Optimización de ensayos celulares para la detección de toxinas marinas responsables de intoxicaciones alimentarias. Aplicación en extractos lipofílicos de muestras naturales de *Mytilus galloprovincialis*

Elisabet Cañete Ortiz

3.3 Artículo 3

Cell-based assay coupled with chromatographic fractioning: A strategy for marine toxins detection in natural samples

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RESUMEN

Los ensayos celulares (cell-based assays; CBA) han sido propuestos para la evaluación de la toxicidad causada por toxinas marinas en muestras de origen natural (pescado, marisco y microalgas). No obstante, su aplicación se ha entorpecido por las interferencias presentes en matrices de origen biológico que pueden causar respuesta celular e interferencias en la evaluación toxicológica. Este trabajo revisa, con una extensa introducción, el uso de CBAs para la evaluación toxicológica de toxinas marinas. Posteriormente, se presenta el acoplamiento del fraccionamiento cromatográfico con CBA, con células de neuroblastoma Neuro-2a, para aumentar la aplicabilidad de los CBAs en matrices complejas. Se proporcionan ejemplos de aplicación en muestras de mejillón (*Mytilus galloprovincialis*) y en microalgas (*Gambierdisus* sp.), y los resultados demuestran un gran potencial de la estrategia combinada para una evaluación toxicológica fiable sin problemas éticos asociados. Fraccionando un equivalente de 72 mg eq/ ml de muestra de mejillón permitió la identificación de fracciones tóxicas y no tóxicas mientras que 2,5 mg eq/ ml del extracto de mejillón no purificado era responsable del 20% de la mortalidad celular. Además, la aplicación del CBA permite selectivamente distinguir entre

efectos del tipo ciguatoxina y otras toxicidades inespecíficas en extractos de *Gambierdiscus* sp..

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ABSTRACT

Cell-based assays (CBA) have been proposed for the evaluation of toxicity caused by marine toxins in natural samples (fish, shellfish and microalgae). However, their application has been hindered due to the interferences present in biological matrices that may cause cellular response and interfere in toxicity evaluation. This work reviews in an extensive introduction the use of CBA for toxicity evaluation of marine toxins. Afterwards, the coupling of chromatographic fractioning with neuroblastoma Neuro-2a CBA is presented to enhance the applicability of CBA for complex matrices. Examples of application are provided for mussel samples (*Mytilus galloprovincialis*) and microalgae (*Gambierdiscus* sp.), and the results demonstrated the great potential of the combined strategy for reliable toxicological evaluation without ethical concern. Fractioning of an equivalent of 72 mg eq mL^{-1} of mussel sample allowed the identification of non-toxic and toxic fractions whereas only 2.5 mg eq mL^{-1} of non-purified mussel sample was responsible for 20% of cell mortality. Furthermore, the application of CBA allowed selectively distinguishing between ciguatoxin-like and other unspecific toxicity in *Gambierdiscus* sp. extract.

1. Introduction

The present work is a report of the communication given during the 15th International Congress on *In Vitro* Toxicology (Estiv2008) held at Stockholm (Sweden, September 2008). Applicability of the coupling of the CBA with a chromatographic fractioning of the extracts was presented as a strategy for the detection of marine toxins in natural samples. In order to support this strategy, a detailed introduction on the use of cell-based assays for marine toxins detection is presented. Additionally two examples of application of this approach to natural samples are reported.

Production of toxins by marine microalgae can suppose a potential health risk for humans and can also have an impact on coastal resources. The transfer of microalgal toxins into the food webs and their bioaccumulation and biotransformation in fish and shellfish are potentially causes of human marine food-born intoxications. As an example, paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP) and amnesic shellfish poisoning (ASP) are common food-born intoxications caused by consumption of toxin contaminated shellfish (Hallegraeff, 2003). Ciguatera fish poisoning (CFP) is a common human intoxication due to the presence of ciguatoxins (CTXs) in fish tissue in tropical and sub-tropical areas (Lewis and Holmes, 1993). Direct exposure of humans to some marine toxins by marine aerosols inhalation in coastal areas has also been reported (Backer et al., 2003). The presence of marine toxins in seafood products and in marine waters, in addition to public health issues, is negatively impacting ecosystems, tourism and the fisheries industry.

