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▶ **To cite this version:**

Jordi Molgó, P. Marchot, Rómulo Aráoz, Evelyne Benoit, Bogdan I Iorga, et al.. Cyclic imine toxins from dinoflagellates: a growing family of potent antagonists of the nicotinic acetylcholine receptors. Journal of Neurochemistry, 2017, 142, pp.41 - 51. 10.1111/jnc.13995 . hal-01661784

HAL Id: hal-01661784
https://hal.science/hal-01661784

Submitted on 21 May 2020

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Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2017 | 142 (Suppl. 2) | 41–51 doi: 10.1111/jnc.13995

REVIEW

Cyclic imine toxins from dinoflagellates: a growing family of potent antagonists of the nicotinic acetylcholine receptors

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Abstract

We present an overview of the toxicological profile of the fast-acting, lipophilic macrocyclic imine toxins, an emerging family of organic compounds associated with algal blooms, shellfish contamination and neurotoxicity. Worldwide, shellfish contamination incidents are expanding; therefore, the significance of these toxins for the shellfish food industry deserves further study. Emphasis is directed to the dinoflagellate species involved in their production, their chemical structures, and their specific mode of interaction with their principal natural molecular targets, the nicotinic acetylcholine receptors, or with the soluble acetylcholine-binding protein, used as a surrogate receptor model. The dinoflagellates *Karenia selliformis* and *Alexandrium ostenfeldii* / *A. peruvianum* have been implicated in the biosynthesis of gymnodimines and spirolides, while *Vulcanodinium rugosum* is the producer of pinnatoxins and portimine. The cyclic imine toxins are characterized by a macrocyclic skeleton comprising 14–27 carbon atoms, flanked by two conserved moieties, the cyclic imine and the spiroketal ring system. These phycotoxins generally display high affinity and broad specificity for the muscle type and neuronal nicotinic acetylcholine receptors, a feature consistent with their binding site at the receptor subunit interfaces, composed of residues highly conserved among all nAChRs, and explaining the diverse toxicity among animal species.

Keywords: acetylcholine-binding protein, dinoflagellates, gymnodimines, marine phycotoxins, muscarinic acetylcholine receptor, nicotinic acetylcholine receptor, pinnatoxins, spirolides.

J. Neurochem. (2017) **142** (Suppl. 2), 41–51.

This is an article for the special issue XVth International Symposium on Cholinergic Mechanisms.

Received December 16, 2016; revised manuscript received February 13, 2017; accepted February 15, 2017.

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Abbreviations used: ACh, acetylcholine; AChBP, acetylcholine-binding protein; mAChRs, muscarinic ACh receptors; nAChRs, nicotinic ACh receptors.

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The cyclic imine toxins comprise a growing family of lipophilic organic compounds produced by some species of marine dinoflagellate microorganisms. These neurotoxins have been found in extracts from contaminated shellfish (mainly bivalves), from natural plankton assemblages, from clonal cultures of toxic dinoflagellates, and as resulting products of the shellfish metabolism (fatty acid acyl esters) (for reviews, see Molgó *et al.* 2007; Guéret and Brimble 2010; Stivala *et al.* 2015). Currently, the cyclic imine family of toxins is exemplified by 40 molecules (not taking into account the acylated esters) and comprises the prorocentrolides, spiro-prorocentrimine, gymnodimines, spirolides, pinnatoxins, pteriatoxins, and portimine (Stivala *et al.* 2015). Spirolides represent the largest family.

Cyclic imine toxins can accumulate in bivalve mollusks and are considered as emergent toxicants affecting food safety in the shellfish food industry. Although their potent neurotoxicity led concern on their potential risks to shellfish consumers, currently these toxins are not regulated.

This short review aims at providing an overview of the origin, toxicological profile, chemical structure, and mode of action of the lipophilic cyclic imine toxins, with particular emphasis on their specificity of interaction with muscle-type and neuronal nicotinic acetylcholine (ACh) receptors (nAChR), which are the main molecular targets involved in their toxicity. The nAChRs are prototypical cation-selective, ligand-gated ion channels that mediate fast neurotransmission in the central and peripheral nervous systems (reviewed by Albuquerque *et al.* 2009). They belong to the Cys-loop superfamily of ligand-gated ion channels, and are formed by distinct combinations of five subunits that confer selectivity in pharmacological properties and cellular location (Corringer *et al.* 2000; Tsetlin *et al.* 2011).

Dinoflagellate species involved in the production of cyclic imine toxins

Several species of dinoflagellates, distributed worldwide in tropical, temperate, and cold marine waters, have been confirmed to be responsible for the production of cyclic imine toxins. Among the *Prorocentrum* species, *P. lima*, and *P. maculosum* have been linked to the production of prorocentrolides A and B and spiro-prorocentrimine (Torigoe *et al.* 1988; Hu *et al.* 1996b; Lu *et al.* 2001).

Gymnodimines A, B, and C have been shown to be produced by a species of dinoflagellates named *Karenia selliformis* (Miles *et al.* 2000, 2003; Haywood *et al.* 2004). However, other gymnodimine analogs like 12-methyl gymnodimine A and 12-methyl gymnodimine B are produced by the dinoflagellate *Alexandrium peruvianum* and *A. ostenfeldii* (Van Wagoner *et al.* 2011; Van de Waal *et al.* 2015; Strangman *et al.* 2016). Also, a new gymnodimine D was found in *A. ostenfeldii* clonal cultures isolated from the Northern Baltic Sea (Harju *et al.* 2016). The production of these various gymnodimine analogs indicates that common biosynthetic pathways are shared by distinct species of dinoflagellates.

