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Computational Determination of the Binding Mode of α-Conotoxin to Nicotinic Acetylcholine Receptor

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Abstract Conotoxins belong to the large families of disulfide-rich peptide toxins from cone snail venom, and can act on a broad spectrum of ion channels and receptors. They are classified into different subtypes based on their targets. The α-conotoxins selectively inhibit the current of the nicotinic acetylcholine receptors. Because of their unique selectivity towards distinct nAChR subtypes, α-conotoxins become valuable tools in nAChR study. In addition to the X-ray structures of α-conotoxins in complex with acetylcholine-binding protein, a homolog of the nAChR ligand-binding domain, the high-resolution crystal structures of the extracellular domain of the α1 and α9 subunits are also obtained. Such structures not only revealed the details of the configuration of nAChR, but also provided higher sequence identity templates for modeling the binding modes of α-conotoxins to nAChR. This mini-review summarizes recent modeling studies for the determination of the binding modes of α-conotoxins to nAChR. As there are not crystal structures of the nAChR in complex with conotoxins, computational modeling in combination of mutagenesis data is expected to reveal the molecular recognition mechanisms that govern the interactions between α-conotoxins and nAChR at molecular level. An accurate determination of the binding modes of α-conotoxins on AChRs allows rational design of α-conotoxin analogues with improved potency or selectivity to nAChRs.

Key words Nicotinic acetylcholine receptor; α-conotoxin; acetylcholine binding protein; docking; homology modeling; molecular dynamics simulation; mutational energy

1 Conotoxins

Conotoxins are disulfide rich peptide toxins from the venoms of cone snail acting extensively on ion channels and receptors (Craik and Adams, 2007; Terlau *et al.*, 2004). These peptides are small and highly constrained, with 10–40 residues and up to five disulfide bonds (Terlau *et al.*, 2004; Adams *et al.*, 1999; Lewis *et al.*, 2012). Most conotoxins discovered so far contain two or three disulfide bonds, and have a wide range of targets, including calcium channel, sodium channel, nAChR, noradrenaline transporter, NMDA receptor, and neurotensin receptor (Lewis *et al.*, 2004). Conotoxins are classified into different subtypes, such as α, δ, κ, μ, and ω conotoxins, based on their target receptors. The therapeutic potential of conotoxins is currently focused on the treatment of pain.

α-Conotoxins (α-CTXs) specifically target nAChR subtypes, and mainly act as antagonist potently inhibiting the opening of the channels (Terlau *et al.*, 2004; Halai and Craik, 2009). Most of the α-CTXs have similar three-dimensional (3D) structure, containing a small helical fragment restrained by two disulfide bonds (Fig.1). α-CTXs have a characteristic framework of CC-X_m-C-X_n-C, where the four cysteines can yield three possible isomers: ribbon (I–IV, II–III), beads (I–II, III–IV) and globular (I–III, II–IV) (Millard *et al.*, 2004). The latter is the most common conformation with potent biological activity (Janes *et al.*, 2005). The number of amino acids presented within the two loops (m, n) of α-conotoxins is the basis for dividing α-CTXs into several subgroups (m/n: 4/7, 4/6, 4/5, 3/5, 4/4 and 4/3) (Fig.1). In general, α-CTXs of the 3/5 class are isolated from fish-hunting snails and show activity toward fish and/or mammalian neuromuscular nAChRs, while conotoxins from the 4/7, 4/6, 4/5, 4/4 or 4/3 classes mainly interact with mammalian neuronal nAChRs (Terlau *et al.*, 2004). Up to now, the most commonly reported framework is 4/7 subgroup. Within this subgroup, one of the most investigated α-CTXs is called Vc1.1, which potently inhibits the α9α10 nAChR (Livett *et al.*, 2006; Vincler *et al.*, 2006). α-CTXs PnIA and TxIA also belong to 4/7 subtype, while the former potently blocks α7 nAChR and the latter selectively inhibits α3β2 nAChR subtype (Hopping *et al.*, 2014; Dutertre *et al.*, 2007). Other α-CTXs like ImI and BuIA belong to 4/3 and 4/4 subgroups, respectively, and they potently inhibit the α7 nAChR and α3β2 nAChR, respec-

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tively (Chi *et al*., 2006; Johnson *et al*., 1995). Because of their exquisite specificity on nAChR subtypes, they are used as neurochemical tools to investigate the functional differences among the nAChR subtypes.

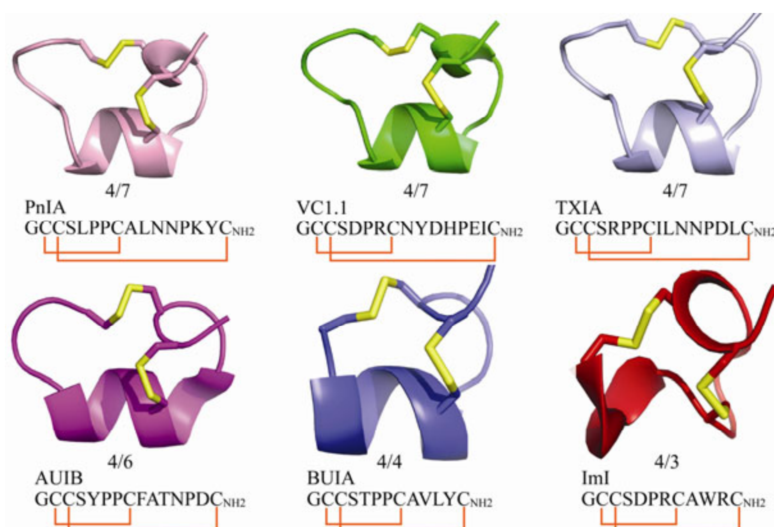

Fig.1 Structures and sequences of α-CTXs. The globular form of the 4/7, 4/6, 4/4, and 4/3 α-CTXs contain a rigid helix structure sustained by two disulfide-bonds (yellow).