Diversity in the nature, target and mechanisms of action of marine toxins explains the variability of symptoms encountered among food-born intoxications. Neurotoxic shellfish poisoning (NSP), PSP and CFP involve the presence of toxins which target voltage-gated sodium channels (VGSC) (Cestèle and Catterall, 2000). Saxitoxins (PSP toxins) as well as tetrodotoxins are VGSC blockers whereas brevetoxins and CTXs are VGSC activators. Activity of proteins phosphatases 1 and 2 are inhibited by the okadaic acid (OA) and dinophysistoxins (DTXs) (Bialojan and Takai, 1988) which are considered potent tumor–promoter agents (Suganuma

Abbreviations: ASP, amnesic shellfish poisoning; CBA, cell-based assay; CFP, ciguatera fish poisoning; CTXs, ciguatoxins; DSP, diarrheic shellfish poisoning; DTXs, dinophysistoxins; EC, European Commission; HPLC, high-performance liquid chromatography; IC50, 50% inhibitory concentration; K^+, potassium; LD50, 50% lethal dose; MBA, mouse bioassay; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium); MTXs, maitotoxins; Na^+, sodium; NSP, neurotoxic shellfish poisoning; OA, okadaic acid; P-CTX1B, pacific ciguatoxin 1B; PSP, paralytic shellfish poisoning; 3R, reduce, refine, replace; SPE, solid-phase extraction; VGCC, voltage-gated calcium channel; VGSC, voltage-gated sodium channel.

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et al., 1988) and which are implicated in DSP. Many other toxins have been identified in marine food, i.e., palytoxin, yessotoxins, azaspiracids, spirolids, maitotoxins (MTXs), domoic acid, pectenotoxins or gymnodimine; for some of them elucidation of their mechanism of action is still under development.

In many countries, the presence of toxins in seafood products is regulated in order to assess consumers' safety. European regulations on marine toxins set the maximum permitted levels of some toxins in seafood products (Regulation (EC) No. 853/2004) and also establish the official methods of analysis that have to be applied for the detection of these toxins (Regulation (EC) No. 2074/2005). Presently, the official testing methods for toxin detection in seafood products are still based on the mouse bioassay (MBA) for PSP toxins and some lipophilic toxins as the DSP toxins. Analytical methods using high-performance liquid chromatography (HPLC) are applied for ASP toxins, and recently accepted also for PSP toxins determination (Regulation (EC) No. 1664/2006, Turner et al., 2009). A competitive enzyme-linked immunosorbent assay has been accepted as an alternative to the HPLC method for ASP toxins (Regulation (CE) No. 1244/2007). Additionally, the presence of toxin-producing phytoplankton in shellfish harvesting areas and biotoxins content in live bivalve molluscs are routinely monitored in order to fulfil European legislations (Regulation (EC) No. 854/2004). Development of alternative or complementary methods to the MBA is supported by the European Union (Directive 86/609/EEC) and others countries in order to decrease the number of animals used in these assays.

In vitro bioassays have been proposed as possible approaches for replacing living animals in toxicity assays. For many years, cell-based assay (CBA) has been used for the identification of bioactive compounds from marine organism which may have a therapeutic potential or, on the contrary, deleterious effects according to the context of application (Mayer and Gustafson, 2003). Application of CBA was extended for the detection, toxicity evaluation and study of the mechanism of action of numerous marine toxins (Rossini, 2005). Many established mammalian cell lines have been used for toxin detection i.e. neuroblastoma cell lines (Jellett et al., 1992, 1995; Leira et al., 2001a, 2002; Cañete and Diogène, 2008), fibroblasts cell lines (Leira et al., 2001b) or myoblasts cell lines (Korsnes et al., 2006). Cell viability evaluation has been one of the major parameters used for toxin detection with CBA (Rossini, 2005). Various staining methods can be used such as the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) test or the neutral red uptake test for viable cell quantification (Borenfreund and Puerner, 1985; Manger et al., 1993; Fessard et al., 1994). Apoptosis (Leira et al., 2001b; Romano et al., 2003; Korsnes et al., 2006) and morphological alterations like the F-actin microfilament distribution (Diogène et al., 1995; Leira et al., 2001a, 2002) were also used as an approach for toxin detection or quantification. Specificity of cell-based assay for VGSC toxins detection has been developed with the use of neuroblastoma cell culture in association with a previous sensitisation of cells with ouabain and veratridine (Catterall, 1985; Kogure et al., 1988; Gallacher and Birbeck, 1992; Jellett et al., 1992, 1995; Manger et al., 1993, 1995). Ouabain blocks sodium efflux through an inhibition of the ATP dependent Na^+/K^+ pump (Catterall, 1986) and veratridine increases Na^+ permeability through a blockage of the voltage-gate Na^+ channel in an open position (Catterall and Nirenberg, 1973). Exposure of cells to ouabain and veratridine results in an increase in the concentration of intracellular Na^+ and additional presence of VGSC activating toxins increments the intracellular Na^+ concentration resulting in higher cell mortality.

Efforts exist for the development of cell-based assays which can specifically detect some groups of toxins. For example, palytoxin toxic effects antagonised by the ouabain has been improved for the development of specific CBA for palytoxin detection as reported in recent studies (Belloci et al., 2008; Espiña et al., 2009).