The toxigenic dinoflagellate *A. ostenfeldii* and the closely related *A. peruvianum* have been reported to produce the large family of spirolides (Cembella *et al.* 1999, 2000; Touzet *et al.* 2008; Borkman *et al.* 2012). However, the profile of toxin production seems to differ with environmental conditions, genetic factors, and the location where the dinoflagellates are collected (Otero *et al.* 2010; Gu 2011; Suikkanen *et al.* 2013; Almandoz *et al.* 2014; Kremp *et al.* 2014; Salgado *et al.* 2015). *Alexandrium ostenfeldii/A. peruvianum* appear capable of producing saxitoxin and analogs (Anderson *et al.* 2012; Hakanen *et al.* 2012; Van de Waal *et al.* 2015; Kremp *et al.* 2016; Savela *et al.* 2016) that bind and block voltage-gated sodium channels (Catterall 1980), yet the cyclic imine toxins appear as the primary toxic agent in most of the world regions. During blooms of *A. ostenfeldii/A. peruvianum,* morphological uniqueness of the dinoflagellates can no longer be used as the indicator of the presumed toxin produced, and should always be accompanied by the chemical characterization of the toxin profile to identify the type(s) of toxins.

Indistinguishable peridinoid dinoflagellate strains were reported to be responsible for the production of pinnatoxins E, F, and G in Australia, pinnatoxin G in Japan, and pinnatoxins E and F in New Zealand (Rhodes *et al.* 2010, 2011a,b; Smith *et al.* 2011). Then, identification of the dinoflagellate producers of pinnatoxins was associated with the discovery, in coastal waters of France, of a novel dinoflagellate species, *Vulcanodinium rugosum* (Nézan and Chomérat 2011), that produced mainly pinnatoxin G (Hess *et al.* 2013). The smallest cyclic imine toxin so far isolated from *V. rugosum* was named portimine (Fig. 1), which is distinguished by a five-membered cyclic imine ring and a macrocycle of only 14 carbon atoms (Selwood *et al.* 2013), while pinnatoxins contain a seven-membered cyclic imine ring. In contrast to other cyclic imine toxins, portimine exhibits very low acute toxicity in the mouse, but it is extremely toxic to human Jurkat T-lymphoma cells and mouse embryonic fibroblasts cells in culture. Cells treated with portimine display rapid caspase activation and phosphatidylserine exposure, suggestive of apoptotic cell death (Cuddihy *et al.* 2016). To the best of our knowledge, none of the known cyclic imine toxins has been reported to activate caspase-3 and initiate apoptosis, as portimine does. Among toxins produced by the dinoflagellate *V. rugosum*, neither pinnatoxin F nor pinnatoxin G were reported to induce apoptosis (Cuddihy *et al.* 2016).

Another pinnatoxin congener (pinnatoxin H) was purified from cultures of *V. rugosum* collected from the South China Sea (Selwood *et al.* 2014) and in the marine waters of Qatar, Arabian Gulf (Al Muftah *et al.* 2016). *Vulcanodinium rugosum* strains of various origins have been

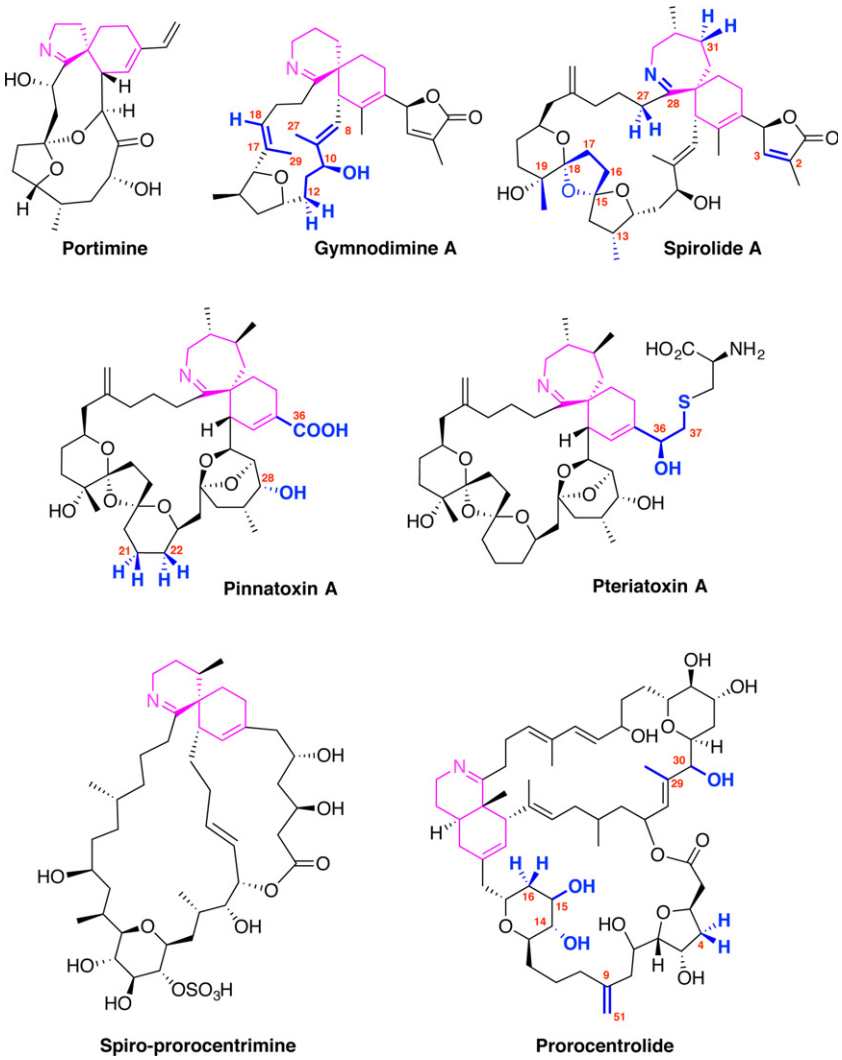


Fig. 1 Chemical structures of cyclic imine toxins. Only the first-described member of each family is represented. The positions where variations in structure were reported are numbered and displayed in blue. The cyclic imine moieties and attached rings are displayed in magenta.

