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# Mode of action of cyclic imine toxins: 20-methyl spirolide-G antagonizes rat neuronal $\alpha 3\beta 2$ nicotinic acetylcholine receptor

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## Abstract

Cyclic imine toxins produced by marine dinoflagellate species move up fast the food chain through shellfish and constitute a potential threat for marine wildlife and public health because of their potent antagonistic activity against nicotinic acetylcholine receptors of muscle and neuronal types. Although not regulated, the lethality of 13-desmethyl spirolide-C does compare the lethality of saxitoxin in mouse bioassay. Besides, cyclic imine toxins are often found in shellfish destined for human consumption. Herein, the unveiling of the mode of action of cyclic imine toxins is reviewed. To illustrate the review, the electrophysiological characterization of the antagonistic capacity of 20-methyl spirolide-G on rat  $\alpha 3\beta 2$  nicotinic acetylcholine receptor is described.

**Keywords:** Cyclic imine toxins, nicotinic acetylcholine receptors, 20-methyl spirolide-G, two-electrodes voltage clamp electrophysiology (TEVC), *Xenopus laevis* oocytes

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## Introduction

Cyclic imines toxins (CiTXs; ~44 members) are marine emergent neurotoxins worldwide distributed among coastal environments. Of dinoflagellate origin, CiTXs move up fast the food chain through shellfish and may constitute a potential threat for public health (Aráoz *et al.*, 2020). CiTXs are potent antagonists of nicotinic acetylcholine receptors (nAChRs). They display a cyclic imine in their structure necessary for interacting with nAChRs (Bourne *et al.*, 2010). Portimines (A-B) produced by *Vulcanodinium rugosum*, display a 5-membered imine ring (Selwood *et al.*, 2013). The following CiTXs display a 6-membered cyclic imine ring: gymnodimines (GYM) (seven congeners), that are produced by *Karenia selliformis* and *Alexandrium peruvianum* (Seki *et al.*, 1995; Van Wagoner *et al.*, 2011), prorocentrolides (six members) produced by *Prorocentrum lima* (Torigoe *et al.*, 1988), spiroprorocentrimine produced by *Prorocentrum* sp. (Lu *et al.*, 2001), and kabirimine produced by *V. rugosum* (Hermawan *et al.*, 2019). Spirolides (SPX) (16 members) produced by *A. ostenfeldii*, reviewed by Gueret and Brimble, 2010, pinnatoxins (PnTX) (A-H) produced by *V. rugosum* (Rhodes *et al.*, 2010) and pteriatoxins (PtTX) (A-C) display a 7-membered cyclic imine ring (Takada *et al.*, 2001).

### Mode of action (MOA) of CiTXs

#### MOA of CiTXs from mouse assay experiments

CiTXs are fast-acting neurotoxic compounds that kill mice by respiratory arrest within minutes after intraperitoneal injection or gavage at lethal toxin doses. The clinical signs preceding mice death induced by CiTXs

were similar to those evoked by curare: the administration of neostigmine, a reversible acetylcholinesterase inhibitor, increases the concentration of acetylcholine (ACh) at the neuromuscular synapse, competitively displacing curare from muscle nAChRs (Leeuwijn *et al.*, 1981). Thus, the administration of two times the LD<sub>50</sub> of GYM-A killed all the mice in 3-7 min. However, neostigmine pre-treated rodents survived GYM-A administration at the referred dose (Munday *et al.*, 2012). This was a strong indication that muscle-type nAChR may be a molecular target of GYM-A.

Another key event was the discovery of keto-amine derivatives of SPX-A and -B, namely SPX-E and -F, in the digestive glands of scallops. The cyclic imine rings in SPX-E and -F were open and showed no activity by mouse assay. The hydrolysis of the cyclic imine ring of SPX-A and -B was associated with the presence of one methyl group on the cyclic imine ring. Thus, SPX-C, 13-desmethyl SPX-C and SPX-D were resistant to oxalic acid hydrolysis suggesting that the double methylation of the spiroimine ring confers a better chemical stability to spiroptides (Hu *et al.*, 2001). SPXs (excluding SPX-A and B), PnTXs and PtTXs, have a double methylated cyclic imine ring, reviewed by Gueret and Brimble (2010) and Stivala *et al.* (2015). Further, the amino-keto PnTX-A analogue, bearing an open form of the imine ring, was inactive on muscle and neuronal nAChRs when tested by electrophysiology, confirming that the cyclic imine is critical for CiTX interaction with nAChR (Aráoz *et al.*, 2011).



