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Colorimetric microtiter plate receptor-binding assay for the detection of freshwater and marine neurotoxins targeting the nicotinic acetylcholine receptors

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ABSTRACT

Anatoxin-a and homoanatoxin-a, produced by cyanobacteria, are agonists of nicotinic acetylcholine receptors (nAChRs). Pinnatoxins, spirolides, and gymnodimines, produced by dinoflagellates, are antagonists of nAChRs. In this study we describe the development and validation of a competitive colorimetric, high throughput functional assay based on the mechanism of action of freshwater and marine toxins against nAChRs. *Torpedo* electrocyte membranes (rich in muscle-type nAChR) were immobilized and stabilized on the surface of 96-well microtiter plates. Biotinylated α-bungarotoxin (the tracer) and streptavidin-horseradish peroxidase (the detector) enabled the detection and quantitation of anatoxin-a in surface waters and cyclic imine toxins in shellfish extracts that were obtained from different locations across the US. The method compares favorably to LC/MS/MS and provides accurate results for anatoxin-a and cyclic imine toxins monitoring. Study of common constituents at the concentrations normally found in drinking and environmental waters, as well as the tolerance to pH, salt, solvents, organic and inorganic compounds did not significantly affect toxin detection. The assay allowed the simultaneous analysis of up to 25 samples within 3.5 h and it is well suited for on-site or laboratory monitoring of low levels of toxins in drinking, surface, and ground water as well as in shellfish extracts.

1. Introduction

Microalgae are natural components of marine and freshwater environments. Excessive growth of algae becomes a nuisance and in some instances a public health threat through consumption of contaminated fish/mollusks and drinking water, and from the use of water for recreational activities. Thus, some members of cyanobacteria, dinoflagellates or diatoms may cause harm through the release of toxins. In continental waters, cyanobacteria harmful algal blooms (CyanoHABs) are being increasingly reported worldwide due to eutrophication and global climate change (Carmichael, 2001; Paerl and Huisman, 2008). Water managers have expressed serious concerns about public health and environmental quality as a result of CyanoHAB toxins in recreational and drinking waters.

CyanoHABs occur in freshwater lakes, ponds, rivers, reservoirs, and brackish waters throughout the world. Organisms responsible include an estimated 40 species,

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primarily belonging to the genera *Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Microcystis, Nostoc,* and *Oscillatoria* (*Planktothrix*) (Carpenter and Carmichael, 1995). Cyanobacterial toxins (cyanotoxins) include cytotoxins and biotoxins responsible for acute lethal, acute chronic, and sub-chronic poisoning of both wild and domestic animals as well as humans. Cyanotoxins include the neurotoxins anatoxin-a, anatoxin-a(s), and saxitoxin, and the hepatotoxins microcystins, nodularins and cylindrospermopsin (Carmichael, 1997).

Marine biotoxins are naturally occurring chemicals produced by a type of microscopic algae known as phytoplankton. These toxins can cause widespread harm to or death of sea life and can also affect humans through multiple routes of exposure (oral, respiratory, skin), making them a growing cause of concern for public health (Landsberg et al., 2005). Marine toxins can accumulate in fish and shellfish and can lead to several types of poisoning: amnesic shellfish poisoning (ASP), diarrhetic shellfish poisoning (DSP), neurologic shellfish poisoning (NSP), and paralytic shellfish poisoning (PSP). More recently, another syndrome, azaspiracid poisoning (AZP) produced by ingestion of azaspiracid toxins (AZA), have been reported after consumption of shellfish (Twiner et al., 2008). The emerging cyclic imine toxins gymnodimines, spirolides, and pinnatoxins have been reported to act as potent antagonists of nicotinic acetylcholine receptors (Kharrat et al., 2008; Bourne et al., 2010; Aráoz et al., 2011; Hu et al., 1995; Uemura et al., 1995). Following a typical toxicity by mouse bioassay, shellfish farms in New Zealand and France have been forced to close due to harmful algal blooms dominated by *Karenia selliformis,* the producer of gymnodimine A, and by *Alexandrium ostenfeldii,* the producer of 13-desmethyl spirolide C. Pinnatoxins have been isolated from shellfish of the genus *Pinna*, and were shown to be produced by *Vulcanodium rugosum* (Rhodes et al., 2011; Nézan and Chomérat, 2011; Hess et al., 2013).