reported to produce not only different pinnatoxins, but also to differ from each other in partial large subunit rDNA, internal transcribed spacer regions, and 5.8S rDNA sequences (Rhodes *et al.* 2011b), suggesting a complex variation in splicing options. At present, no definitive evidence has been published regarding a dinoflagellate origin of pteriatoxins.

Chemical structure

The general chemical structure of cyclic imine toxins encompasses a macrocyclic ring of 14–27 carbon atoms, and two highly conserved moieties: the cyclic imine group (mainly found as a spiroimine) and the spiroketal ring system. The cyclic imines are composed of a 5-membered (portimine), 6-membered (gymnodimines, spiroprorocentrimine, prorocentrolides), or 7-membered rings (spirolides, pinnatoxins, pteriatoxins); all are considered as essential components for bio-activity. Amino-ketone derivatives with an open imine ring, for example, spirolides E and F (Hu *et al.* 1996a) or the amino-ketone form of pinnatoxin A (Aráoz *et al.* 2011; Bourne *et al.* 2015) are devoid of biological activity. The other features of the ring system can be a simple tetrahydrofuran (in portimine and gymnodimines) or a tetrahydropyran group (prorocentrolides and spiroprorocentrimine), but also more complex 6,5- (spirolides H and I), 6,6,5- (spirolide G), 6,5,5- (spirolides A-F), or 6,5,6-spiroketal moieties (pinnatoxins and pteriatoxins), as shown in Fig. 1.

Several approaches for the synthesis of cyclic imine toxins were reported during the last few years (Beaumont *et al.* 2010; for reviews, see Molgó *et al.* 2014; Stivala *et al.* 2015), which were focused mainly on pinnatoxin A (McCauley *et al.* 1998; Sakamoto *et al.* 2004; Nakamura *et al.* 2008; Stivala and Zakarian 2008; Aráoz *et al.* 2011), pinnatoxins B and C (Matsuura *et al.* 2006a), pteriatoxins (Matsuura *et al.* 2006b), and gymnodimine (Kong *et al.* 2009, 2011). A fragment of gymnodimine A containing the

6,6-spiroimine core induced significant inhibition of ACh-evoked nicotinic currents generated by the nAChRs, a feature revealing the critical role of this moiety for the biological activity of the toxin (Duroure *et al.* 2011). The unusual stability to hydrolysis of the spiroimine group in pinnatoxin A, which is related to the high oral toxicity of this phycotoxin compared to its congeners (Molgó *et al.* 2015), was demonstrated by *in vitro* experiments performed under conditions addressing stability and by computational studies (Jackson *et al.* 2012).

Mode of action on muscle-type nAChRs

The nAChR from the *Torpedo* electric organ, a prototype of the vertebrate skeletal muscle nAChR, is a transmembrane heteropentameric molecule composed of four homologous subunits with a $\alpha 1_2\beta 1\gamma\delta$ stoichiometry. Two binding sites with distinct binding affinities for the nicotinic agonists and competitive antagonists are located at the α–γ and α–δ subunit interfaces. Functional studies revealed that nanomolar concentrations of gymnodimine A (Kharrat *et al.* 2008), 13-desmethyl spirolide C and 13,19-didesmethyl spirolide C (Aráoz *et al.* 2015), 20-methyl spirolide G (Couesnon *et al.* 2016), and pinnatoxins E, F, and G (Hellyer *et al.* 2013) blocked, in a concentration- and time-dependent manner, muscle twitches evoked by nerve stimulation in isolated mouse or rat nerve-muscle preparations without affecting the directly elicited muscle twitches, a feature indicating that these phycotoxins alter neuromuscular transmission without affecting the excitation-contracting coupling process. This interpretation was confirmed by electrophysiological recordings performed at single neuromuscular junctions, which showed that gymnodimine A (Kharrat *et al.* 2008), 13-desmethyl spirolide C and 13,19-didesmethyl spirolide C (Aráoz *et al.* 2015), and pinnatoxins E, F, and G (Hellyer *et al.* 2013), either reduced or completely blocked (depending on concentration) the amplitude of spontaneous miniature endplate potentials, an effect suggesting that the toxins impeded the interaction of ACh quanta with endplate nAChRs. In addition, the toxins blocked nerve-evoked endplate potentials without affecting the resting membrane potential of muscle fibers, so that endplate potentials could no longer reach the threshold for opening voltage-gated sodium channels in muscle fibers, and could not trigger a muscle action potential. These *in vitro* findings are consistent with *in vivo* studies in anesthetized mice showing that a local injection of toxins caused a dose-dependent block of the maximal compound muscle action potential amplitude, evoked by nerve stimulation. The effective doses needed to block 50% of the maximal compound muscle action potential amplitude (ED_{50}) were 1.7 ng/kg for 20-methyl spirolide G (Couesnon *et al.* 2016) and 6 ng/kg for 13-desmethyl spirolide C, which in turn was about 300-fold more active than gymnodimine A on an equimolar basis (Marrouchi *et al.* 2013). Overall, these studies indicated that these cyclic imine toxins potently block neuromuscular transmission on junctions expressing the mature muscle-type ($\alpha 1_2\beta 1\delta\varepsilon$) nAChR.

