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Suitability of the Neuro-2a cell line for the detection of palytoxin and analogues (neurotoxic phycotoxins)

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ABSTRACT

Palytoxin and related compounds are neurotoxic phycotoxins produced by benthic microalgae belonging to the genus *Ostreopsis*. For several years this family of phycotoxins has been posing a threat to human health since they can bioaccumulate in shellfish. With the aim of replacing current biological assays, such as the mouse or hemolytic assays, we investigated using the Neuro-2a neuroblastoma cell line to detect palytoxin and related compounds. Cell death induced by the effects of PlTX and analogues on Na^+, K^+-ATPase were measured using the 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay for mitochondrial reductase activity as a surrogate for cell number. The specificity of the Neuro-2a cell-based assay for palytoxin detection was confirmed by using ouabain, which also acts on Na^+, K^+-ATPase. Pre-treatment of the Neuro-2a cells with ouabain minimizes the effects of palytoxin. The specificity of the Neuro-2a assay was confirmed by the finding that cell death was not detected when Neuro-2a cells were exposed to other phycotoxins with unrelated cellular targets. When the Neuro-2a assay was used to detect palytoxin in mussel extracts spiked with levels of palytoxin around the proposed regulatory value of 250 μg palytoxin/kg shellfish, a good correlation was observed between the levels found and the expected values.

We conclude by proposing an experimental design for functional assays using the Neuro-2a cell line for the specific detection of four neurotoxic phycotoxin families: saxitoxins, brevetoxins, ciguatoxins and palytoxins.

1. Introduction

Palytoxin (PlTX) is one of the most potent marine toxins produced by zoanthids (soft corals) of the genus *Palythoa* (Moore and Scheuer, 1971). The structure of this large and complex polyhydroxylated molecule has been elucidated by Uemura et al. (1981), and Moore and Bartolini (1981).

The origin of palytoxin in the zoanthids has long been a matter of speculation, and several possible origins have been suggested (e.g. microorganisms associated with zoanthids; Halstead, 2002), including dinoflagellates belonging to the genus *Ostreopsis* (Usami et al., 1995; Taniyama et al., 2003). This hypothesis is supported by the implication of *Ostreopsis siamensis* in a case of clupeotoxism in Madagascar (Onuma et al., 1999). To date, four species of *Ostreopsis*: *Ostreopsis lenticularis* (Mercado et al., 1994), *Ostreopsis siamensis* (Usami et al., 1995), *Ostreopsis mascarenensis* (Lenoir et al., 2004) and *Ostreopsis ovata* (Ciminiello et al., 2008) are known to be capable of producing palytoxin and related (PlTX-like) compounds. The chemical structure of some of the analogues of PlTX has been elucidated (Usami et al., 1995; Ciminiello et al., 2008).

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For several years, PlTX-like compounds have occurred in the Mediterranean Sea, and have been linked to respiratory irritation in people exposed to seawater spray, especially in Italy and Spain (Durando et al., 2007). Recent studies have demonstrated that the species *O. ovata* observed in Italy produces ovatoxin-a, an analogue of PlTX (Ciminiello et al., 2008), and that PlTX-like toxins can bioaccumulate in shellfish (Aligizaki et al., 2008). This possible bioaccumulation poses a threat to human health, and has economic consequences for shellfish producers.

Palytoxin and its analogues can be detected by recently developed chromatographic methods (Ciminiello et al., 2006; Riobo et al., 2006). These methods are very efficient, but are not suitable for assessing the biological activity of extracts that may contain several related active compounds, as in the case of *Ostreopsis* toxins (Ciminiello et al., 2008). Like other neurotoxins, PlTX can be detected by a mouse bioassay, which was developed over 30 years ago (Teh and Gardiner, 1974), and has remained unchanged ever since. Both for ethical reasons, and because of the lack of specificity and sensitivity of the mouse bioassays, alternative methods are now being sought for use in monitoring PlTX and its analogues in shellfish.

Functional assays offer an alternative to chemical measurements and the non-specific mouse bioassay. In the case of neurotoxic phycotoxins, these tests exploit the binding of the toxin to its receptor (for review, see Rossini, 2005). The intensity of the response is related to the concentration of the toxin and its analogues interacting with their specific receptors.

Habermann and Chhatwal (1982) showed that in mammalian erythrocytes, PlTX causes a rapid depletion of K^+ in the cells followed by hemolysis. This hemolysis is specifically inhibited by ouabain (Habermann and Chhatwal, 1982), a glycoside that binds to the sodium pump (Lingrel et al., 1998). The effect of PlTX is attributed to its ability to bind to the outer part of Na^+, K^+-ATPase, leading to its conversion into a cationic channel (Habermann, 1989; Artigas and Gadsby, 2003).