### *MOA of CiTXs from electrophysiological research*

Electrophysiological recordings using *ex vivo* neuromuscular preparations confirmed that cyclic imine toxins act on nAChRs, reviewed by Molgó *et al.* (2017). Thus, GYM-A produced a concentration- and time-dependent block of twitch responses evoked by nerve stimulation without affecting elicited muscle twitches in isolated mouse phrenic-hemidiaphragm preparations. This suggested that GYM-A blocked muscle nAChR. The reduction of the amplitude of miniature-endsplate resulting from quantal ACh-release and subsequent activation of post-synaptic nAChR, confirmed the blockade of muscle-nAChR by GYM-A (Kharrat *et al.*, 2008). Similar effects on the neuromuscular junction were obtained for 13,19-desmethyl SPX-C and 13-didesmethyl SPX-C (Aráoz *et al.*, 2015; Bourne *et al.*, 2010), 20-methyl SPX-G (Couesnon *et al.*, 2016), PnTX -A, -E, -F and -G (Aráoz *et al.*, 2011; Hellyer *et al.*, 2011), and procontrolide-A (Amar *et al.*, 2018). These findings are consistent with *in vivo* electrophysiological recordings in sedated mice showing that a local injection of PnTX-A or PnTX-G induced the blockade of the maximal compound muscle action potential amplitude, evoked by nerve stimulation (Benoit *et al.*, 2019).

*In vitro* two-electrodes voltage clamp (TEVC) recordings to determine the mode of action of CiTXs were performed on *Xenopus laevis* oocytes which is a successful electrophysiological model for heterologous *de novo* expression of human ion channels and receptors and for the transplantation of membranes rich in receptors and ion channels for pharmacological studies (Miledi *et al.*, 2004). Binding of ACh to the ligand-

binding sites rapidly (microseconds) shifts the receptor conformation from the resting (closed state) to the open state, initiating the ion flux through the cation-selective pore for ~2 milliseconds, and the receptor channel closes (desensitized state) (Giniatullin *et al.*, 2005). The perfusion of GYM-A, 13-desmethyl SPX-C, 13-19-didesmethyl SPX-C, 20-methyl SPX-G, PnTX-A, -E, -F, and -G or procontrolide does not evoke inward currents on muscle and neuronal nAChRs regardless of subtype composition or toxin concentration. When these CiTXs are co-applied with ACh, the ACh-elicited currents are inhibited in a concentration-dependent manner. The inhibition degree is dependent on the nAChR subtype and on the CiTX tested. The blockade of muscle and neuronal receptors is not voltage-dependent. Indeed, CiTXs are competitive antagonists of muscle and neuronal nAChRs with broad subtype selectivity and high potency. Spirolides showed sub-nanomolar affinities for *Torpedo* ( $\alpha 1$ )<sub>2</sub> $\beta 1\gamma\delta$  nAChR (Aráoz *et al.*, 2015), while the affinities of GYM-A and PnTX-A and -G were in the nanomolar range (Aráoz *et al.*, 2011; Kharrat *et al.*, 2008). The affinities of the studied CiTXs for human  $\alpha 4\beta 2$  nAChRs are in the nanomolar range (Stivala *et al.*, 2015). Except for PnTX-G, GYM-A, 13-desmethyl SPX-C, 13-19 didesmethyl SPX-C, 20-methyl SPX-G and PnTX-A showed picomolar affinities for  $\alpha 7$  nAChR (Bourne *et al.*, 2015). Portimine-A and procontrolide-A were less active on muscle and neuronal nAChR (Amar *et al.*, 2018; Lamoise *et al.*, 2017). PnTX-A shows the best selectivity profile: the affinity of PnTX-A for  $\alpha 7$  nAChR is 50-times higher than its affinity for *Torpedo* ( $\alpha 1$ )<sub>2</sub> $\beta 1\gamma\delta$  nAChR, and 300-times higher than its affinity for  $\alpha 4\beta 2$  nAChR (Aráoz *et al.*, 2011).