Further studies were carried out using *Xenopus* skeletal myocytes expressing the embryonic muscle-type $\alpha 1_2\beta 1\gamma\delta$ nAChR at their membrane surface, and the patch-clamp technique. Under these conditions, it was disclosed that gymnodimine A blocked nicotinic currents caused by short (5 ms) iontophoretic pulses of ACh, and that the block of ACh-evoked current exhibited no voltage dependency, and was persistent but reversible (Kharrat *et al.* 2008). Comparable results were obtained with 13-desmethyl spirolide C (Aráoz *et al.* 2015).

To gain further insight into the interaction between cyclic imine toxins and nAChRs, studies were performed on *Xenopus* oocytes microtransplanted with purified electrocyte membranes prepared from the electric organ of the fish *Torpedo marmorata* that expresses muscle-type $\alpha 1_2\beta 1\gamma\delta$ nAChR. Microinjection of the purified electrocyte membranes to the oocyte cytosol allows rapid incorporation of native and functional $\alpha 1_2\beta 1\gamma\delta$ nAChR into the oocyte membrane (for reviews, see Miledi *et al.* 2006; Eusebi *et al.* 2009; Bourne *et al.* 2010). Using this approach and the two-microelectrode voltage-clamp technique, the actions of gymnodimine A, 13-desmethyl spirolide C (Aráoz *et al.* 2009; Bourne *et al.* 2010), 13,19-didesmethyl spirolide C (Aráoz *et al.* 2015), 20-methyl spirolide G (Couesnon *et al.* 2016), and pinnatoxins A and G (Aráoz *et al.* 2011; Bourne *et al.* 2015) were studied. None of the cyclic imines analyzed exhibited by themselves an agonist action on the $\alpha 1_2\beta 1\gamma\delta$ nAChR incorporated to the *Xenopus* oocyte membrane, but they decreased the peak amplitudes of the ACh-elicited nicotinic current in a concentration-dependent manner. The IC_{50} for the various toxins studied are reported in Table 1. The 6,6-spiroimine synthetic analog of gymnodimine A also blocked ACh-evoked currents in *Xenopus* oocytes having incorporated the $\alpha 1_2\beta 1\gamma\delta$ nAChR (Duroure *et al.* 2011), but it was much less active than the native toxin.

Reversibility of antagonism of the nAChR was relatively fast with gymnodimine A and pinnatoxin A, but it was extremely slow with 13-desmethyl spirolide C, 13,19-didesmethyl spirolide C, and 20-methyl spirolide G; the latter compounds exhibited much higher potency than the other phycotoxins (see Table 1).

Competition binding studies revealed that the six cyclic imine toxins studied interact with high affinity with the $\alpha 1_2\beta 1\gamma\delta$ nAChR, and totally displaced [^{125}I]α-bungarotoxin from its binding site with dissociation constants in the nanomolar or subnanomolar ranges, as reported in Table 2.

Several methods for detecting cyclic imine toxins have been set up to substitute the conventional mouse bioassay, which has several weaknesses including specificity, sensitivity, and ethical concerns (reviewed in Daneshian *et al.*

Table 1 Inhibition constants (IC_{50}, nM) for the action of some cyclic imine dinoflagellate toxins on ACh-evoked nicotinic currents, recorded from oocytes micro-transplanted with the muscle-type $\alpha1_2\beta1\gamma\delta$ nicotinic ACh receptor, or expressing the human neuronal $\alpha7$ or $\alpha4\beta2$ nAChR subtypes (various subunit stoichiometries for the $\alpha4\beta2$)

Cyclic imine toxin	$\alpha1_2\beta1\gamma\delta$ (*Torpedo*)	$\alpha7$ (human)	$\alpha4\beta2$ (human)	References
20-meSPX-G[a]	0.36 (0.29–0.45)[j]	0.48 (0.15–1.4)	2.1 (1.4–3.1)	Couesnon *et al.* (2016)
13,19-ddmeSPX-C[b]	0.20 (0.16–0.26)	0.25 (0.24–0.27)	6.26 (4.7–8.3)	Aráoz *et al.* (2015)
13-SPX-C[c]	0.51 (0.4–0.6)	0.18 (0.16–0.21)	3.9 (2.9–5.1)	Bourne *et al.* (2010)
GYM-A[d]	2.8 (1.9–4.1)		0.9 (0.6–1.2)	Kharrat *et al.* (2008)
PnTX-A[e]	5.53 (4.5–6.8)	0.107 (0.086–0.132)	30.4 (19.4–47.5)	Aráoz *et al.* (2011)
PnTX-G[f]	3.82 (2.99–4.88)	5.06 (3.84–6.67)	4.90 (3.97–6.06)	Bourne *et al.* (2015)
PnTx-A AK[g]	24,760 (9,771–62,750)	182,500 (2,213–1,505,000)	> 1,000,000	Bourne *et al.* (2015)
PnTx-F[h]		6.8 ± 1.09 [k]	16 ± 0.44 [l] 24 ± 2.8 [m]	Hellyer *et al.* (2015)
PnTX-G[i]		10 ± 2.4 [k]	230 ± 15 [l] 105 ± 8.7 [m]	Hellyer *et al.* (2015)

[a]20-Methyl spirolide G; [b]13,19-Didesmethyl spirolide C; [c]13-Desmethyl spirolide C; [d]Gymnodimine A; [e]Synthetic pinnatoxin A; [f]Pinnatoxin G; [g]Synthetic pinnatoxin A amino ketone; [h]Pinnatoxin F; [i]Pinnatoxin G; [j]Mean values from concentration–response curves recorded from 46 to 50 oocytes for each experimental condition, 95% confidence intervals are indicated in parentheses; [k]Mean ± SEM; [l]Data obtained with $(\alpha4)_3(\beta2)_2$; [m]Data obtained with $(\alpha4)_2(\beta2)_3$.

