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

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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