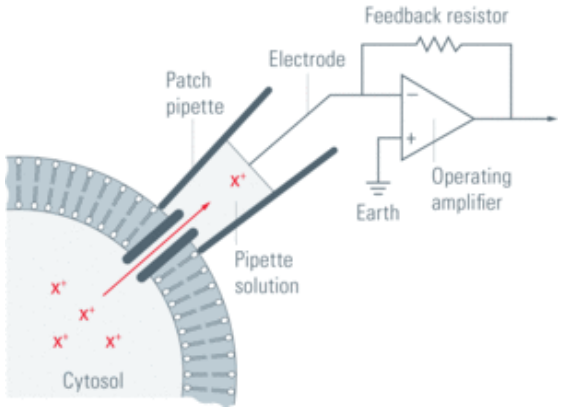


Figure 8. Schematic representation of automated patch-clamp technique

Apart from APC, fluorometric CBAs are also a field to explore. They are based in the incorporation of fluorescent probes that are sensitive to intracellular messengers that are altered by a toxin. For instance, Fura-2 is a ratiometric calcium-sensitive probe that has been used to assess neurotoxic and neuroprotective effects [18] that can also be adapted for marine toxin detection [19]. SBFI is a sodium-sensitive probe that can also be used[20]. Both approaches can be adapted to immortal cell lines offering a potential high-throughput and fast response.

HYPOTHESIS AND OBJECTIVES

Our hypothesis is that new CBA assays can be developed and adapted to cell lines to detect marine toxins.

Fluorometric cell bioassays are sensitive methods that can be used to detect the depolarization of cells that express VDIC like the Neuro2A cell line. Moreover, the automated patch clamp technology can also be used as an efficient, reliable, and fast detection method to detect marine toxins.

Therefore, our main objective is:

To prove the applicability and efficiency of fluorescence-based methods or APC using the Neuro2A cell line to detect activity of marine toxins acting at VGDCs.

MATERIALS AND METHODS

1. CULTURE AND MAINTENANCE OF NEURO-2A CELL LINE

The substrate used during our experiments were the Neuro-2a cells (figure 9). These are neuroblastoma cells from *Mus musculus* obtained originally from the American Type Culture Collection (ATCC number: CCL-131). The protocol followed for the maintenance of this culture is the one provided by Institute of Agrifood Research and Technology (IRTA) based in La Ràpita, Montsià, Spain.

The use of this cell line is justified since it has been widely used for the detection of sodium channel toxins and cytotoxicity assays [9], [13], [21]. This cell line expresses both sodium and calcium voltage-gated channels [22], and can be used to investigate the impact of different toxins acting on these ion channels by applying fluorometric CBAs.

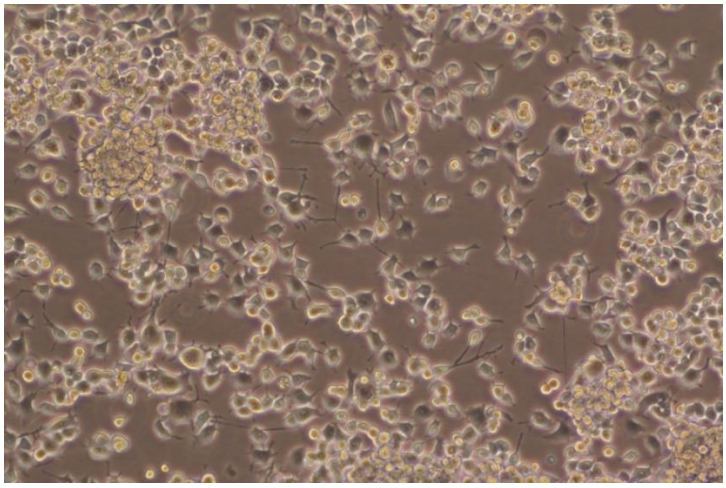


Figure 9. Neuro-2a cells in 75 cm^2 flask, six days in vitro FBS 10%

1.1. MATERIALS AND REAGENTS

- Sterile pipettes (10 and 25 mL) (Nalgene)
- Sterile centrifuge tubes (15 and 50 mL) (Nalgene)
- 75 cm^2 sterile flasks for cell culture (Nalgene)
- Cell culture incubator (Panasonic) (37 °C and 5% CO2)
- Laminar flow hood (Telstar AV-100)
- Inverted microscope (Nikon TE200)

- Swinging bucket rotor Centrifuge (VWR Mega Star 1.6 R)
- Water bath at 37 °C
- Vacuum pump and aspiration system
- Liquid Nitrogen Dewar

- Ethanol 70% and Milli-Q water
- RPMI medium 1640 (Gibco, Cat. No 21875-059)
- Inactivated FBS (Gibco, Cat. No 10082-147) stored at -20 °C
- Sodium pyruvate solution (100 mM) (Gibco, Cat. No 11360-070) stored at -4 °C
- Penicillin Streptomycin (Gibco, Cat. No 15140-122) stored at -20 °C
- PBS (Sigma-Aldrich, Cat. No P3813)
- Trypsin-EDTA 1X solution (Gibco, Cat. No 15400-054) stored at -20 °C
- Accutase Stempro® (Thermo Fisher, Cat. No A1110501) stored at -20 °C
- 2 mL Cryogenic vials

1.2. CULTURE MEDIUM PREPARATION

For cell maintenance, a Complete Medium (CM) containing RPMI 1640 in 10% FBS was prepared. The medium was supplemented with sodium pyruvate solution (1 mM) and streptomycin-penicillin. Once prepared, CM was stored at 4°C for 10 days maximum.

1.3. THAWING AND PLATING CELLS

The cells were originally stored in liquid nitrogen containers. To use the cells, one vial was thawed in a 37 °C water bath and resuspend it in 9 mL CM. Afterwards, centrifuged 5 to 7 minutes at 125 g. Supernatant was discarded and the pellet resuspended in 14 mL of CM and plated them in a 75 cm^2 flask. Neuro-2a cells were maintained at 37 °C in a cell 5% CO_2 incubator (5% CO_2).

1.4. PROTOCOL FOR PASSAGING CELLS

When the cell's confluency of the flask reached the 80-90%, cells were harvested and passaged into new cell culture flasks. To proceed, medium was discarded by vacuum

aspiration and cells washed with 10 mL sterile PBS per flask. Trypsin-EDTA was added (4mL per flask) and cells were pipetted thoroughly until cells detach from the flask wall. After centrifugation (5 minutes at 125 g) cells were resuspended in 14 mL CM per new flask.

1.5. PROTOCOL FOR FREEZING CELLS

For freezing cells, after retrieving medium and washing with 10 mL of PBS per flask, 2 mL of Accutase were added for 2 minutes at CO_2 incubator. After centrifugation, cells were resuspended in 9 mL CM and 0.45 mL DMSO and distributed in five cryogenic vials placed in a cuvette full of ice. The cuvette to a -20°C freezer and after 2 hours to the -80ºC freezer. After 24 h, the vials were transferred to a Dewar in N_2 for storage.

2. CELL BIOASSAYS

2.1. FLUOROMETRIC ASSAYS

Fluorescent indicators were used to determine intracellular ion concentrations. These exist in a variety of affinity, brightness, or spectral characteristics.