Table 2 Inhibition constants (K_i ± SEM, nM) obtained from competition binding assays at equilibrium for some cyclic imine toxins on various nicotinic ACh receptors, and comparisons with the nicotinic antagonists α-toxin, methyllycaconitine (MLA), and epibatidine

Cyclic imine toxin	$\alpha1_2\beta1\gamma\delta$ (*Torpedo*)	$\alpha7$-5HT$_3$ (chick)	$\alpha3\beta2$ (human)	$\alpha4\beta2$ (human)	Reference
20-meSPX-G[a]	0.028 ± 0.005[i]	0.11 ± 0.08	0.040 ± 0.001	3.6 ± 0.7	Couesnon *et al.* (2016)
13,19-ddmeSPX-C[b]	0.017 ± 0.003	0.22 ± 0.06	0.51 ± 0.14	53 ± 25	Aráoz *et al.* (2015)
13-SPX-C[c]	0.080 ± 0.002	0.53 ± 0.08	0.021 ± 0.005	0.58 ± 0.07	Bourne *et al.* (2010)
GYM-A[d]	0.23 ± 0.08	0.33 ± 0.08	0.24 ± 0.09	0.62 ± 0.07	Kharrat *et al.* (2008)
PnTX-A[e]	2.80 ± 0.03	0.35 ± 0.04	9.4 ± 1.9	15.6 ± 5.2	Aráoz *et al.* (2011)
PnTX-G[f]	0.11 ± 0.04	0.72 ± 0.03	64 ± 2	101 ± 30	Bourne *et al.* (2015)
α-Toxin[g]	0.011 ± 0.002				Couesnon *et al.* (2016)
MLA[h]		0.83 ± 0.12			Couesnon *et al.* (2016)
Epibatidine			0.034 ± 0.002	0.054 ± 0.011	Couesnon *et al.* (2016)

[a]20-Methyl spirolide G; [b]13,19-Didesmethyl spirolide C; [c]13-Desmethyl spirolide C; [d]Gymnodimine A; [e]Synthetic pinnatoxin A; [f]Synthetic pinnatoxin G; [g]Synthetic peptidic α-toxin (*Naja nigricollis)*; [h]Methyllycaconitine; [i]Values expressed as the mean ± SEM from 3 to 4 distinct experiments performed in duplicate.

2013). The new methods are based on competition assays where the cyclic imine toxins prevent the interaction of fluorescent- or biotin-labeled α-bungarotoxin with the $\alpha1_2\beta1\gamma\delta$ nAChR contained in purified *Torpedo* membranes (Vilariño *et al.* 2009; Fonfría *et al.* 2010; Otero *et al.* 2011; Rodríguez *et al.* 2011, 2013a; Aráoz *et al.* 2012; Rubio *et al.* 2014). Also, a receptor-based detection method was developed using *Torpedo* nAChR, or *Lymnaea stagnalis* ACh-binding protein immobilized on the surface of carboxylated microspheres, and the competition of cyclic imines toxins with biotin-α-bungarotoxin for binding to these proteins (Rodríguez *et al.* 2013b).

Available evidence indicates that both mature ($\alpha1_2\beta1\delta\varepsilon$) and embryonic ($\alpha1_2\beta1\gamma\delta$) muscle-type nAChRs are important targets for cyclic imine toxins, and are certainly responsible for the fast depression of respiratory neuromuscular transmission during acute toxicity assays in rodents (Munday *et al.* 2004, 2012).

Mode of action on neuronal types of nAChRs

Cyclic imine toxins are among the few organic compounds produced by dinoflagellates known to interact with the major neuronal nAChRs, as assessed by functional and ligand-binding assays. Vertebrate neuronal nAChRs comprise a varied population of receptors with assorted subunit assemblies of α2–α10 and β2–β4 subunits (Millar and Gotti 2009). The human $\alpha3\beta2$ and $\alpha4\beta2$ subtypes, which play a

predominant role in both pre- and post-synaptic functions in the central and peripheral nervous systems, may have variable stoichiometries of 2 α/3 β versus 3 α/2 β subunits, with higher affinity ligand binding at the α/β subunit interfaces and lower affinity ligand binding at the α/α subunit interfaces (Shahsavar *et al.* 2015).

Under voltage-clamp conditions, gymnodimine A, 13-desmethyl spirolide C, 13,19-didesmethyl spirolide C, 20-methyl spirolide G, or pinnatoxins A, E, F, and G do not activate the human homomeric α7 or heteromeric α4β2 nAChRs expressed in *Xenopus* oocytes, and therefore do not exert the agonist action typically observed with ACh or nicotine. In contrast, the toxins reduce the inward peak current elicited by ACh in both human α7 and α4β2 nAChR subtypes (Kharrat *et al.* 2008; Bourne *et al.* 2010, 2015; Aráoz *et al.* 2011, 2015; Hellyer *et al.* 2015; Couesnon *et al.* 2016). The IC_{50} values are reported in Table 1.

