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► **To cite this version:**

Romulo Aráoz, Jordi Molgó, Denis Servent. Bioassays for detection and identification of cyclic imine toxins. The 18th International Conference on Harmful Algae: From Ecosystems to Socio-Ecosystems., Oct 2018, Nantes, France. hal-02413151

**HAL Id: hal-02413151**

**<https://hal.science/hal-02413151>**

Submitted on 16 Dec 2019

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# Bioassays for detection and identification of cyclic imine toxins

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## Abstract

Marine neurotoxins move-up fast in the food chain through shellfish that filter-feed on toxic dinoflagellate species constituting a serious hazard for Public Health. Monitoring of regulated marine toxins is performed by physicochemical analyses. It is essential however to develop functional bioassays as an alternative to mouse bioassay for high-throughput detection of novel and emergent marine toxins. Cyclic imine toxins are very-fast acting neurotoxins highly lethal to mice acting as potent antagonists of nicotinic acetylcholine receptors. Although not regulated, chronic exposition to cyclic imine toxins is of concern given their high affinity for neuronal and muscle nicotinic receptors and their capacity to cross the intestinal and the brain blood barriers. A series of bioassays based on the mode of action of cyclic imine toxins are here reviewed.

*Keywords:* Cyclic imine toxins, gymnodimines, spirolides, pinnatoxin, receptor-binding assay, toxin fishing.

## Introduction

Cyclic imines toxins (CiTXs) constitute a large family of emergent marine phycotoxins associated with harmful algal blooms and shellfish toxicity (Gueret and Brimble 2010; Stivala et al., 2015). The growing family of CiTXs purified from toxic dinoflagellates and/or contaminated shellfish includes 43 toxic members: **7 gymnodimines** (A-E and 12-methylgymnodimine), **16 spirolides** (SPX) (A-D, G-I, 13-desmethyl SPX-C, 27-hydroxy-13-desmethyl SPX-C, 13,19-didesmethyl SPX-C, 20-hydroxy-13,19-didesmethyl-SPX-C, 27-hydroxy-13,19-SPX-C, 27-oxo-13,19-didesmethyl SPX-C, 13-desmethyl SPX-D, 20-hydroxy-13,19-didesmethyl-SPX-D and 20-methyl-SPX-G), **8 pinnatoxins** (A-H), **3 pteriatoxins** (A-C), **1 spiroporocentrimine**, **6 prorocentrolides** and **2 portimines** (A and B). *Karenia selliformis* synthesises gymnodimines. *Alexandrium ostenfeldii* strains produce spirolides and 12-methylgymnodimine. *Prorocentrum* sp. produce prorocentrolides. *Vulcanodinium rugosum* produces pinnatoxins and portimines. (For references see : Fribley et al., 2018; Zurhelle et al., 2018; Molgó et al., 2017; Stivala et al., 2015; Gueret and Brimble 2010; Torigoe et al., 1988). Early reports on CiTXs go back to the eighties (Torigoe et al., 1988) and their physicochemical characterization back to the nineties (Hu et al., 1995; Seki et al., 1995; Uemura et al., 1995).

CiTXs exhibit strong neurotoxicity to mice by oral or intraperitoneal administration at lethal doses provoking transient hyperactivity, followed by a decrease of the respiratory rate with prominent abdominal breathing leading to death by respiratory arrest within 3-5 minutes following i.p. administration (Kharrat et al., 2008; Selwood et al., 2010; Takada et al., 2001). In fact, cyclic imine toxins were long-time considered as false positives for lipophilic toxin monitoring by mouse bioassay forcing in the past the prophylactic closure of conchylicultural activities in Foveaux Strait, New Zealand (Seki et al., 1995) and Arcachon, France (Amzil et al., 2007). CiTXs display potent antagonism and broad selectivity towards muscle and neuronal nicotinic acetylcholine receptors (nAChRs) (Aráoz et al., 2011; Molgó et al., 2017; Stivala et al., 2015). Although not regulated in Europe, chronic exposition to CiTXs is of concern given their high affinity for neuronal and muscle nAChRs and their capacity to cross the intestinal and the brain blood barriers (Alonso et al., 2013; Munday et al., 2012).