Fura-2 AM is a membrane-permeable, non-invasive derivative of the ratiometric calcium indicator fura-2. Fura-2 AM crosses cell membranes and once inside the cell, the cellular esterases remove the acetoxymethyl group. This dye has an excitation spectrum at 380 nm as a Ca^{2+} free form. When the dye binds to Ca^{2+}, the excitation is shifted to 340 nm. Thus, the increase of intracellular Ca^{2+} excites the dye in the Ca^{2+} binding form at 340 nm, along with a decrease in fluorescence intensity at 380 nm from the free form of the dye [23].

SBFI is a UV-excitable, ratiometric green indicator for intracellular sodium (Na^+) measurements. Excitation spectrum in the Na^+- bound SBFI form is at 340 nm, whereas the excitation which can be used to detect Na^+- free SBFI is 380 nm. It is about 18 times more selective for Na^+ over K^+.

The solutions studied in this assay are sodium channels activators that allow the entry of ions into the cytoplasm, which at the same time, bind themselves to the dyes and exhibit the fluorescence read by the fluorometer.

2.1.1. MATERIALS AND REAGENTS

- LS-55 Fluorescence Spectrometer (PerkinElmer)
- Water bath (Selecta)
- Fluorescence quartz cuvette (Hellma)
- Cell culture 35 mm plates (Corning)=
- 10 mm round glass coverslips (Menzel glaser)
- Special coverslip holder for fluorescence cuvettes (PerkinElmer)
- CM: for preparation protocol, see point 1.2
- LH buffer 1X: get a 50 mL graduated cylinder and add 33 mL Milli-Q water, 10 mL $CaCl_2$ ($X2H_2O$) 13 mM (Panreac, Cat. No 141215) stock solution, 5 mL Locke-Hepes stock solution 10X (to prepare, weight 7,01 g NaCl, 745,6 mg KCl, 1,26 mg NaHCO3, 670,9 mg $MgCl_2$ ($x6H_2O$) and 2,383 g HEPES and dissolve in 100 mL MQH_2O) and 25,2 mg D-glucose (Panreac, Cat. No 141341) for a 2,8 mM final concentration or 150,4 mg for a 16,7 mM final concentration. Mix using a magnetic stirrer in a 100 mL beaker. Adjust pH to 7.4 and bring up to 50 mL. Keep at 4ºC for a week.
- Fura-2 solution 2 mM: add 25 µL DMSO anhydrous (Sigma-Aldrich, Cat. No 276855) to 1 vial containing 50 µg of Fura-2 AM (Thermo Fisher, Cat. No F1221). Stock at -20ºC for a maximum of 1 month.
- SBFI solution 5 mM: weight 1 mg of SBFI (Sigma-Aldrich, Cat. No S1148) in 177 µL DMSO anhydrous (Sigma-Aldrich, Cat. No 276855). Stock at -20ºC for a maximum of 1 month.
- Pluronic (Sigma-Aldrich, Cat. No P2443) 5 % in H_2Od. Stock at -20ºC for a maximum of 1 month.
- Veratridine solution: prepare Veratridine (Sigma-Aldrich, Cat. No 676950-5MG) solution 1 mM in HCl. For a final concentration of 10 µM in the sample, add 13 µL to a total volume of 1300 µL in LH buffer. Keep at -20ºC.

- Ouabain solution: prepare Ouabain (Sigma-Aldrich, Cat. No O3125) 0,1 mM in HCl. For a final concentration of 100 µM in the sample, add 13 µL to a total volume of 1300 µL in LH buffer. Keep at -20ºC.
- CTX solution: for the experiment, take 2 µg/mL CTX extract (kindly donated by Mònica Campàs from IRTA) and add 13 µL of it to a total volume of 1300 µL for a final concentration of 1,54 ng/mL in LH buffer. Keep at -20ºC.
- KCl solution 3M: weight out 223 mg KCl (Panreac, Cat. No 141494) and dissolve in 1 mL. Add 26 µL for the assay. Keep at 4ºC.
- Ionomycin solution: dissolve 1 mg Ionomycin (Thermo Fisher, Cat. No J60628) in 1,34 mL DMSO anhydrous (Sigma-Aldrich, Cat. No 276855) for a final concentration of 1 mM. For the assay, add 13 µL of this aliquot to a total volume of 1300 µL in LH buffer for a final concentration of 10 µM. Keep at -20ºC.
- Monensin solution: dissolve 2 mg Monensin (Thermo Fisher, Cat. No J61669) in 2,88 mL DMSO anhydrous (Sigma-Aldrich, Cat. No 276855) for a final concentration of 1 mM. For the assay, add 13 µL of this aliquot to a total volume of 1300 µL in LH buffer for a final concentration of 10 µM. Keep at -20ºC.

2.1.2. FURA-2 ASSAYS

For Fura-2 assays, cells were seeded in glass coverslips as needed (approximately at a density of 400.000 cells/mL). After 24-48 hours of plating Fura-2AM was added in CM (4 µM final concentration) and incubated for 45 minutes in the cell incubator. Afterwards coverslip was inserted on the special holder and to a quartz cuvette containing 1.3 mL LH buffer at 37°C and a magnetic stirrer. Ratio of emission fluorescence (510 nm) was recorded during 30 min at excitation fluorescence of 340 and 380 nm under gently swirling.

2.1.3. SBFI ASSAY

SBFI was added to cells similarly as in the Fura-2 assay but at a final concentration of 10 µM plus 10 µL Pluronic solution and incubated for 2,5 hours in the cell incubator.

2.2. AUTOMATED PATCH CLAMP

2.2.1. MATERIALS AND REAGENTS

- 100 mm cell culture plates
- Patchliner from Nanion and accessories [16]
- PBS (Sigma-Aldrich, Cat. No P3813)
- Accutase Stempro® (Gibco, Cat. No A11105-01)
- RPMI medium 1640 (Gibco, Cat. No 21875-059)
- External solution for Na^+ channels (Nanion, Cat. No 08 3001): 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM D-Glucose monohydrate, 10 Mm Hepes/NaOH ph 7.4, Osmolarity: 298 mOsmol.
- Internal solution for Na^+ channels (Nanion, Cat. No 08 3008): 50 mM CsCl, 10 mM NaCl, 60 mM Cs-Fluoride, 20 mM EGTA, 10 mM Hepes/CsOH, pH 7.2, Osmolarity: 285 mOsmol.
- Seal enhancer solution (Nanion, Cat. No 08 3012): 80 Mm NaCl, 3mM KCl, 10 mM $MgCl_2$, 35 mM $CaCl_2$, 10 mM Hepes (Na^+-salt) / HCl pH 7.4, Osmolarity: 298 mOsmol
- Lidocaine solution: prepare 3 mM, 1 mM, 300 µM, 100 µM, 10 µM and 1 µM aliquots from stock solution 73,8 mM from Lidocaine medication 20 mg/mL (B Braun, Cat. No 345504)
- Tetracaine solution: prepare 300 µM, 100 µM, 30 µM, 10 µM, 3 µM, 1 µM and 0,1 µM aliquots from the Tetracaine (Thermo Fisher, Cat. No 460820050) stock solution 20 mM.
- TTX solution: prepare 1 µM, 100 nm, 30 nm, 10 nm, 3 nm, 1 nm and 0,1 nm aliquots from 1 mg/mL TTX extract (kindly donated by Mònica Campàs from IRTA).