1.1. Marine toxin evaluation in natural samples

Detection and quantification of marine toxins from fish and shellfish tissues using CBA is a challenge for numerous laboratories which are in charge of the monitoring programmes of marine toxins in seafood harvesting areas. For toxins present in fish and shellfish tissues, purification steps previous to toxin detection have been proposed in order to avoid negative matrix effects during the detection procedure. According to our experience, these steps are crucial for the successful implementation of CBA for the detection of marine toxins in seafood, and deserve detailed description. Many protocols have been used for the extraction, purification, separation and detection of toxins from fish and shellfish tissues depending on the nature and polarity of toxins. For example, liquid/liquid partitions have been used to extract CTXs from fish tissue. This first purification step was used for toxicity evaluation using the MBA and further characterization of CTXs compounds (Legrand et al., 1989). Separation of OA, DTXs and azaspiracids from mussel samples has been performed using solid-phase extraction (SPE) cartridges to improve their analytical determination (Alfonso et al., 2008). SPE clean-up was also applied to mussels extracts for PSP toxins detection using CBA (Jellett et al., 1992), while carbon black was used to remove organic material from mussel samples followed by detection of marine toxins using the CBA (Croci et al., 2001).

Application of CBA for the detection of marine toxins directly produced by microalgae is an interesting tool which allows a preventive geographical risk assessment by toxicity evaluation of novels species or recently reported in a specific area. This approach can also facilitate the detection of unknown toxins or bioactive compounds. Moreover, identification of the toxins produced by microalgae can improve the species description and contribute for the taxonomic classification of microalgae. Production of CTXs by the dinoflagellate *Gambierdiscus* sp. supposes a potential risk of ciguatera in tropical and sub-tropical areas. Toxicity evaluation of *Gambierdiscus* sp. strains will help to assess the ciguatera risk (Darius et al., 2007) in areas where this genus is present. However, purification steps are required for toxin characterization of *Gambierdiscus* sp. strains due to the production of two concomitant groups of toxins, MTXs and CTXs. As an example, liquid/liquid partition for the separation of MTXs versus CTXs was reported for the CTXs toxicity evaluation of a strain of *Gambierdiscus toxicus* by MBA and for CTXs identification (Legrand et al., 1989).

The neuroblastoma Neuro-2a cells have been widely used for VGSC toxins detection (Jellett et al., 1992, 1995; Manger et al., 1993, 1995) and their utilization has been extended for the study of non VGSC toxins (Dragunow et al., 2005; Twiner et al., 2005; Dickey R. and Jester E.L.E./Gulf COAST Seafood Laboratory, USA, personal communication). Recently, Cañete and Diogène (2008) verified the suitability of the response of the Neuro-2a cells to detect a wide range of marine toxins for its possible application for routine toxin detection in seafood products. Here two examples are presented on the application of the Neuro-2a CBA for the detection of toxic compounds in natural samples: mussel samples (*Mytilus galloprovincialis*) and microalgal sample (*Gambierdiscus* sp.). Studies on fish matrices are currently ongoing in our laboratory. Neuro-2a cells response after a 24 h exposure to a non-purified extract of a non-toxic mussel sample is presented in order to evaluate the matrix effect. A chromatographic fractioning previous to the Neuro-2a CBA was used as a purification step in order to reduce the negative matrix effects and be able to detect the toxic compounds in the semi-purified

extracts of mussels as well as from the microalgae *Gambierdiscus* sp.

2. Material and methods

2.1. Neuroblastoma Neuro-2a cell culture maintenance and seeding of cells for the cytotoxicity assay

Neuro-2a cells (ATCC, CCL131) were maintained in 10% foetal bovine serum (FBS) RPMI medium (Sigma) at 37 °C in a 5% CO_2 humid atmosphere (Binder, Tuttlingen, Germany). For experiments, cells were seeded in a 96-well microplate in 5% FBS RPMI medium at an approximate density of 35,000 cells per well. Cells were incubated 24 h before exposure in the same conditions of temperature and atmosphere as described for cell maintenance.

2.2. Cytotoxicity assays

A corresponding concentration of mussel crude extract and of the different fractions (obtained throughout the fractionation of mussel and microalgal samples described below) were firstly evaporated to dryness under N_2 flux at 40 °C (TurboVap, Caliper, Hopkinton, USA) to completely remove the methanol from extracts previous to exposure of the Neuro-2a cells. Evaporated extracts were then dissolved in 5% FBS RPMI medium and 10μL of the corresponding extract was added to the well.

In order to detect the presence of CTXs, specificity of the Neuro-2a CBA to detect VGSC toxins was obtained with a previous treatment of the Neuro-2a cells with 0.1 mM of ouabain and 0.01 mM of veratridine; those concentrations were set in order to reduce an approximate 20% of cell viability (Cañete and Diogène, 2008).

For each cell treatment (with or without ouabain or veratridine) Neuro-2a cells were exposed in triplicates to 5% RPMI medium as "cell control".