In the European Union, liquid chromatography coupled to mass spectrometry (LC-MS/MS) have replaced mouse bioassay by for the simultaneous monitoring of internationally regulated marine biotoxins. Nevertheless, novel functional assays are needed to replace mouse bioassay for rapid detection of novel marine neurotoxins directed against key receptors targets of the Nervous

System (Campbell et al., 2011; Davidson et al., 2015). Herein is presented a short overview of receptor-based assays for the detection of CiTXs.

## The receptor target of CiTXs

nAChR are ligand-gated cation-selective transmembrane pentameric proteins activated by acetylcholine. Muscle ( $\alpha 1$ ) $2\beta 1\gamma\epsilon/\delta$  nAChR that mediate fast transmission at the skeletal neuromuscular junction necessary for muscle contraction, respiration and escape from predation, are a primary target for a wide array of neurotoxins including CiTXs (Albuquerque et al., 2009; Ar oz et al., 2015; Changeux 2010). Neuronal nAChRs display a higher structural and functional diversity and broad brain occupancy. Neuronal nAChRs are homopentameric or heteropentameric and result from the assembly of  $\alpha 2$ - $\alpha 7$ ,  $\alpha 9$  and  $\alpha 10$  subunits with  $\beta 2$ - $\beta 4$  subunits. Neuronal nAChRs modulate neurotransmitters release participating in fundamental aspects of synaptic plasticity involved in attention, learning, memory, and development

(Bertrand et al., 2015; Changeux and Christopoulos 2016).  $\alpha 4\beta 2$  and  $\alpha 7$  nAChR subtypes are major pharmacological targets for the treatment of Alzheimer disease, Parkinson disease, schizophrenia, epilepsy, and addiction.

## Receptor-based methods for detection of CiTX

The development of ligand binding assays for nAChRs was headed by two important discoveries: *i*) the finding of biological tissues extremely rich in nAChRs, namely the electric organ of *Torpedo californica* and *T. marmorata*: nAChRs represent ~40% of the total protein content of *Torpedo*-electrocyte membranes, and are surrounded by their natural lipids and clustering proteins. Thereby, the receptors are stable and functional. *ii*) The characterization of  $\alpha$ -bungarotoxin from the venom of the snake *Bungarus multicinctus*. (Changeux 2010). This peptide toxin is a highly potent competitive antagonist of nAChR and is used as toxin tracer even after functionalization with radioactive elements, biotin or fluorescent