2.2.2. CELL HARVESTING

Medium from a plate was removed and cells were washed with 8 mL PBS. Afterwards, 4 mL Accutase were added and incubated in the CO_2 incubator for 10 minutes. 1 mL of cells were then diluted in 3 mL external solution plus 3 mL RPMI. Cell density was

adjusted to 200.000-400.000 cells/mL with the same RPMI/External solution (50/50) mixture.

2.2.3. AUTOMATED PATCH CLAMP ASSAY

The cell suspension was placed in the white container (CellHotel) and all the recording buffers and drug solutions were placed in their positions (figure 10). PatchControl HT was programmed using standard procedures for the measurement of the activity of antagonists. VDSC Chips of medium resistance were used and 8 channels were recorded simultaneously. Each Patchliner chip (NPC-16) has 16 recording chambers (figure 11), therefore, the 8-channel Patchliner uses half a chip for each run. Two HEKA amplifiers were used for recordings and the PatchControl and PatchMaster software were used for programming and registering all the data.

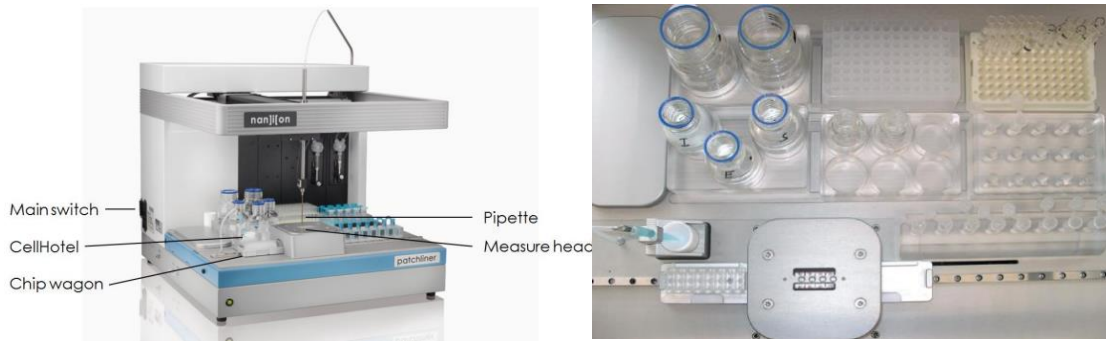


Figure 10. Nanion's Patchliner equipment and location of the solutions for the experiment [16]

Figure 11. Patchliner chip (NPC-16). The external fluidic channel is indicated in red and the internal fluidic channel in blue [16].

3. MEMBRANE FRACTIONS ISOLATION FOR LIGAND BINDING ASSAY

Although the aim of this work was to set up experiments to detect toxins, throughout my stay in the Pharmacology Unit I had the opportunity to learn other techniques not directly related to the study of the depolarization of voltage-gated sodium channels. In this case, the task consisted in isolating membrane protein from N2a cells for ligand binding assays and afterwards determining the final concentration of protein in the sample.

3.1. BIOLOGICAL SUBSTRATE

The substrate used for this technique were N2a cells from 5 different 75 cm^2 flasks at a 90-95% confluency, in order to extract as much protein as possible.

3.2. MATERIALS AND REAGENTS

- Ultraturrax-type homogenizer (VWR)
- Swinging bucket rotor Centrifuge (VWR Mega Star 1.6 R)
- 50 Ti rotor Ultracentrifuge (Beckman)
- Ultracentrifuge Kontron
- PBS (Sigma-Aldrich, Cat. No P3813)
- Homogenation buffer (HB):
  - Tris (Trizma® Base; Sigma-Aldrich, Cat. No T1503 or equivalent) → 50 mM
  - EDTA disodium salt dihydrate (Sigma-Aldrich, Cat. No E4884 or equivalent) → 5 mM pH = 7

- Assay buffer (AB):
  - Tris (Trizma® Base; Sigma-Aldrich, Cat. No T1503 or equivalent) → 50 mM
  - EDTA disodium salt dihydrate (Sigma-Aldrich, Cat. No E4884 or equivalent) → 1,5 mM pH = 7.4

3.3. CELL MEMBRANE ISOLATION

Medium was discarded and cells were washed with 5 mL PBS. Cells were scraped in 4 mL HB. Then, mix all and distribute in centrifuge tubes 5 mL each. Cells were homogenized using an ultraturrax-type homogenizer (15 s maximum power) and centrifuged 400 x g 5 min at 4°C. Afterwards, supernatant was re-centrifuged in polycarbonate tubes 75000 x g for 20 min at 4 °C. Finally, the pellet was resuspended in 3,5 mL AB and frozen at -80°C. An aliquot was saved for protein assay.

4. BRADFORD PROTEIN ASSAY AND CALIBRATION CURVE PREPARATION

In order to determine the final protein concentration from the isolation procedure explained in section 3.3, it was necessary to prepare a calibration curve and use the Bradford technique. The principle of this technique is that the binding of protein molecules to Coomassie dye under acidic conditions leads to a color change from brown to blue, which absorbance is read at 595 nm.

4.1. MATERIALS AND REAGENTS

- Biotek PowerWave XS2 Microplate Reader
- 96-well microplate
- Bradford Reagent (Sigma-Aldrich, Cat. No B6916)
- Albumin Standard (2 mg/mL) (Thermo Fisher, Cat. No 23210)
- AB solution: for preparation protocol see section 3.2

4.2. CALIBRATION CURVE

For the calibration curve, a solution 2 mg/mL of BSA and AB was used (table 1).

Table 1. Different BSA concentration aliquots in AB for calibration curve preparation

[BSA] (mg/mL)	$V_{BSA2mg/mL}$ (µL)	V_{AB} (µL)
0	0	250
0,1	12,5	237,5
0,2	25	225
0,4	50	200
0,75	93,75	156,25
1	125	125

4.3. BRADFORD ASSAY

For the protein assay, a 96-well microplate was used. To measure the protein concentration in our sample, dilutions of 1/2, 1/4 and 1/8 were prepared from the aliquot saved during protein extraction. Incubation was performed according the instructions from the manufacturer. Absorbance was read at 595 nm.

5. TOXICITY EVALUATION OF NEUROPROTECTIVE DRUG EV-19 BY MTT ASSAY

Another CBA I was able to learn was the MTT viability assay. This was used for the toxicity evaluation of a newly developed drug named EV-19 as a request from a referee for a previous manuscript that was undergoing revision for publication.

Viable cells with active metabolism convert MTT into a blue-purple colored formazan product with a maximum absorbance near 570 nm. When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful indicator of the viable cells exclusively. The exact cellular mechanism of MTT reduction into formazan is not well understood, but it likely involves NADH or similar reducing molecules that transfer electrons to MTT [24].