2.3. Evaluation of the cytotoxic effects

After 24 h exposure of the Neuro-2a cells, cytotoxic effect was evaluated using cell viability measurement. Cell viability was assessed using the colorimetric MTT assay (Manger et al., 1993). Absorbances were read at 570 nm using an automated multi-well scanning spectrophotometer (Biotek, Synergy HT, Winooski, Vermont, USA). Viability of cells exposed to the extracts was expressed in relation to the viability of the corresponding cell control (with or without ouabaine and veratridine). Results were analyzed using the software Prism 4 (GraphPad, San Diego, zCalifornia, USA). A *t*-test was used to identify significant differences between both treatments, with and without ouabain and veratridine.

2.3.1. Application of CBA to mussel samples

2.3.1.1. Preparation of crude extracts of mussel samples. Mussel samples whose toxicity was determined by the MBA, were obtained from the regional monitoring programme for shellfish (Catalan Coast, Spain). Acetone extraction of mussel sample was based on the protocol described by Yasumoto et al. (1978) for the detection of lipophilic toxins with the MBA. In brief, 7 g of mussel tissue were extracted in triplicate with 24 mL of acetone. Supernatants were recovered by centrifugation at 1000*g* for 5 min at 4 °C (Joan MR23i, Saint Herblain, France), filtrated using 0.45 μm nylon filters then pooled and dried at 40 °C using a rotary evaporator (Büchi R-200, Flawil, Switzerland). The residue was finally dissolved in absolute methanol at a final concentration of 600 mg tissue equivalent (mg eq) mL^{-1} in order to obtain the crude extract.

2.3.1.2. Matrix effects evaluation of mussel crude extracts. Dilutions at one half of the mussel crude extract were done in 5% FBS RPMI medium and exposed to the Neuro-2a cells at concentrations ranging between 100 and 0.78 mg eq mL^{-1}.

2.3.1.3. Chromatographic fractioning of mussel crude extracts. Mussel crude extracts were first purified in triplicate by liquid/liquid partition with hexane:methanol in a 1:1 ratio before chromatographic separations performed on a HPLC Alliance 2695 (Waters Corporation, Milford, MA, USA) equipped with an Atlantis C_{18} (4.6 × 150 mm) analytical column with a 5 μm particle size (Waters Corporation, Milford, MA, USA). Flow rate was 0.3 mL min^{-1} and injection volume was 100 μL (60 mg eq). Water 0.1% acetic acid (A) and ammonium acetate 0.1% acetic acid (B) were used as mobile phases using the following chromatographic gradient: the run started with 70% A and 30% B, and it was kept for the next 10 minutes. Afterwards, it was increasing up to 80% B up to 15 min and maintained until minute 20. Then an increasing up to 100% B was run until minute 30, kept until minute 60 with a 0.6 mL min^{-1} flow rate and finally back to initial conditions after 65 min. Fractions ($n = 60$) were automatically collected using a Fraction Collector III (Waters) for 60 min in a ration of 1 fraction min^{-1}.

2.3.1.4. Toxicity evaluation of mussel extract fractions. Neuro-2a cells were exposed to each fraction of the mussel sample at an equivalent of 72 mg eq mL^{-1}.

2.3.2. Application of CBA to microalgal sample

2.3.2.1. Culture of Gambierdiscus sp. One strain of *Gambierdiscus* sp. (Vgo920) isolated from Indonesia was cultured in a 33 psu modified ES medium (Provasoli, 1968) at 24 °C under a light:dark 12 h:12 h regime with a photons flux rate of 78 μEinstein m^{-2} s^{-1}. Culture in the exponential growth phase was harvested through filtration using GF/F filters (Whatman). Filters were stored in absolute methanol at −20 °C until toxin extraction.

2.3.2.2. Extraction procedure. For toxin extraction, filters were sonicated during 30 minutes at 38% amplitude (Sonics VibraCell, Newtown, USA) in absolute methanol with an extraction volume (Ve) proportional to the total cell number (with 1 mL for 10×10^6 cells). Methanol extract was obtained after 5 min of centrifugation at 4 °C at 600 g (Jouan MR23i, Saint Herblain, France). Extraction procedure was repeated 3 times for *Gambierdiscus* sp. extracts: one with absolute methanol and two with methanol:water (50:50, v:v). Supernatants were pooled and dried at 40 °C using a rotary evaporator (Büchi R-200, Flawil, Switzerland). Extract was dissolved in absolute methanol and finally stored at −20 °C.

2.3.2.3. SPE fractioning of Gambierdiscus sp. extract. SPE fractioning was performed using a 1 g Florisil cartridge from BakerBond (JT Baker, Phillipsburg, NJ, USA). Mobile phase were hexane (A), acetone (B) and methanol (C) of HPLC grade. Cartridge was conditioned with 20 mL of (90% A:10% B) and loaded with 1 mL of microalgal extract equivalent to 500,000 cells of *Gambierdiscus* sp. (corresponding to 45 mg microalgal extract dried weigh) dissolved in (90% A:10% B). Each fraction was collected consecutively by elution with 10 mL of solvent with increasing polarity from fractions(#) 1 to 10 (%A/%B/%C): #1 (90/10/0); #2 (0/90/10); #3 (0/80/20); #4 (0/70/30); #5 (0/60/40); #6 (0/50/50); #7 (0/40/60); #8 (0/30/70); #9 (0/20/80); #10 (0/10/90) and with 100% B for fractions 11–12. Fractions were collected without vacuum

using a Vac-Elut SPE vacuum manifold (Varian, Harbor City, CA, USA).