A hemolysis assay including ouabain was originally developed to estimate the PlTX content of algal or shellfish extracts (Taniyama et al., 2001, 2003; Rhodes et al., 2002; Riobo et al., 2002, 2006; Lenoir et al., 2004; Aligizaki et al., 2008). However, the lack of reliable results of this hemolytic assay, and the fact that it cannot be used to identify the toxin involved has led to the development of alternative assays.

In the case of PlTX and its analogues, we need functional assays that are both sensitive and specific, especially in view of the fact that most PlTX-like compounds remain to be identified and toxicologically characterized. Recently, Bellocci et al. (2008) have described a method intended to replace erythrocytes with the established MCF-7 cell line. They propose a cytolytic assay based on the release of cytosolic lactate dehydrogenase in response to PlTX. This response indicates the presence of a severe membrane defect that presages cell lysis. Espiña et al. (2008) have demonstrated that the neuroblastoma cell line BE(2)-M17 is a suitable model for the specific detection of PlTX.

We set out to demonstrate that Neuro-2a neuroblastoma cells could be used to detect several different phycotoxins families. Indeed, this neuroblastoma cell-based bioassay is already being used to detect and distinguish several other neurotoxic phycotoxin families: saxitoxins (Jellett et al., 1992, 1995; Manger et al., 1993, 1995; Humpage et al., 2007), brevetoxins (Truman et al., 2002), and ciguatoxins (Dechraoui et al., 1999). We then investigated the sensitivity, specificity, and reliability of the Neuro-2a bioassay for the detection of palytoxin and its analogues. The method proposed provides an accurate estimate of the PlTX content of biological materials, such as algae and shellfish. Finally we proposed an experimental strategy for detecting a range of seafood toxins using the Neuro-2a cell-based bioassay.

2. Material and methods

2.1. Neuro-2a culture

The Neuro-2a mouse neuroblastoma cell line, obtained from the ATCC (CCL-131), was routinely grown in 75 cm^2 Nunc plastic flasks (Nunc, Denmark) with L-glutamine-free RPMI 1640 medium (Biowest, France) supplemented with 10% newborn calf serum (Biowest, France), 90 mg L^{-1} sodium pyruvate (Sigma–Aldrich, France), 600 mg L^{-1} L-glutamine (Sigma–Aldrich, France), 1000 U L^{-1} penicillin G (Biowest, France) and 1 mg L^{-1} streptomycin sulfate (Biowest, France) at 37 °C in an atmosphere enriched with 5% CO_2.

2.2. Toxins

Palytoxin purified from *Palythoa tuberculosa* was obtained from Wako Chemicals (Germany), and dissolved in 50% ethanol. A certified reference calibration solution of saxitoxin (STX) was obtained from the Institute for Marine Biosciences (Halifax, NS, Canada). Brevetoxin PbTx-3 was obtained from Calbiochem (USA), and prepared in absolute methanol. Microcystin-LR was obtained from Alexis Biochemicals (Switzerland), and diluted in absolute methanol. Aliquots of toxins were stored at −20 °C. Ouabain was purchased from Sigma–Aldrich (France). The stock solution was prepared in pH 7 milliQ water. The working dilutions of toxins and ouabain were prepared in RPMI medium supplemented with 600 mg L^{-1} L-glutamine, 90 mg L^{-1} sodium pyruvate, 1000 U L^{-1} penicillin G and 1 mg L^{-1} streptomycin sulfate (MWS: medium without serum).

2.3. Algal extracts

2.3.1. Epiphytic dinoflagellates

Three macroalgal samples (*Rhodophyta* and *Phaeophyta*) collected from Morgiret (Ile du Frioul, France) and Bain des Dames (Marseille, France) (on August 21st 2007, August 28th and September 5th, kindly provided by Françoise Marco-Miralles and Caroline Lecalard, Ifremer) (Table 1) were vigorously shaken in filtered seawater in order to collect the associated epiphytic dinoflagellates. The seawater was then filtered through plankton netting (20 μm mesh) or centrifuged (3220 g, 20 min, 10 °C). The procedure was repeated twice to obtain a plankton pellet. The pellets were sonicated with an ultrasonic probe in 5 mL methanol/water (3:7, v/v) for 3 min in pulse mode (Sonics