5.1. BIOLOGICAL SUBSTRATE

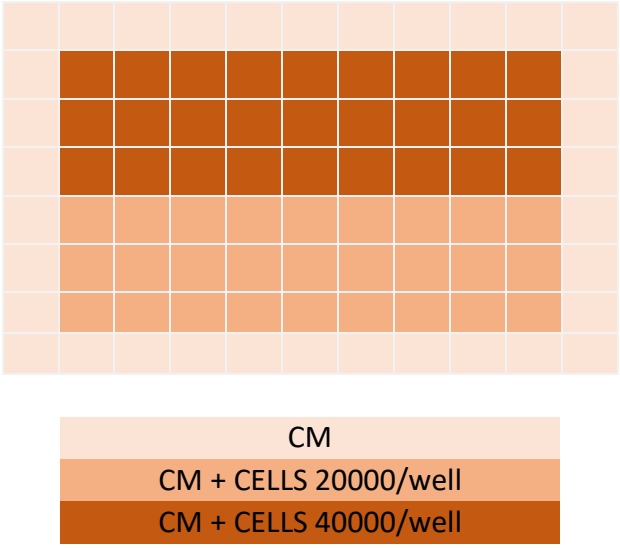
The substrate used for this technique were N2a cells plated in 96-well plates.

5.2. MATERIALS AND REAGENTS

- Biotek PowerWave XS2 Microplate Reader
- LH-BSA buffer: 2 mL LH 10x + 2 mL Ca2+ stock 10x + 22 mg D-glucosa (Panreac, Cat. No 141341) + 20 mg BSA (Thermo Fisher, Cat. No 23210). Add 15 mL H_2Od and adjust pH to 7,4. Complete solution up to 20 mL.
- Aliquots of EV-19 with LH-BSA buffer: starting with a 1 mM EV-19 solution, we prepare a 1/10 dilution and obtain the 100 µM solution. Afterwards, we prepare another 1/10 dilution from the 100 µM solution to get the 10 µM solution.
- MTT solution (5 mg/mL): 25 mg MTT (Sigma-Aldrich, Cat. No M2128) in 5 mL PBS (Sigma-Aldrich, Cat. No P3813), kept at -20 ºC.
- DMSO anhydrous (Sigma-Aldrich, Cat. No 276855)

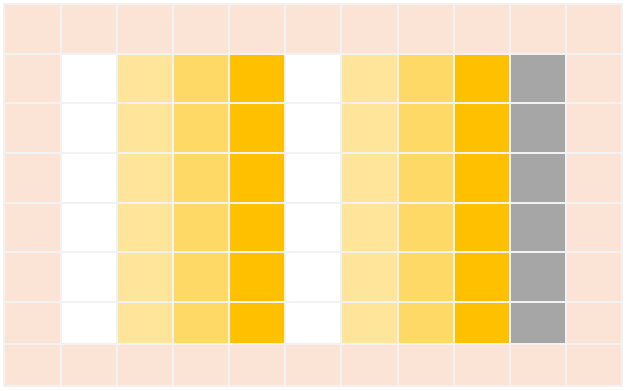
5.3. PROTOCOL FOR PLATING CELLS

Cells were plated in a 96-well plate according to the following diagram (designed for a concentration of 40000 and 20000 cells/well), a final volume of 200 µL/well was added.



5.4. INCUBATION OF DRUGS

During incubation, 20 µL of medium was removed and 20 µL of the different EV-19 solutions prepared previously were added following the diagram. Cells were incubated for 24 h before the MTT viability assay.



Vehicle
EV-19 1 µM
EV-19 10 µM
EV-19 100 µM
HCl 0,1 mM

5.5. MTT VIABILITY ASSAY

After 24 hours of drug incubation, 20 µL of an MTT solution 5 mg/mL was added to each well. After 20 minutes incubation at 37°C 100 µL DMSO were added to each well. Cells were pipetted thoroughly to solubilize formazan blue crystals. Absorbance was measured at 595 nm in a plate reader.

RESULTS

1. FLUOROMETRIC ASSAYS

In this assay we tested the depolarizing effect of different compounds acting on voltage-gated ion channels, present in the membrane of Neuro2a cells. Some of these compounds, such as veratridine and ouabain were used as positive controls because of their proven effect on targets that mediate intracellular ion fluxes and easy accessibility and cost. Afterwards, we checked if ciguatoxin, a sodium channel opener (difficult to obtain and high cost) caused this depolarization of the membrane.

The first compound, veratridine, is known to act as an activator of the sodium channels [25]. On the other hand, ouabain acts as a Na^+/K^+ ATPase inhibitor. Their combined action leads to an increase in the Na^+ intracellular levels, which bind themselves to the SBFI molecule and give a rise in the fluorescent signal.

The entry of sodium ions into the cell, provokes the depolarization of the membrane through less negative potentials. This event produces the activation of the voltage dependent Ca^{2+} channels, which allow the entry of calcium ions into the cell. In case the cell was depolarized, these ions would bind themselves to the Fura-2 molecule present in the inside of the cell, which would imply a rise in the signal read by the fluorometer.

The intracellular Ca^{2+} concentration was calculated by the emission of fluorescence at 510 nm wavelength at two different excitation wavelengths 340/380 nm) [21]. This gave us a ratio value (R) for the intracellular calcium. Intracellular sodium concentration was monitored using SBFI and same wavelengths. Ratio values were measured throughout time, and these are the values represented in the following graphics.

➔ FURA-2 RESULTS

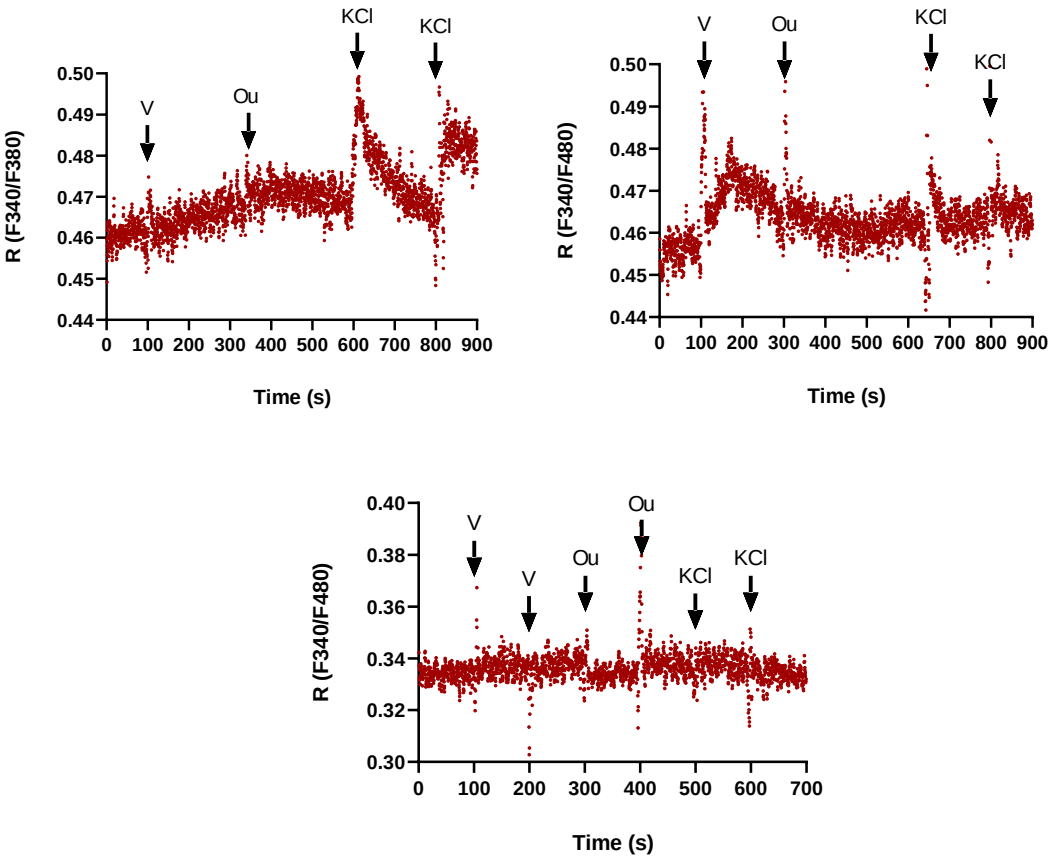


Figure 12. Fura-2 assays with Veratridine (V), Ouabain (Ou) and KCl. Cells at optimal density.