2.3.2.4. Toxicity evaluation of Gambierdiscus sp. fractions. Neuro-2a cells were exposed to an equivalent of 435 cells mL^{-1} for each fraction of the *Gambierdiscus* sp. extract. This concentration was set according to the toxic potency of this strain established in previous CBA studies.

3. Results and discussion

3.1. Application of the CBA to mussel samples

An equivalent of 2.5 mg mL^{-1} of non-toxic (according to the MBA) mussel crude extract produced 20% cell mortality after 24 h of exposure (Fig. 1). Direct exposure of shellfish crude extracts to Neuro-2a CBA without previous purification is limited due to negative matrix effects.

A chromatographic fractioning combined with CBA was therefore applied for the study of the toxicity of two mussel samples. As it can be seen in Fig. 2, fractions obtained around 9–10 and 33–34 min were toxic both for samples 1 and 2. Fractions obtained between 49 and 56 min for sample 1 were toxic to the Neuro-2a CBA. For sample 2 and control mobile phase, these fractions were non-toxic suggesting that the toxicity of the mussel sample 1 was not related to matrix effect. However, many factors like the seasonal variation in feed availability or the sexual status may influence matrix composition among different mussel samples. Analytical measurements are required for the identification of the toxic compounds or bioactive compounds responsible for the toxicity in those fractions. Both mussel samples analysed during this study were found non-toxic according to the MBA but presented some cytotoxicity. Analytical results will be determinant for the confirmation of the toxicity of these mussel samples and for the identification of the compounds.

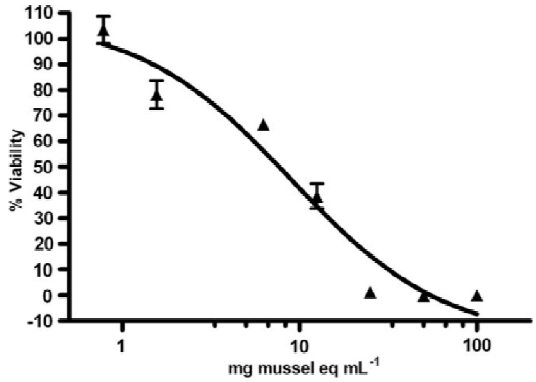

Fig. 1. Dose-response curve of Neuro-2a cells exposed for 24 h to a crude extract of a non-toxic mussel sample according to the MBA.

Fractioning of 72 mg eq mL^{-1} of mussel allowed the discrimination of toxic from non-toxic fractions using CBA. On the contrary, 2.5 mg eq mL^{-1} of a non-purified extract from a non-toxic mussel sample (according to the MBA) was toxic to cells reducing cell viability of 20%. The fractioning approach constitutes a purification step of the extracts that allows separation of toxic and non-toxic compounds, leading to a reduction of interferences. As a consequence, the fractioning protocol increases the amount of extract that can be analysed.

3.2. Application of the CBA to microalgal sample

Preliminary results obtained for the evaluation of the toxicity of fractions of a *Gambierdiscus* sp. showed no toxic effects from fraction 1 to fraction 4, while toxicity was detected from fraction 5 to 12 (Fig. 3). For all of them, pre-treatment with ouabain and veratridine increased significantly (*t*-test) the toxicity to Neuro-2a cells which suggested the presence of VGSC activating toxins in those fractions. As a consequence, CTX-like compounds may elute in a gradient of solvent comprised between (80:20) and (0:100) of acetone:methanol. Non VGSC specific toxicity that is not dependent of a pre-treatment of Neuro-2a cells with O/V (fraction 6, 7, and 8) supposed the presence of MTX-like or others toxic compounds in those fractions according to the types of toxins described in the literature for *Gambierdiscus* spp (Holmes and Lewis, 1994; Cuypers et al., 2008). Modulation of the elution gradient is needed in order to refine the separation of the CTXs-like compounds from the MTXs-like compounds for a future identification of those compounds.