The action of cyclic imines was also studied using a fluorescence Ca^{2+} mobilization assay. In brief, nicotine binding to cell surface nAChRs caused a release of the intracellular calcium (monitored through its binding to the previously loaded calcium-sensitive dye, fluorescence imaging plate reader calcium 4) and thereby increased the cell fluorescence intensity measured with a fluorescence imaging plate reader. Neither gymnodimine A nor 13-desmethyl spirolide C at concentrations up to 10 μM displayed any calcium release from cells expressing the human α4β2 or α4β4 nAChRs (Hauser *et al.* 2012). The antagonistic blockade effect of gymnodimine A and 13-desmethyl spirolide C was also examined using the calcium-flux assay in cells expressing various subtypes of nAChRs by incubating the cells with the toxins for 30 min, and thereafter adding nicotine at an EC_{80} concentration. Under these conditions, the inhibition of nicotine-induced calcium-flux response, determined at each antagonist concentration, revealed the following rank order of potency for 13-desmethyl spirolide C: α7 > low sensitivity form of α4β2 > human α3β4 > high sensitivity form of α4β2 > human α4β4 > rat α3β4, and for gymnodimine A: low sensitivity form of α4β2 > human α3β4 > α7 > high sensitivity form of α4β2 > human α4β4 > rat α3β4. The antagonism of the nicotine-induced calcium mobilization by both toxins was found to be not surmountable (Hauser *et al.* 2012). Furthermore, 13-desmethyl spirolide C and gymnodimine A inhibited nicotine-mediated dopamine release from rat striatal synaptosomes with similar high potency (IC_{50} = 0.2 and 0.3 nM, respectively) (Hauser *et al.* 2012).

Additionally, none of the toxins studied appeared to modify the desensitization kinetics of the α7 nAChR. The antagonistic activity of gymnodimine A on the human α7 and α4β2 nAChR subtypes was found to be readily reversible, whereas those of 13-desmethyl spirolide C and 20-methyl spirolide G were not abolished after a 30–60 min washout (Bourne *et al.* 2010; Couesnon *et al.* 2016). Pinnatoxin A activity on the human α7 nAChR was also reported to be slowly reversible, whereas on α4β2 nAChR it was rapidly reversible (Aráoz *et al.* 2011; Bourne *et al.* 2015).

Functional studies were also performed with the voltage-clamp technique on oocytes expressing the α4β2 nAChR in two stoichiometric forms [the low-affinity $(\alpha4)_3(\beta2)_2$ and high-affinity $(\alpha4)_2(\beta2)_3$ forms] (Nelson *et al.* 2003). Both pinnatoxins F and G inhibited the ACh-evoked responses, yet with different potencies reflected in their IC_{50} values. As shown in Table 1, pinnatoxin F was more active on $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ nAChRs than pinnatoxin G. However, both pinnatoxins displayed similar potencies on the $(\alpha4)_3(\beta2)_2$ versus $(\alpha4)_2(\beta2)_3$ nAChRs (Hellyer *et al.* 2015). Thus, the lower sensitivity of the cyclic imine toxins toward the α4β2 nAChR, compared to the homomeric α7 nAChR, does not result from changes in the subunit stoichiometry and creation of an α–α interface, but rather from essential structural properties in the ligand-binding sites at the respective subunit interfaces.

The spiroimine fragment seems essential for the functional blocking activity of pinnatoxin A, as the open-ring, amino-ketone derivative of pinnatoxin A has no action on the various neuronal nAChR subtypes (Table 1).

A deeper insight into the interaction between cyclic imine toxins and neuronal nAChRs was obtained from competition binding experiments performed at equilibrium on membranes from cells expressing various nAChR subtypes and an α7-5HT3 chimera, and using radiolabeled probes and standard protocols (Servent *et al.* 1997). Gymnodimine A, 13-desmethyl spirolide C, 13,19-didesmethyl spirolide C, 20-methyl spirolide G, and pinnatoxins A and G totally displaced [^{125}I]α-bungarotoxin from its binding site, thereby confirming that these phycotoxins interact with high affinity with the α7-5HT3 chimera. In addition, their property of displacing [^{3}H]epibatidine binding from human α3β2 and α4β2 neuronal nAChRs highlights the broad capacity of cyclic imine toxins to interact with either of the homo- and hetero-pentameric forms of neuronal nAChRs.

The rank order of potency for gymnodimine A (from K_i values) was found to be chicken α7-5HT3 > human α3β2 > human α4β2 nAChR (Kharrat *et al.* 2008). In another study this pharmacological profile was confirmed and detailed, the order of potency for gymnodimine A being: α7, α6β3β4α5 > rat α3β4 > human α3β4, α4β4 > rat α4β2, human α4β2 (Hauser *et al.* 2012). Table 2 summarizes the dissociation constant (K_i) values of several cyclic imine toxins relative to distinct neuronal nAChRs subtypes, recorded under the same experimental conditions.

A comparable broad specificity toward the neuronal nAChR subtypes was also observed with 13-desmethyl spirolide C (Bourne *et al.* 2010). The rank order for inhibition by 13-desmethyl spirolide C was: α7 > α6β3β4α5 ≫ rat α3β4, α4β4, human α3β4 > human α4β2 > rat α4β2 (Hauser *et al.* 2012). The selectivity profile for pinnatoxin

A also exhibited a higher affinity for the human α7 compared to the human α3β2 and α4β2 nAChRs (Aráoz *et al.* 2011), and this was maintained for other pinnatoxins exhibiting the following order of potency: pinnatoxin F > pinnatoxin G > pinnatoxin E (Hellyer *et al.* 2015).