➔ SBFI RESULTS

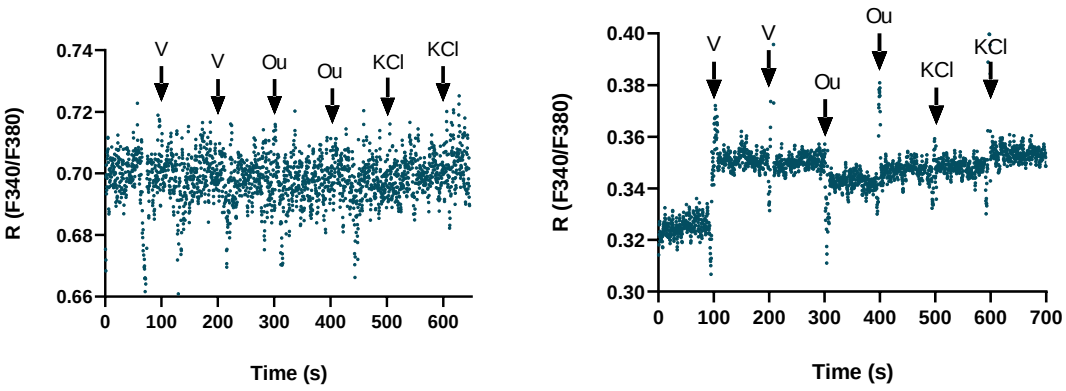


Figure 13. SBFI assays with Veratridine (V), Ouabain (Ou) and KCl. Cells at optimal density.

After veratridine or ouabaine addition we added KCl, which acts depolarizing the cellular membrane because of its permeability to the cytoplasm. This in turn should

open VDSC or VDCC leading to a rise in sodium and/or calcium intracellular concentrations and consequently the R value of Fura-2 or SBFI. However, as we can see in Fura-2 assays (figure 12), only a little depolarization was observed when adding KCl but not with any of the other compounds. On the other hand, in SBFI assays (figure 13) no depolarization was observed when adding any of the compounds, nor KCl.

Lastly, we added a CTX extract to the sample. As mentioned above (introduction section 2), CTX is a marine toxin which acts slowing down the inactivation of the Na^+ current, which facilitates the persistent entry of Na^+ into the cells. Therefore, it is a NaV channel activator and should also provoke a rise in the fluorescent signal.

➔ FURA-2 RESULTS

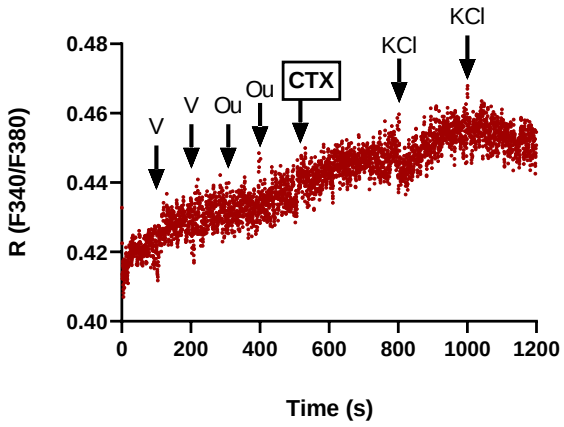


Figure 14. Fura-2 assay with Veratridine (V), Ouabain (Ou), CTX and KCl. Cells at optimal density.

Very little depolarization was observed when adding CTX, but not with any of the previous compounds.

➔ SBFI RESULTS

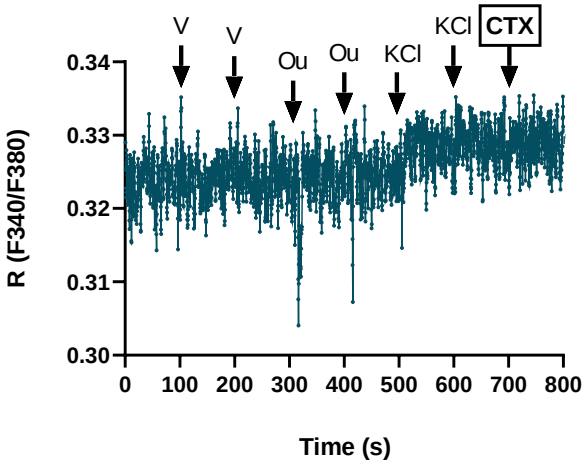
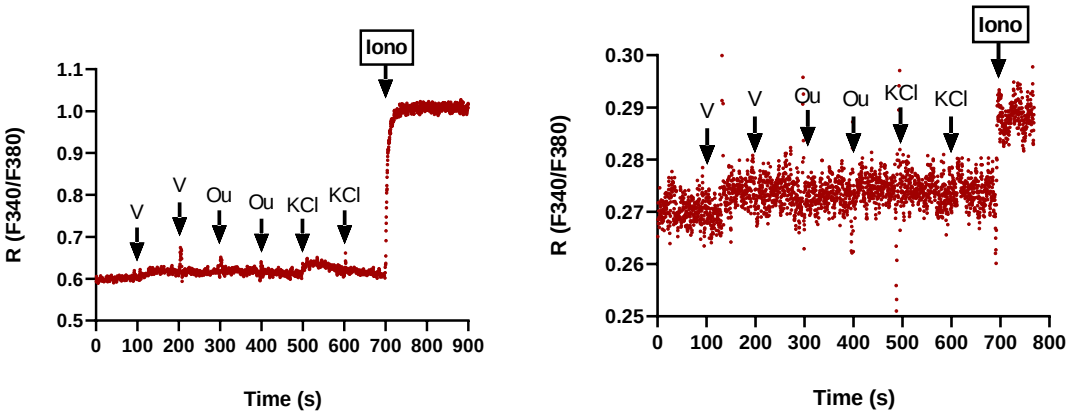


Figure 15. SBFI assay with Veratridine (V), Ouabain (Ou), KCl and CTX. Cells at optimal density.

No depolarization was observed when adding any of the previous compounds nor the CTX.

As shown in the graphics above, the results of our experiments did not demonstrate that different agents that act directly or indirectly on VDSC or VDCC can be monitored through fluorometric analysis. In order to dismiss a lack of loading of the dye, we added Ionomycin or monensin, for Fura-2 and SBFI, respectively. These ionophores transport ions across the cell membrane. For ionomycin, it allows the transport of Ca^{2+} into the cell, and if loading were successful, we should see a rise in the fluorescent signal.