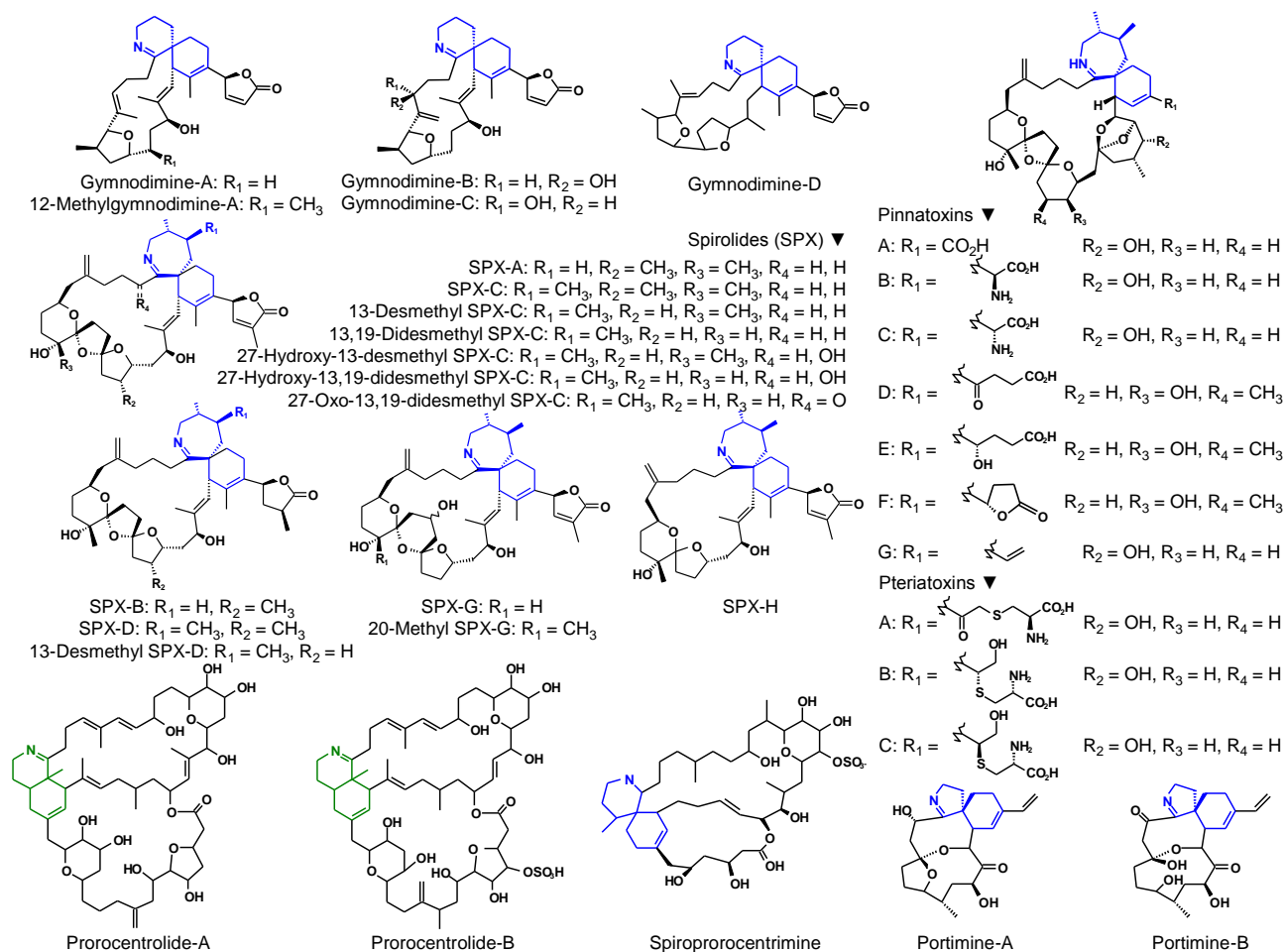


Figure 1. Chemical structure of CiTXs.

compounds. Since CiTXs are potent antagonists of nAChRs, several receptor assays were developed.

#### *Filtration radioactive ligand-binding assay.*

This is the reference method for screening nAChR ligands. CiTXs are incubated to equilibrium with *Torpedo*-nAChR\*. CiTXs inhibit the binding of <sup>125</sup>I-bungarotoxin to the ACh binding site of the receptor. Following radio ligand displacement, the complex receptor-radio ligand is retained on a filter membrane. Successive washing steps remove unbound radio ligand prior to scintillation counting from dried filters. Radioactive binding assay was essential to show the affinity and selectivity of CiTXs for muscle and neuronal nAChRs subtypes using eukaryotic cellular lines expressing neuronal nAChRs (Coesnon et al., 2016; Kharrat et al., 2008; Stivala et al., 2015).

#### *Fluorescence polarization binding assay.*

Upon excitation by plane polarized light, a given fluorophore will emit fluorescence in the same plane as the exciting light: *i.e.*,  $\alpha$ -bungarotoxin Alexa Fluor 488 conjugate tumbles rapidly in solution (4.1 ns fluorescent lifetime) and when excited by plane polarized light, the resulting emission fluorescence is low. The binding of  $\alpha$ -bungarotoxin Alexa Fluor 488 to *Torpedo*-nAChR strongly decreases the rotation of the fluorophore tracer, and when excited by plane polarized light, the resultant emitted fluorescence will be high. The presence of gymnodimine-A, 13-desmethyl SPX-C or 13,19-didesmethyl SPX-C in the reaction mixture inhibited the binding of the fluorescent tracer to *Torpedo*-nAChR in a concentration-dependent manner. (Vilariño et al., 2009; Fonfria et al., 2010a). The use of fluorescein fluorophores to label *Torpedo*-nAChRs also enabled the detection of 13-desmethyl SPX-C and 13,19-didesmethyl SPX-C by Fluorescence polarization (Otero et al., 2011). The matrix effect of mussels, clams, cockles and scallops on the performance of the competitive fluorescence polarization assay was negative (Fonfria et al., 2010b).