Interaction with mAChRs

Muscarinic ACh receptors (mAChRs) have been proposed in early studies to explain part of the acute toxicological mode of action of 13-desmethyl spirolide C in rats (Gill *et al.* 2003). Indeed, both transcriptional alterations for early injury markers (c-jun and HSP-72) and for mAChRs and nAChRs were revealed. The *M1*, *M4*, and *M5 mAChR* genes, as well as the *α2* and *β4 nAChR* genes were altogether up-regulated, implying that both types of cholinergic receptors could be potential molecular targets for the 13-desmethyl spirolide C. Studies carried out in the human neuroblastoma cell line BE(2)-M17, expressing mAChRs subtypes, reported that 13-desmethyl spirolide C inhibited ACh-induced Ca^{2+} signals, while the reversible competitive antagonist atropine diminished the inhibitory effect of the spirolide. Also, the spirolide at 0.5 μM reduced the [^{3}H]*N*-methyl scopolamine specific binding to the cells by *ca*. 53%. Similar inhibition of [^{3}H]quinuclidinyl benzilate binding (59%) was observed under the same experimental conditions. Such data suggested that the spirolide binds to the orthosteric binding site of mAChRs (Wandscheer *et al.* 2010). However, later competition binding assays, performed on membrane embedded mAChRs from TE671/RD clonal cells and rat cortices using radiolabeled [^{3}H]quinuclidinyl benzilate and both gymnodimine A and 13-desmethyl spirolide C, failed to show any significant interaction with the mAChRs (Hauser *et al.* 2012).

Further work performed with CHO cells stably expressing each of the five human mAChRs subtypes revealed that pinnatoxin A (1 μM) had no significant action on [^{3}H]*N*-methyl scopolamine binding to M1, M2, M3, and M4 mAChRs, whereas it displaced radiotracer binding to the M5 mAChR subtype by 35%, a value reflecting interaction in the low micromolar range (Aráoz *et al.* 2011). This property was not observed using the pinnatoxin A amino-ketone derivative. In a similar assay, 13-desmethyl spirolide C and 13,19-didesmethyl spirolide were found to interact with very low affinity (in the micromolar range) with the five mAChR subtypes, yielding affinities 3–4 orders of magnitude lower than those for the nAChR subtypes (Aráoz *et al.* 2015).

Structural studies

The absolute stereochemistry of gymnodimine A was unambiguously assigned from the crystal structure of the *p*-bromobenzamide derivative of the reduced form of gymnodimine A (Stewart *et al.* 1997), whereas the relative stereochemistry of 13-desmethyl spirolide C, except for one chiral center, has been determined using the ConGen molecular modeling method, from NOESY and ROESY NMR data (Falk *et al.* 2001). Later on, the crystal structures of acetylcholine-binding protein (AChBP) complexes with gymnodimine A (Bourne *et al.* 2010), 13-desmethyl spirolide C (Bourne *et al.* 2010), and pinnatoxins A and G (Bourne *et al.* 2015) unveiled the molecular determinants of toxin-binding selectivity. The soluble AChBPs, with their overall pentameric architecture and their amino acid residues forming a binding pocket for the nicotinic ligands at subunit interfaces, have been extensively used as functional and structural surrogates for the ligand-binding domains of the nAChRs and have provided valuable information on how ligands interact with the various nAChR subtypes (Brejc *et al.* 2001; Celie *et al.* 2004; for a recent review, see Shahsavar *et al.* 2016).

In addition to the overall structural features of the subunits, the aromatic side chains that form the ligand-binding pocket at the subunit interfaces are well conserved in the nAChR family, with greater variability for residues at the complementary [or (−)] face than the principal [or (+)] face of each interface. In fact, the binding pocket of AChBP possesses all the functional residues identified in the nAChR ligand-binding domain. Hence, the ligand-binding pocket encompasses a nest of five electron-rich aromatic side chains provided by residues Tyr93, Trp147, Tyr188, Tyr195 on the (+) face and residue Tyr55 on the (−) face of the interface.

In each complex, the toxin is imbedded within this aromatic nest contributed by loops C and F on opposing faces of the subunit interface and display exquisite shape and chemical complementarity with the ligand-binding pocket (Fig. 2). The orientation and conformation of the toxin carbon skeleton, with its long axis roughly aligned parallel with the pentamer five-fold axis, ideally position the protonated cyclic imine donor, similar to the anabaseines (Talley *et al.* 2006; Hibbs *et al.* 2009), to be within H-bond distances to the carbonyl oxygen of Trp147 (loop B). At the apical side of the interface, the tetrahydrofuran ring (gymnodimine A) or bulky and more rigid *bis*-spiroacetal ring system (13-desmethyl spirolide C, pinnatoxin A) abuts against the tip of loop C to localize the binding interface. At the opposing subunit face, the variable terminal γ-butyrolactone (gymnodimine A) or cyclohexene (13-desmethyl spirolide C, pinnatoxin A) rings promote additional interactions including the conserved Tyr93 from the (+) face. In fact, pinnatoxin A contains a bulky bridged 5,6-bicycloketal substructure instead of a smaller allylic alcohol linker found in gymnodimine A and 13-desmethyl spirolide C. This unique substructure in pinnatoxins (also found in pteriatoxins) extends radially from the interfacial binding pocket to interact with the sequence-variable loop F and governs nAChR subtype selectivity (Bourne *et al.* 2010, 2015).