➔ FURA-2 RESULTS



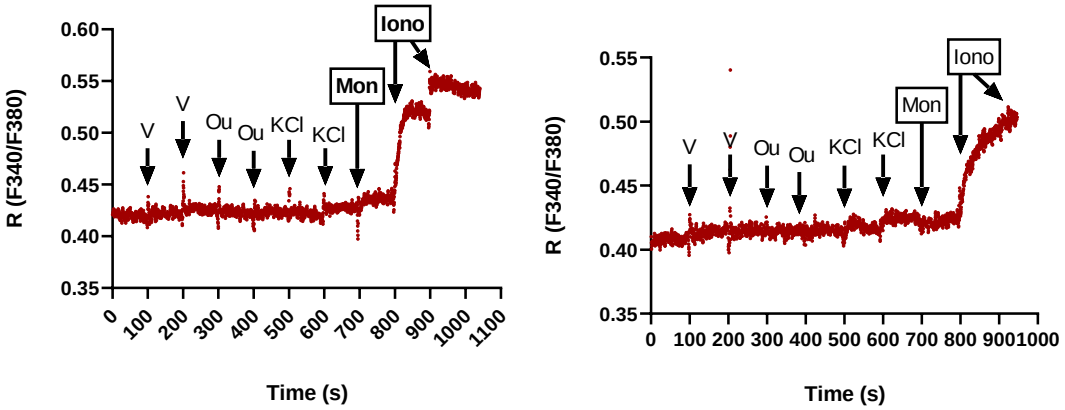


Figure 16. Fura-2 assays with veratridine, ouabain, KCl and ionomycin. Monensin was also added in the last two graphics to check if the entry of Na^+ provoked the entry of Ca^{2+}.

As we can see in the figure 16, we obtained an important rise in the signal when adding the ionomycin, much more intense than with KCl.

➔ <u>SBFI RESULTS</u>

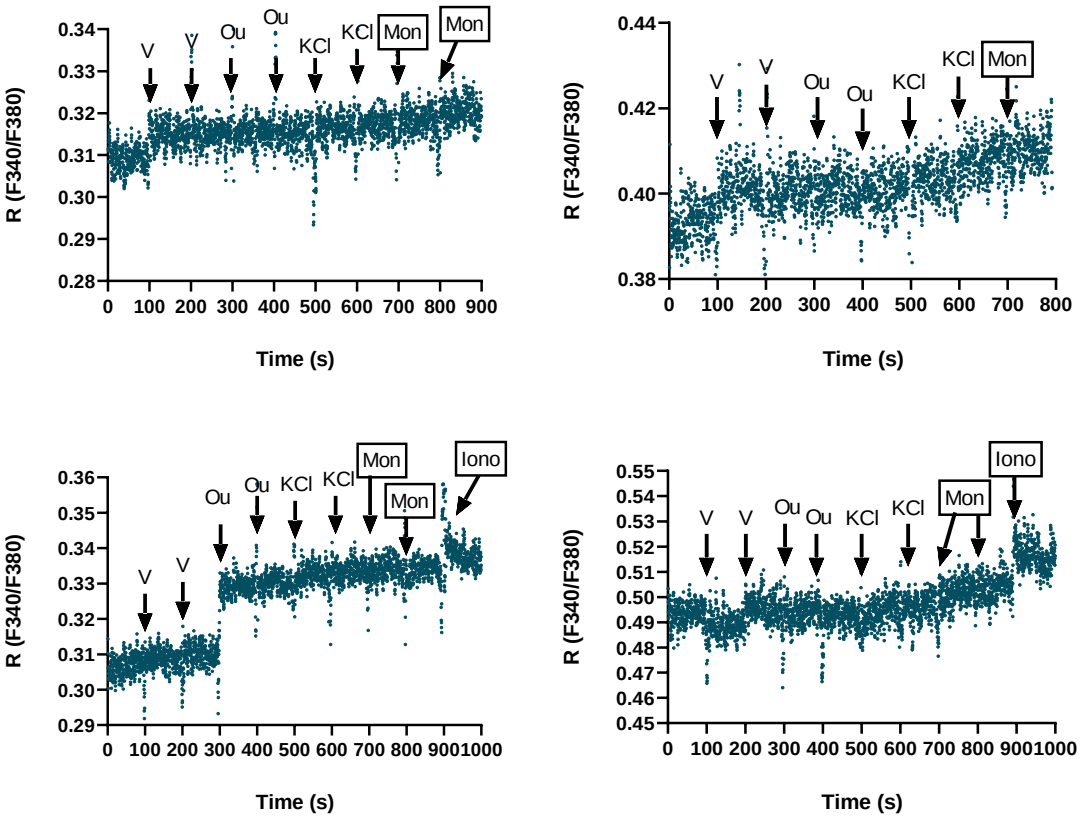


Figure 17. SBFI assays with veratridine, ouabain, KCl, monensin an ionomycin

Contrary to Fura-2, SBFI did not show any rise in signal when adding the ionophore monensin (figure 17).

To corroborate these results, we observed the fluorescence of the loaded, as specified in the protocol.

➔ FURA-2 RESULTS

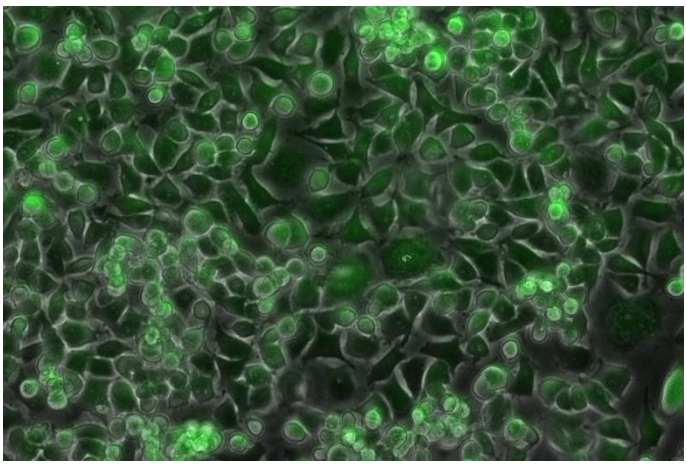


Figure 18. Merge of phase contrast and fluorescence pictures of N2a five days in vitro, after 45 minutes incubation in Fura-2.

➔ SBFI RESULTS

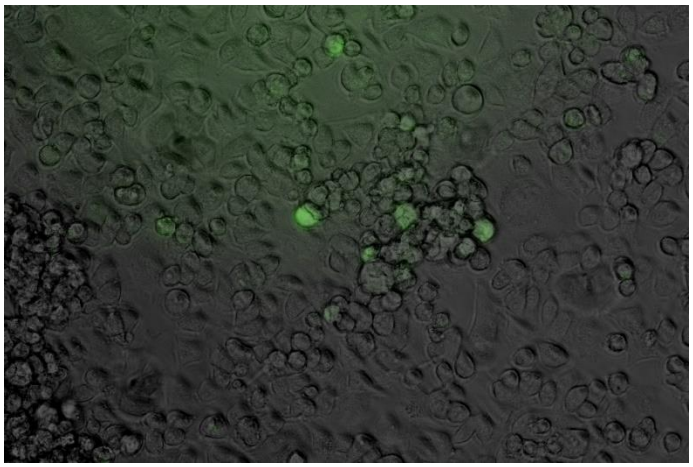


Figure 19. Merge image of phase contrast and fluorescence pictures of N2a two days in vitro, after 2,5 hours incubation in SBFI.