#### *Solid-phase receptor-based assay.*

The method consists on the immobilization of biotin- $\alpha$ -bungarotoxin on 96-well microplates coated with streptavidin. In the absence of CiTXs, *Torpedo*-nAChRs bind the toxin tracer to form the

complex streptavidin-[biotin- $\alpha$ -bungarotoxin-*Torpedo*-nAChR]. The receptors are detected with primary antibodies anti-nAChR from *T. marmorata*. A conjugated secondary antibody allows the visualization of the captured receptors by chemiluminescence, fluorescence, or colorimetry (Rodriguez et al., 2011). The presence of CiTXs in the reaction mixture inhibits the binding of biotin- $\alpha$ -bungarotoxin to *Torpedo*-nAChR reducing the secondary antibody-evoked signal. The method is suited for high-throughput survey of CiTXs using 384-well microplates on shellfish samples (Rodriguez et al., 2013a).

#### *Flow cytometry receptor-based assay.*

Carboxylated microspheres are conjugated with *Torpedo*-nAChR or acetylcholine binding protein from the freshwater snail *Lymnaea stagnalis*. The conjugated microspheres are incubated with shellfish samples and later with biotin- $\alpha$ -bungarotoxin. After filtration, the fluorophore streptavidin-R-Phycoerythrin is added. The competitive binding is recorded by flow cytometry. The presence of CiTXs in the samples reduces the fluorescence yield in a concentration dependent manner. Further, it is possible to use microspheres beads owing different intrinsic fluorescence emission, consequently, different receptors or channel subtypes could be simultaneously analysed in the same sample (Rodriguez et al., 2013b).

#### *Torpedo-microplate receptor binding-assay.*

*Torpedo*-nAChR are immobilized on the wells of plastic microplates. Biotinylated  $\alpha$ -bungarotoxin is used as toxin tracer and streptavidin-horseradish peroxidase enables the detection and quantitation of anatoxin-a in surface waters, and of CiTXs in shellfish extracts (Aráoz et al., 2012; Rubio et al., 2014). CiTXs competitively inhibit biotinylated- $\alpha$ -bungarotoxin binding to *Torpedo*-nAChR in a concentration-dependent manner. The method compares favourably in terms of sensitivity to LC-MS/MS and provides accurate results for CiTXs monitoring (Aráoz et al., 2012). This functional assay is a high throughput method for rapid detection of known and emerging neurotoxins from marine and freshwater organisms that target nAChR directly in environmental samples with minimal sample handling, high sensitivity, reduced matrix effect and low cross-reactivity.

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\* *Torpedo*-nAChR refers to *Torpedo marmorata*-electrocyte membranes rich in nAChR of muscle type.

### *Neurotoxin fishing.*

A methodology for capturing novel ligands directed against nAChRs from complex mixtures containing small size alkaloids or large peptides was developed by using *Torpedo*-nAChR immobilized on the wells of plastic microplates (Aráoz et al., 2012) or *in-solution* (Echterbille et al., 2017). This methodology allows the direct capture of a toxin by the nAChR target. After washing under stringent conditions, the captured toxins are eluted by using solvents or by changing the pH. Afterwards, the eluates could be analysed for activity using electrophysiological techniques or by mass spectrometry to determine the chemical nature of the captured toxins.

### **Concluding remarks**

The European Food Safety Agency that prompted the use of LC-MS/MS for monitoring internationally regulated marine biotoxins also encourages the development of novel functional assays. The bioassays presented here are rapid, cost-effective, high-throughput and user-friendly for the detection of cyclic imine toxins in complex matrices that could be used in routine monitoring of CiTXs and later confirmed by LC-MS/MS.

### **Acknowledgements**

The authors acknowledge the LABEX LERMIT for funding DETECTNEUROTOX project and to Interreg/ Atlantic Area for funding the ALERTOX-NET EAPA\_317/2016 project.

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