During the assay with *Gambierdiscus* sp. extract, the specificity of the CBA for the detection of the VGSC toxins with a pre-treatment of the cells with O/V is required for the detection of the fractions containing VGSC activating toxins. This response is a very valuable tool that will help to optimize the chromatographic separation of CTXs from MTXs. The isolation and selective detection of CTXs and MTXs compounds is the first step required for the characterization of the toxins produced by this microalga. Therefore, the combination of chromatographic fractioning with CBA used in this work proved to be essential and can be a key step previous

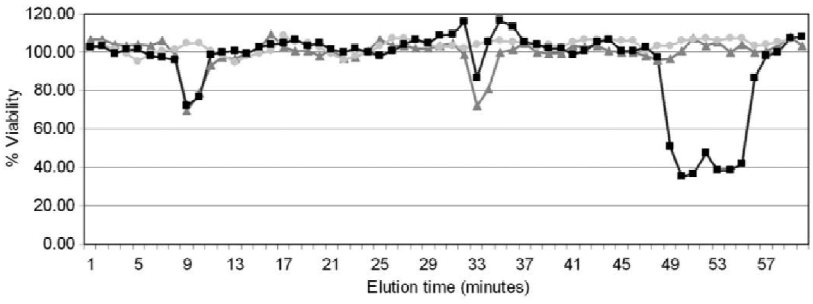

Fig. 2. Toxicity on Neuro-2a cells after a 24 h exposure to the fractions of an equivalent of 72 mg of mussel mL^{-1} obtained after fractioning with high-performance liquid chromatography (HPLC): (■) mussel sample 1, (▲) mussel sample 2, (●) mobile phase.

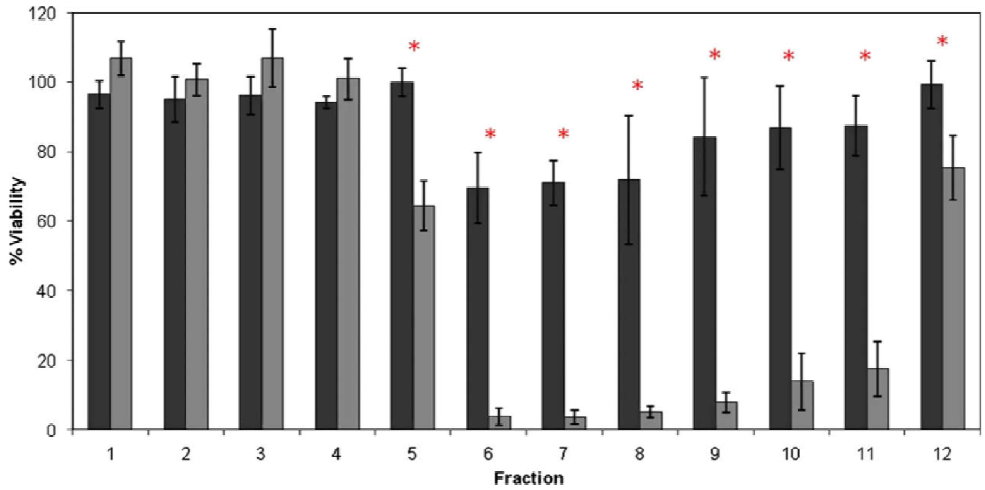

Fig. 3. Toxicity on Neuro-2a cells with (grey) and without (black) pre-treatment of the cells with ouabain and veratridine, after 24 h exposure to the fractions of an equivalent of 435 cells mL^{-1} obtained after SPE fractioning of *Gambierdiscus* sp. extract. *significant differences between both treatments, with and without ouabain and veratridine ($p < 0.05$, *t*-test).

to chemical structural characterization studies with mass spectrometry or NMR spectroscopy of new toxic compounds.

4. Conclusions

In vitro cell toxicity has been reviewed and discussed in this work showing a great potential to be used as an alternative or complementary approach to the mouse bioassay (MBA) for toxicity evaluation in microalgae and seafood products. Experimentally, we showed the application of CBA for the toxicological evaluation of natural samples, shellfish and microalgae. The combination of CBA with chromatographic fractioning improved the performance of the Neuro-2a CBA for the detection of toxic compounds in mussels and *Gambierdiscus* sp. samples, by minimizing the matrix effects.

On mussels extracts, the chromatographic fractioning performed allowed the distribution of the interfering compounds among fractions, consequently decreasing their negative effects on CBA and allowing the detection of toxic compounds by cellular response.

On phytoplankton extracts from the dinoflagellate *Gambierdiscus* sp., the chromatographic fractioning implemented allowed the distinction between toxic and non-toxic fractions by the CBA, but should be improved for better separation of toxic compounds. For the strain of *Gambierdiscus* sp. studied, the specificity of the response of Neuro-2a for the detection of VGSC toxins associated with the use of ouabain and veratridine, strongly suggested the presence of CTXs compounds in the microalgal extract.

Specificity of the CBA is of interest for the determination of toxins based on their mechanism of action. While specificity of the CBA response is still a challenge for the detection of several toxins, the combination of chromatographic fractioning with toxicity evaluation by CBA is a powerful tool which may be used as a complementary approach with other chemical or biochemical methods for the eventual "3R's rule: reduce, refine, replace" encouraged by European Union (Directive No. 86/609/EEC).

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References

Alfonso, C., Alfonso, S., Otero, P., Rodríguez, P., Vieytes, M.R., Elliot, C., Higgins, C., Botanna, L.M., 2008. Purification of five azaspiracids from mussel samples contaminated with DSP toxins and azaspiracids. Journal of Chromatography B 865, 133–140.