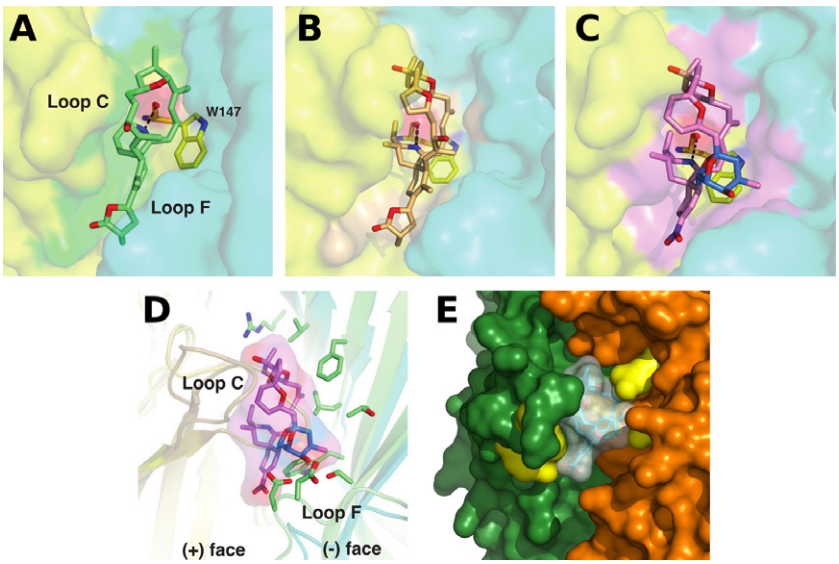


Fig. 2 Close-up views of the binding interfaces of several cyclic imine toxins. Crystal structures of the AChBP subunit interface [the (+) and (−) faces are displayed in yellow and cyan, respectively] with bound gymnodimine A (A), 13-desmethyl spirolide C (B), and pinnatoxin A (C) (Protein Data Bank accession codes 2X00, 2WZY, and 4XHE; Bourne *et al.* 2010, 2015); (D) overlay of the binding interfaces of AChBP with bound pinnatoxin A and of the human α4β2 receptor (5KXI; Morales-Perez *et al.* 2016) (the α and β subunits are displayed in light orange and green, respectively). Those side chains from the β2 subunit [(−) face] that could contribute to pinnatoxin A binding are shown in green; (E) docking complex of pinnatoxin A with the α7 nAChR structure generated by homology modeling (Aráoz *et al.* 2011). The α7 residues Trp147, Arg186, Tyr188, Tyr195 [in the (+) subunit] and Gln57, Gln116 [in the (−) subunit], which are predicted to establish hydrogen bonds with the toxin, are displayed in yellow.

In addition to the crystal structures of AChBP–toxin complexes, additional complexes between different nAChR subtypes (human α7, α4β2, α3β2, and $\alpha 1_2\beta 1\gamma\delta$) and pinnatoxin A (Aráoz *et al.* 2011), 13-desmethyl spirolide C (Aráoz *et al.* 2015), and 13,19-didesmethyl spirolide C (Aráoz *et al.* 2015) generated by *in silico* molecular docking, provided complementary information for the identification of key residues responsible for the differences in binding affinities and subtype specificities that were determined experimentally (Fig. 2) (Aráoz *et al.* 2011, 2015).

Conclusion

In conclusion, the globally distributed and chemically well-characterized cyclic imine toxins, from toxic dinoflagellate species, represent a novel source of potent antagonists of muscle- and neuronal-type nAChRs. The distinctive chemical signature of these phycotoxins is related to the presence of a cyclic imine moiety in their structure, and their toxicological profile is predominantly associated to their specific interaction with the nAChRs.

Taking advantage of the competitive binding of cyclic imine toxins to nAChRs, several tests have been developed to detect spirolides, gymnodimines, and pinnatoxins in contaminated shellfish with better accuracy than the broad spectrum mouse bioassay. These tests are important in the food safety field, because shellfish represents a rich food resource that may be contaminated by toxins produced by toxic dinoflagellates.

Substantial progress has been obtained on the characterization of the dinoflagellates producing the cyclic imine toxins, but the genes involved in their production, and the pathways leading to the biosynthesis of the various families of toxins remain, at present, elusive. Furthermore, the ecological factors favoring dinoflagellate blooming need to be determined.

Shellfish regularly contain variable amounts of cyclic imine toxins in their edible tissues and can transfer these phycotoxins through the marine food chain. Although cyclic imine levels are not regulated, it has become a matter of concern to assess the risks for human health. Thus, a consensus is emerging that further studies should be conducted to enhance our understanding of the gastrointestinal absorption, tissue disposition, and crossing of the blood–brain and placental barriers. Also, more information is needed on the environmental distribution and risks of chronic exposure to these phycotoxins.

Acknowledgements and conflict of interest disclosure

The works described here were funded by the Agence Nationale de la Recherche (France, grant AQUANEUROTOX ANR-12-ASTR-0037

to J. M. and D. S.), the French Infrastructure for Integrated Structural Biology (FRISBI) ANR-10-INSB-05-01 (to the AFMB laboratory), a CNRS-DRI PICS grant (to Y. B. and P. M.), and the National Institutes of Health (USA, grants NIGMS R01 GM077379 to A. Z. and GM18360 to P. T.). The authors declare no conflicts of interest.

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