The nicotinic acetylcholine receptors (nAChRs) belong to the family of ligand-gated ion channels. These receptors are widely expressed by various non-neural cell types including muscle, skin, pancreas, lungs and by neural cells in the central and peripheral nervous system. The transmembrane nAChRs are assembled from five subunits that are arranged around a central water-filled pore which opens following acetylcholine binding to its putative binding site. In vertebrate mammals, muscle nAChRs are of two types: embryonic ($\alpha 1_2\beta 1\gamma\delta$) or mature receptor type ($\alpha 1_2\beta 1\delta\varepsilon$). In contrast, neuronal nAChRs show more variability in terms of subunit composition: αβ nAChR are made up from a combination of α2-α6 and β2-β4 subunits. Muscle nAChR mediate fast synaptic transmission at the neuromuscular junction playing a central role in regulating functions vital for life and escaping from predation such as muscle contraction, and neuronal nAChRs control autonomic and central nervous system function (Albuquerque et al., 2009). In addition, the basic structure of the ligand binding site of nAChRs has been retained with little variability throughout evolution, making it an excellent structural target for a toxin. There are many examples of compounds that target nAChRs that are used as both predatory weapons and defensive measures against predation (Daly, 2005).

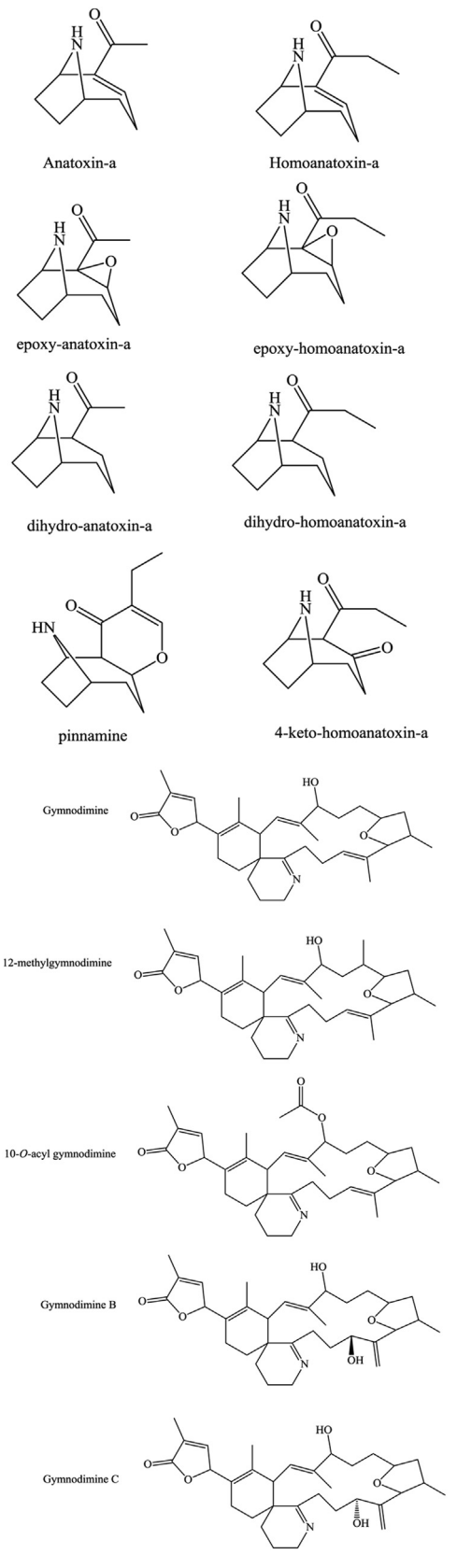

Fig. 1. Anatoxins and cylic imine toxins chemical structures.