Backer, L.C., Fleming, L.E., Rowan, A., Cheng, Y., Benson, J., Pierce, R.H., Zaias, J., Bean, J., Bossart, G.D., Johnson, D., Quimboc, R., Baden, D.G., 2003. Recreational exposure to aerosolized brevetoxins during Florida red tide events. Harmful Algae 2, 19–28.

Belloci, M., Ronzitti, G., Milandri, A., Melchiorre, N., Grillo, C., Poletti, R., Yasumoto, T., Rossini, G.P., 2008. A cytolytic assay for the measurement of palytoxin based on a cultured monolayer cell line. Analytical Biochemistry 377, 48–55.

Bialojan, C., Takai, A., 1988. Inhibitory effects of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochemical Journal 256, 283–290.

Borenfreund, E., Puerner, J.A., 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicology Letters 24, 119–124.

Cañete, E., Diogène, J., 2008. Comparative study of the use of neuroblastoma cells (Neuro-2a) and neuroblastoma x glioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins. Toxicon 52, 541–550.

Catterall, W.A., 1985. The voltage sensitive sodium channel: a receptor for multiple neurotoxins. In: Anderson, D., White, A., Baden, D. (Eds.), Toxic Dinoflagellates. Elsevier, New York, pp. 329–344.

Catterall, W.A., 1986. Molecular properties of voltage-sensitive sodium channels. Annual Review of Biochemistry 55, 953–985.

Catterall, W.A., Nirenberg, M., 1973. Sodium uptake associated with activation of action potential ionophores of cultures neuroblastoma and muscle cells. Proceedings of the National Academy of Science USA 70 (12), 3759–3763.

Cestèle, S., Catterall, W.A., 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. Biochimie 82, 883–892.

Croci, L., Stacchini, A., Cozzi, L., Ciccaglioni, G., Mazzei, F., Botre, F., Toti, L., 2001. Evaluation of rapid methods for the determination of okadaic acid in mussels. Journal of Applied Microbiology 90 (1), 73–77.

Cuypers, E., Abdel-Mottaleb, Y., Kopljar, I., Rainier, J.D., Raes, A.L., Snyders, D.J., Tytgat, J., 2008. Gambierol, a toxin produced by the dinoflagellate Gambierdiscus toxicus, is a potent blocker of voltage-gated potassium channels. Toxicon 51, 974–983.

Darius, H.T., Ponton, D., Revel, T., Cruchet, P., Ung, A., Tchou Fouc, M., Chinain, M., 2007. Ciguatera risk assessment in two toxic sites of French Polynesia using receptor-binding assay. Toxicon 50, 612–626.