*CiTXs' target: nicotinic acetylcholine receptor*

nAChRs are allosteric transmembrane pentameric proteins belonging to the cysteine-loop ligand-gated ion channel superfamily. Muscle nAChR ensures fast neurotransmission at the neuromuscular junction that is essential for respiration and muscle contraction for daily life, mobility, or escape from predation (Albuquerque *et al.*, 2009). At the central nervous system (CNS), neuronal nAChRs modulate neurotransmitters release, hence participating in fundamental aspects of synaptic plasticity involved in attention, learning, memory, and neurodegenerative disorders. Non-neuronal nAChRs are ubiquitously expressed in the human body from epithelial cells, to primary immune organs such as the bone marrow, thymus and macrophages (Bertrand *et al.*,

2015). Human genomic studies revealed 16 homologous genes coding for nAChR-subunits. The presence of the Cys-pair characterizes  $\alpha$  subunits ( $\alpha 1$ - $\alpha 10$ ), and its absence characterizes  $\beta 1$ - $\beta 4$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$  subunits. Muscle-nAChRs can be fetal ( $\alpha 1$ )<sub>2</sub> $\beta 1\gamma\delta$  or adult ( $\alpha 1$ )<sub>2</sub> $\beta 1\gamma\epsilon$ . Neuronal nAChRs show more variability resulting from the combination of  $\alpha 2$ - $\alpha 7$  with  $\beta 2$ - $\beta 4$  subunits. The  $\alpha 7$  and  $\alpha 9$  form monomeric receptors, and  $\alpha 8$  subunit was found only in avian tissues. The heteropentameric  $\alpha 7\beta 2$  and  $\alpha 9\alpha 10$  nAChRs were found in the CNS (Bertrand *et al.*, 2015). Different receptor configurations display distinct pharmacological profiles and receptor distribution patterns across the brain.

*MOA of CiTXs from crystallographic studies*  
GYM-A & 13-desmethyl SPX-C, and PnTX-A & PnTX-G were co-crystalized with the ACh-binding

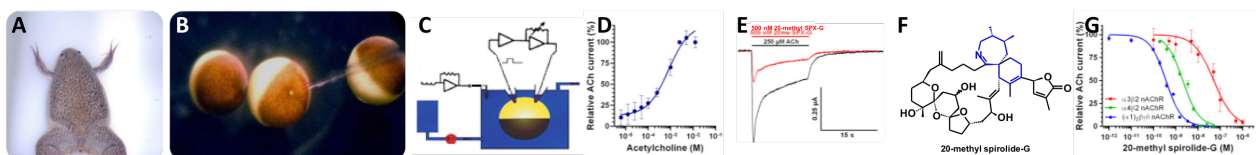


Fig. 1. MOA of 20-methyl SPX-G on  $\alpha 3\beta 2$  nAChR. (A) *Xenopus laevis* frog. (B) *Xenopus* oocyte microinjected with mRNA. (C) Schema of TEVC: a *Xenopus* oocyte is impaled with two electrodes. ACh-evoked currents are recorded at a holding potential of -60 mV. (D) ACh dose-response curve on oocytes expressing  $\alpha 3\beta 2$  nAChR. (E) Electrophysiological recordings showing ACh-current (black) and the antagonistic effect of 500 nM 20-methyl SPX-G (red). (F) 20-methyl SPX-G. (G) Concentration-dependent inhibition of ACh-evoked currents by 20-methyl SPX-G in *Xenopus* oocytes having incorporated *Torpedo*-( $\alpha 1$ )<sub>2</sub> $\beta \gamma \delta$  nAChR (blue), or expressing the *ha4* $\beta 2$  (green) (Couesnon *et al.*, 2016), and the  $\alpha 3\beta 2$  (red; this work). Amplitudes of the ACh-current peak (mean  $\pm$  SEM; 5 oocytes per concentration), were normalized to control currents and fitted to the Hill equation.



protein from *Lymnaea stagnalis* and *Aplysia californica* to define the structural determinants responsible for their with the orthosteric site of  $ha7$  nAChR, (Bourne *et al.*, 2010; 2015). These proteins are structural surrogates of the extracellular ligand-binding domain of  $ha7$  nAChR. Overall, the co-crystallized CiTXs share a similar binding mode where the conserved loop C envelops the bound toxins buried at the binding interface with high surface complementary. The protonated imine nitrogen of the six- or seven-membered cyclic imines is positioned to hydrogen bond to the backbone carbonyl oxygen of loop C Trp147, a key residue for agonists and antagonists binding. The bis-spiroketal substituent of PnTX-A and PnTX-G binds between the loop C and the complementary side of the subunit interface, as observed for 13-desmethyl SPX-C and for the tetrahydrofuran group of GYM-A. The cyclohexene-butyrolactone of 13-desmethyl SPX-C interacts with Y188 and K143, while in the case of GYM-A, it interacts with Y188 and Q186. In the case of the two latter toxins, very limited interaction is observed with the sequence-variable loop F. On the contrary, the bridged ketal ring specific to PnTXs extends out of the binding pocket underneath loop C to interact with loop F on the negative side of the interface. Specific interactions between the charged carboxylate group of PnTX-A and the vinyl group of PnTX-G, may explain the selectivity towards  $ha7$  and *Torpedo*- $(\alpha1)_2\beta1\gamma\delta$  nAChRs (Bourne *et al.*, 2015).