As shown in figure 18, loading with Fura-2 was successful. However, SBFI failed to load a high proportion of Neuro2A cells (figure 19), which ultimately could be responsible for the lack of effect of the different compounds in the SBFI assays.

2. AUTOMATED PATCH CLAMP

Different concentrations of several sodium channel blockers were prepared to determine the IC50 from each compound. The IC50 stands for the half maximal inhibitory concentration, which means the concentration of the compound at which half of the current through channels is blocked. It is a measure of the potency of the compounds.

Dose-response curves were constructed by a non-lineal Schild regression, in which IC50 and Hill coefficient were calculated using the Igor software. For graphical purposes, Prism 5.0 was used.

Table 2. Results obtained from 14 experiments with lidocaine in Patchliner.

Cell	IC50 (µM)	Hill	IC50 (µM)	Hill	IC50 (µM)	Hill
1	850	2,1	560	1,1		
2						
3	980	1,8	590	1,8	780	2,3
4	1000	30			830	1,7
5	840	1,5	350	1,3		
6	910	2,5			500	2,8
7	490	1,1			420	1,2
8			740	1,1		

Mean IC50	**SD**	**N**
702,86	214,13	14
Mean Hill	**SD**	**N**
3,74	7,58	14

Table 3. Results obtained from 10 experiments with tetracaine in Patchliner.

Cell	IC50 (µM)	Hill	IC50 (µM)	Hill	IC50 (µM)	Hill
1	7,2	1,1				
2						
3	16	0,75			9,8	0,8
4	38	0,86	43	0,73		
5						
6	7,1	0,47	76	0,63	21	0,9
7			12	0,58		
8					75	3,1

Mean	SD	N
30,51	26,72	10
Mean Hill	**SD**	**N**
0,99	0,76	10

Table 4. Results obtained from 13 experiments with TTX in Patchliner.

Cell	IC50 (µM)	Hill	IC50 (µM)	Hill	IC50 (µM)	Hill
1					**9,9**	**1**
2						
3			**5,9**	1,5	**13**	0,7
4	**5**	0,87	**0,54**	0,54	**6,2**	1,1
5	**3,1**	1,1			**4**	0,83
6	**4,6**	1,1	**5,4**	1,2		
7	**3,7**	0,93	**1,3**	0,74		
8					**6,9**	1,8

Mean	SD	n
5,35	3,32	13
Mean Hill	**SD**	**n**
1,03	0,34	13

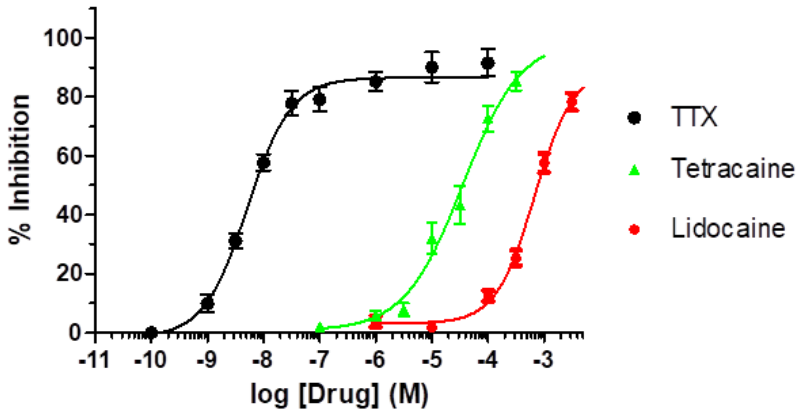


Figure 20. % Inhibition of the VGSC by TTX, tetracaine and lidocaine in different concentrations

3. MEMBRANE ISOLATION FOR LIGAND-BINDING ASSAY

The calibration curve obtained with the aliquots prepared in section 4.2 was the following:

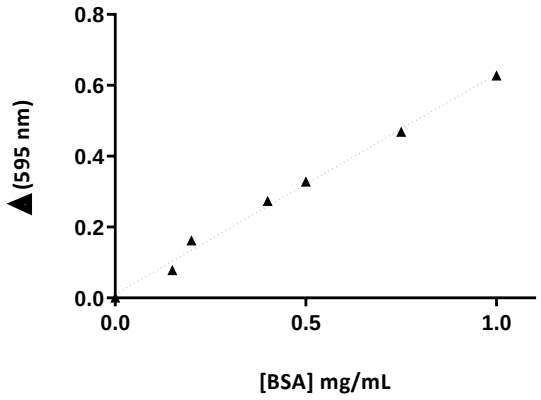


Figure 21. Calibration curve with BSA aliquots for protein concentration assay. R^2= 0,9937

After membrane protein isolation and Bradford technique determination, the absorbance values of our 1/2, 1/4 and 1/8 dilutions were:

Abs 1/2	Abs 1/4	Abs 1/8
0,2355	0,1065	0,071

With the equation obtained from the calibration curve, we calculate the concentration from the different aliquots and the average concentration. The total concentration of our sample is 2,84 mg/mL.

y = 0,6215x + 0,0101

[ ] 1/2 (mg/mL)	[ ] 1/4 (mg/mL)	[ ] 1/8 (mg/mL)	Average (mg/mL)
2,901367659	2,481737731	3,135639582	2,839581657

4. MTT ASSAY

After checking the absorbance levels of the different concentrations of EV-19, we represented the percentage of viability of the cells after the treatment with the drug, by calculating the mean and SD data in an interleaved bar graphic:

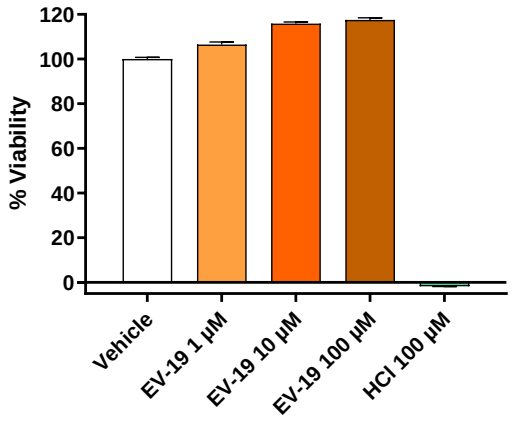


Figure 22. % Of viability of N2a after 24h incubation with EV-19 1, 10, 100 µM and HCl µM

The viability of the cells treated with EV-19 drug in any of its concentrations, was 100%, like in the vehicle solution. In comparison, HCl solution killed all the cells, and its viability percentage was 0%.

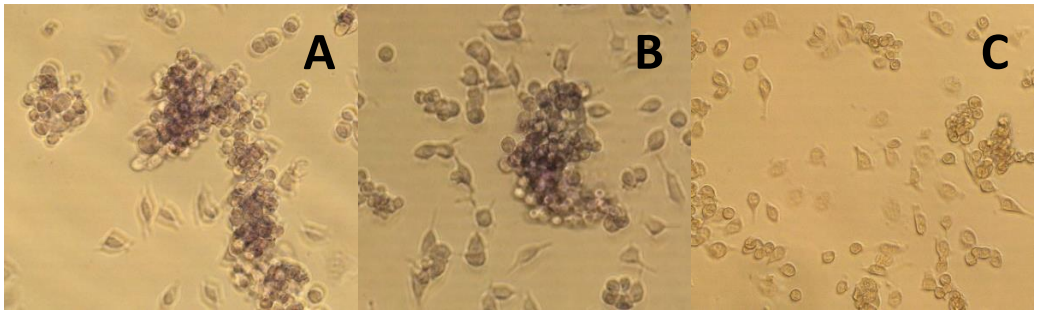


Figure 23. N2a cells after 20 minutes incubation in vehicle medium (A), 100 µM MTT solution (B) and 100 µM HCl (C).