Diogène, G., Fessard, V., Dubreuil, A., Puiseux-Dao, S., 1995. Comparative studies of the actin cytoskeleton response to maitotoxin and okadaic acid. Toxicology In Vitro 9, 1–10.
Dragunow, M., Trzoss, M., Brimble, M.A., Cameron, R., Beuzenberg, V., Holland, P., Mountfort, D., 2005. Investigations into the cellular actions of the shellfish toxin gymnodimine and analogues. Environmental Toxicology and Pharmacology 20 (2), 305–312.
Espiña, B., Cagide, E., Louzao, M.C., Martinez-Fernandez, M., Vieytes, M.R., Katikou, P., Villar, A., Jaen, D., Maman, L., Botana, L.M., 2009. Palytoxin specific and dynamic detection by in vitro microplate assay with human neuroblastoma cells. Bioscience Reports Immediate Publication 29, 13–23.
European Union Commission Regulation, 1986. EC 86/609/EEC. Official Journal of the European Union L 358, 1–28.
European Union Commission Regulation, 2004. EC 853/2004. Official Journal of the European Union L 226, 22–82.
European Union Commission Regulation, 2004. EC 854/2004. Official Journal of the European Union L 226, 83–127.
European Union Commission Regulation, 2005. EC 2074/2005. Official Journal of the European Union L 338, 27–59.
European Union Commission Regulation, 2006. EC 1664/2006. Official Journal of the European Union L 320, 13–45.
European Union Commission Regulation, 2007. EC 1244/2007. Official Journal of the European Union L 281, 12–18.
Fessard, V., Diogène, G., Dubreuil, A., Durand-Clément, M., Legay, C., Puiseux-Dao, S., 1994. Selection of cytotoxic responses to maitotoxin and okadaic acid and evaluation of toxicity of dinoflagellate extracts. Natural Toxins 2, 322–328.
Gallacher, S., Birbeck, T.H., 1992. A tissue culture assay for direct detection of sodium channel blocking toxins in bacterial culture supernates. FEMS Microbiology Letters 92, 101–108.
Hallegraeff, G.M., 2003. Harmful algal blooms: a global overview. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. UNESCO, pp. 25–49.
Holmes, M.J., Lewis, R.J., 1994. Purification and characterization of large and small maitotoxins from cultured Gambierdiscus toxicus. Natural Toxins 2, 64–72.
Jellett, J.F., Wood, Stewart, J.E., Laycock, M.V., 1995. Toxicological evaluation of saxitoxin, neosaxitoxin, gonyautoxin II, gonyautoxin II plus III and decarbamoylsaxitoxin with the mouse neuroblastoma cell bioassay. Toxicology In Vitro 9, 57–65.
Jellett, J.F., Marks, L.J., Stewart, J.E., Dorey, M.L., Watson-Wright, W., Lawrence, J.F., 1992. Paralytic shellfish poison (saxitoxin family) bioassay : automated endpoint determination and standardization of the in vitro tissue culture bioassay, and comparison with the standard mouse bioassay. Toxicon 30, 1143–1156.
Kogure, K., Tamplin, M.L., Simidu, U., Colwell, R.R., 1988. A tissue culture assay for tetrodotoxin, saxitoxin and related toxins. Toxicon 26, 191–197.
Korsnes, M.S., Hetland, D.L., Espenes, A., Tranulis, M.A., Aune, T., 2006. Apoptotic events induced by yessotoxin in myoblast cell lines from rat and mouse. Toxicology In Vitro 20 (7), 1077–1087.
Legrand, A.M., Litaudon, M., Genthon, J.N., Bagnis, R., Yasumoto, T., 1989. Isolation and some properties of ciguatoxin. Journal of Applied Phycology 1, 183–188.
Leira, F., Alvarez, C., Vieites, J.M., Vieytes, M.R., Botana, L.M., 2001a. Study of cytosqueletal changes induced by okadaic acid in BE(2)-M17 cells by means of a quantitative fluorimetric microplate assay. Toxicology In Vitro 15, 277–282.
Leira, F., Vieites, J.M., Vieytes, M.R., Botana, L.M., 2001b. Apoptotic events induced by the phosphatase inhibitor okadaic acid in normal human lung fibroblasts. Toxicology In Vitro 15, 199–208.
Leira, F., Cabado, A.G., Vieytes, M.R., Roman, Y., Alfonso, A., Botana, L.M., Yasumoto, T., Malaguti, C., Rossini, G.P., 2002. Characterization of F-actin depolymerisation as a major toxic event induced by pectenotoxin-6 in neuroblastoma cells. Biochemical Pharmacology 63, 1979–1988.
Lewis, R.J., Holmes, M.J., 1993. Origin and transfer of toxins involved in ciguatera. Comparative Biochemistry and Physiology C 106 (3), 615–628.
Manger, R.L., Leja, S., Lee, S.Y., Hungerford, J.M., Hokama, Y., Dickey, R.W., Granade, H.R., Lewis, R., Yasumoto, T., Wekell, M.M., 1995. Detection of sodium channel toxins : directed cytotoxicity assays of purified ciguatoxins, brevetoxins, saxitoxins and seafood extracts. Journal of AOAC International 78, 521–527.
Manger, R.L., Leja, S., Lee, S.Y., Hungerford, J.M., Wekell, M.M., 1993. Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Analytical Biochemistry 214, 190–194.
Mayer, A.M.S., Gustafson, K.R., 2003. Mini review marine pharmacology in 2000: antitumor and cytotoxic compounds. International Journal of Cancer 105, 291–299.
Provasoli, L., 1968. Media and prospects for the cultivation of marine algae. In: Watanabe, A., Hattori, A. (Eds.), Cultures and Collection of Algae, Proceedings of the US-Japanese Conference, Hakone, September 1966, pp. 63–75.
Romano, E., Cannata, S., Di Bartolomeo, S., Spinedi, A., 2003. Caspase inhibition shifts neuroepithelioma cell response to okadaic acid from apoptosis to an apoptotic-like form of death. Biochemical and Biophysical Research Communication 303, 469–474.
Rossini, G.P., 2005. Functional assays in marine biotoxin detection. Toxicology 207, 451–462.
Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K., Yamada, K., Sugimura, T., 1988. Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. Proceedings of the National Academy of Sciences 85, 1768–1771.
Turner, A.D., Norton, D.M., Hatfield, R.G., Morris, S., Reese, A.R., Algoet, M., Lees, D.N., 2009. Refinement and extension of AOAC method 2005.06 to include additional toxins in mussels: single-laboratory validation. Journal of AOAC International 92 (1), 190–207.
Twiner, M.J., Hess, P., Bottein Dechraoui, M.Y., McMahon, T., Samons, S.M., Satake, M., Yasumoto, T., Ramsdell, J.S., Doucette, G.J., 2005. Cytotoxic and cytoskeletal effects of azaspiracid-1 on mamalian cell lines. Toxicon 45 (7), 891–900.
Yasumoto, T., Oshima, Y., Yamaguchi, M., 1978. Occurrence of a new type of shellfish poisoning in the Tohoku district. Bulletin of the Japanese Society of Scientific Fisheries 44, 1249–1255.