### 20-methyl SPX-G antagonizes rat $\alpha3\beta2$ nAChR

The  $\alpha3$  nAChR subunit was the first to be cloned and the  $\alpha3\beta2$  nAChR subtype was among the first subtypes to be heterologously expressed in *X. laevis* oocytes for electrophysiology

(Boulter *et al.*, 1987).  $\alpha3\beta2$  nAChR is expressed in the CNS, the spinal cord and the heart, and as presynaptic receptor in the neuromuscular junction (Young *et al.*, 2008; Fagerlund and Eriksson, 2009). The MOA of 20-methyl SPX-G (CIFGA, Spain) on  $\alpha3\beta2$  nAChRs determined by TEVC is here shown.

The mRNAs coding for the rat  $\alpha3$  and  $\beta2$  nAChR subtypes were synthesized from the plasmids PKN $\Omega\alpha3$  and PKN $\Omega\beta2$  kindly provided by Annette Nicke (LMU, Munich, Germany), and injected into *X. laevis* oocytes (Fig. 1A-B). TEVC electrophysiology was performed as described in (Aráoz *et al.*, 2011) (Fig. 1C). The dose-response curve for ACh gave an  $EC_{50}$  of 250  $\mu$ M ( $nH = 0.945 \pm 0.15$ ; Fig. 1D). The perfusion of 250  $\mu$ M ACh induced a rapid opening of the  $\alpha3\beta2$  nAChR's channel (microseconds) followed by a two-phase desensitization pattern: a very fast (milliseconds) after the peak amplitude was reached and a slow desensitization phase (Fig. 1E). The perfusion of a mix containing 250  $\mu$ M ACh and 20-methyl SPX-G (Figs. 1E, F) at a given concentration in the range of 100 pM – 1  $\mu$ M induced a competitive blockade of the ACh-evoked currents with an  $IC_{50}$  of 50.9 nM ( $nH = -0.98 \pm 0.14$  Fig. 1E, 1G), without affecting the desensitization pattern of the receptor. 20-methyl SPX-G is not an agonist of  $\alpha3\beta2$  nAChR.

20-methyl SPX-G showed picomolar affinities for *Torpedo*- $(\alpha1)_2\beta1\gamma\delta$  nAChR ( $IC_{50} = 360$  pM) and for  $ha7$  nAChR ( $IC_{50} = 480$  pM). Its affinity for the neuronal  $ha4\beta2$  nAChR was 2.1 nM (Fig. 1G) while for rat  $\alpha3\beta2$  nAChR was 50.9 nM. Unlike *Torpedo*- $(\alpha1)_2\beta1\gamma\delta$  and  $ha7$  subtypes, the  $\beta2$  containing  $\alpha4\beta2$  and  $\alpha3\beta2$  nAChRs are resistant to  $\alpha$ -bungarotoxin, but sensitive to kappa-



bungarotoxin (Boulter *et al.*, 1987). Molecular docking to predict the structural determinants within  $h\alpha 3\beta 2$  nAChR and 20-methyl SPX-G were performed (Couesnon *et al.*, 2016). Key amino acids explaining the antagonism of 20-methyl SPX-G towards  $h\alpha 3\beta 2$  and  $h\alpha 4\beta 2$  nAChRs are conserved in the orthosteric site. The lower affinity of both nAChR subtypes for 20-methyl SPX-G relies on the bulky K77 which provokes steric clashes displacing the spiroketal moiety of the toxin to the  $\beta 2$  subunit (Couesnon *et al.*, 2016). Further, the butyrolactone of 20-methyl SPX-G hydrogen bond with R186 of  $h\alpha 4\beta 2$  nAChR while it hydrogen bond with K143 of  $r\alpha 3\beta 2$  nAChR.

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