As we can see in figure 23, in both figures A and B the cells showed a blue-purple color from the Formazan reagent. However, figure C does not. As we know, only viable cells can metabolize the MTT reagent into the Formazan product, so this also proves that EV-19 was not toxic, since the cells in 100 µM EV-19 solution also showed the color, which means they remained viable after drug incubation. Nevertheless, the cells in HCl solution were dead and that is why we could not see any color.

DISCUSSION

1. FLUOROMETRIC ASSAYS

In our fluorometric assays, we wanted to check that the effects of CTX over the Neuro2a cells, implied the entry of sodium and calcium ions through the VGIC. As shown in figures 14 and 15, no depolarizing signal was observed. This could be due to different factors. The first one could be related to the expression of NaV channels or the level of heterogeneity of these channels in our cells (existence of channel subtypes, each of them having a certain sensitivity to the different compounds and toxins). Another one could be related to a failure of our probes to load.

After adding ionomycin and monensin to the samples, we observed that in Fura-2 assays, there was an increase in the ratio of fluorescence, which implies that the Fura-2 molecule entered into the cells correctly and that the dye molecule was not damaged during the incubation procedure. However, in the SBFI assays, we did not see any raise in the fluorescent signal, not even after adding monensin. Therefore, in this case we do not know if the sodium ions or the SBFI molecule were unable to enter the cell. We do not know if the dye was damaged either.

To check the results we were obtaining, we observed the cells in the microscope. As we can see in figure 18, in Fura-2 assay there was a good signal of green fluorescence, which means the dye entered the cell and bound itself to the calcium ions. We can say the loading of Fura-2 from our cells was correct, and the dye was working as expected.
The dye entered the cells correctly, but when adding our compounds (veratridine, ouabain, KCl and CTX) we did not see the typical depolarization curve obtained from previous research carried out in our laboratory. In these assays, a different extract was studied and a rise in the Fura-2 signal was observed when adding the compound to the cells (figure 24).

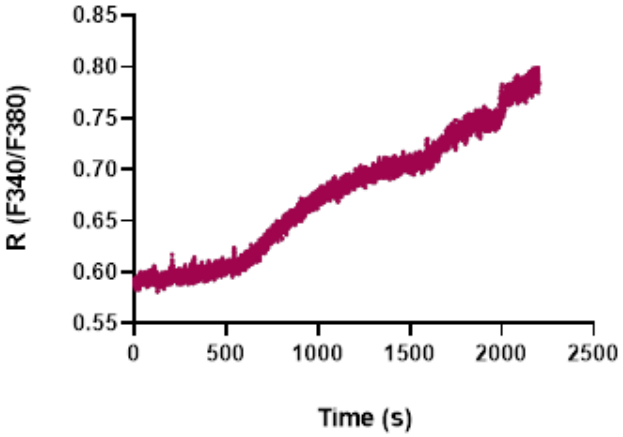


Figure 24. Previous Fura-2 assay carried out in our laboratory from *Fukuyoa* microalgae extract (added at 500, 1500 and 2000 s).

To sum up, we could not prove that the effect of CTX could be measured through the measurement of Fura-2 or SBFI after entering through VGIC.

SBFI could not enter appropriately into the cell (figure 19). Only a reduced number of cells showed SBFI fluorescence. We believe that cells that contained SBFI could be the death ones instead of the living ones. These cells would have a higher permeability of their membranes and would let the probe enter into the cytoplasm. On the contrary, viable cells would not, due to a preserved impermeability of the cytoplasmic membrane. Although monensin should cause an important increase in intracellular sodium ions, an increase of fluorescence was not detected so the most probable cause was that SBFI did not load successfully to the cells, in spite long incubation times and Pluronic® were used. In this case, the cells were not containing the dye during the assay, so we could not see any significant change after addition of any of our compounds (veratridine, ouabain, KCl and CTX) during the experiment. This means we cannot prove our hypothesis on the feasibility of fluorometric techniques to detect neurotoxins.

After several fluorometric experiments, we concluded that fluorometric assays were not sensitive enough to determine the effect of marine toxins in N2a cell line. Therefore, and taking advantage of the Patchliner system, that was donated by Almirall Laboratories we kept our research going using the automated patch clamp technology.

2. AUTOMATED PATCH CLAMP

After analyzing the blocking effect of lidocaine, tetracaine and tetrodotoxin, we could conclude that in all cases, their blocking effect was both detectable and concentration dependent.

The IC_{50} obtained from lidocaine, tetracaine and TTX in our experiments agree with the literature existing for these compounds. For instance, IC_{50} for lidocaine seems to be between 10-990 µM [26]. Our result was 702,82 µM (table 2), which is inside the range. For tetracaine, IC_{50} values are between 35-74 µM [27]. In our experiments, this value was 30,51 µM (table 3). In case of TTX, reference values of IC50 are between 1 and 25 nM [28], meanwhile our data was 5,35 µM (table 4). These values may vary depending on the level of expression of the different NaV channels subtypes. Differences in the subtype imply a higher or lower affinity for the ligand binding. Also, the state of depolarization of the ligand at which the experiment takes place affects its activity. For this reason, our values may vary from reference values.

As we can see in figure 20, the most potent compound was tetrodotoxin, since it was the one which blocked the channel at the lowest concentration, followed by tetracaine and lastly lidocaine. In this graphic we can also see that it seems that TTX has a lower capacity to totally inhibit VGSCs compared to lidocaine or tetracaine, since the maximum value of inhibition is around 90%. Contrary, it looks like lidocaine and tetracaine were able to inhibit the 100% of the channels at higher concentrations. This could be explained because of the existence of a VGSC subtype which is TTX insensitive, the NaV 1.8 [29], [30]. The higher presence of this subtype in some of our cells would make them more resistant to the depolarizing effect of TTX.

On the whole, we believe that APC with Patchliner yields good inhibition curves from sodium channel antagonists, among them a marine toxine; therefore, this innovative and automated patch clamp technology would be a precise method to study the activity of VGIC in the N2a cell line. This technology shows many advantages compared to the fluorometric technique, such as the higher sensitivity, which allowed us to obtain inhibition results quickly, easily and with a medium throughput (500 data points per day).

We could obtain signals from our cells with an efficiency of 40-60% from the total number of experiments, so we can say automated patch clamp technology is an accurate technique to study depolarizing effects of marine toxins in N2a.

CONCLUSION

In conclusion, we can assure that automated patch clamp technology is an accurate technique to determine marine toxins activity in N2a cell line, demonstrating a high sensitivity and efficiency. On the other side, the sensitivity of fluorometric assays is not enough to detect the depolarization effect caused by marine toxins in N2a, so this technique would not be so suitable for these assays.

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