2 nAChR

Nicotinic acetylcholine receptors (nAChRs) belong to the Cys-loop superfamily of ligand-gated ion channels (LGICs) that rapidly mediate synaptic signal transduction throughout the nervous system (Taly *et al*., 2009). The nAChRs are pentameric ion selective channels and exist as different subtypes via variation of the subunits in the pentamer. Up to now, different subunits of the nAChR have been identified, including α1−10, β1−4, γ, δ, or ε subunits (Gotti *et al*., 2007). The nAChRs are homo- or heteropentamers formed from either single or different types of homologous subunits. Each subunit has an extracellular domain (ECD) which contains a conserved Cys loop and a ligand binding domain (LBD), a transmembrane domain (TMD), and an intracellular domain [ICD] (Fig.2) (Unwin *et al*., 2005). The ligand binding site of the nAChR lies between two adjacent subunits of the ECD.

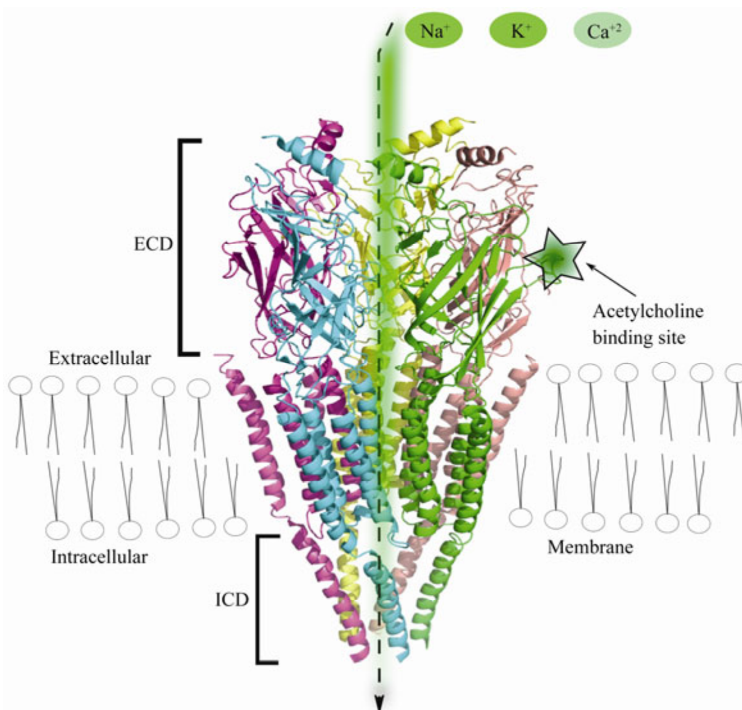

Fig.2 The structure of the *Torpedo* nicotinic acetylcholine receptor. The nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels. Their structure consists of a ligand binding domain, a transmembrane domain, and an intracellular domain. nAChRs are permeable to Na^+, K^+, and Ca^{2+}. The opening of the channel is triggered by binding small agonists, such as acetylcholine and nicotine. One of the acetylcholine binding sites is indicated with a star.

The binding of the agonist at the subunit interfaces triggers a conformational perturbation that is transmitted to the TMD where the channel gate is opened (Sine and Engel, 2006). The abnormal opening and closing of these receptors can initiate different neurophysiological diseases, including Parkinson's disease, Alzheimer's disease, schizophrenia, neuropathic pain, memory loss, and stress (Taly *et al*., 2009; Sine and Engel, 2006; Colomer *et al*., 2010; Lester *et al*., 2004; Arneric *et al*., 2007; Levin and Rezvani, 2007; Romanelli *et al*., 2007). Because of their broad involvement of neurophysiological diseases or disorders, nAChRs have become important targets for drug design.

3 Computational Modeling the Binding Modes of α-Conotoxins

Up to now, several breakthroughs were made for studying the structure of nAChR, and they were prerequisite for computational modeling of the nAChRs. The first crystal structure of the acetylcholine binding protein (AChBP), a homologue of the extracellular domain of the acetylcholine receptor, was determined by Brejc and colleagues in 2001 (Brejc *et al*., 2001). The AChBP was used directly as surrogate to study the interactions between nAChR and α-CTXs, and also as template for homology modeling of the nAChR due to its high structural similarity to the ECD of nAChR (Sine and Engel, 2006; Karlin *et al*., 2002; Sine *et al*., 2002; Smit *et al*., 2001). Sebstian *et al*. first built the homology model of the α7 nAChR using AChBP as template, and investigated the binding modes of the α-CTX ImI to the α7 nAChR (Dutertre *et al*., 2004). The computational docking of ImI into the homology model of neuronal nAChR provided structure activity relationship (SAR) of ImI with regards to the α7 nAChR subtype, and discovered a discrete binding site above loop C. In their model, the toxin entered into the acetylcholine (ACh) pocket, placing the triad D5-P6-R7 in van der Waals contacts with (+)Y193 and (+)W147, while the C-terminus made contacts with the (–) face of α7 nAChR, bridging the two subunits.

The crystal structures of the complexes between AChBP and α-conotoxins (Fig.3), such as ImI, PnIA variant, and TxIA variant, showed more details on the pairwise interactions between α-conotoxins and nAChRs (Dutertre *et al*., 2007; Ulens *et al*., 2006; Celie *et al*., 2005). Surprisingly, in the crystal structure of ImI bound with AChBP, the center of ImI was discovered to be deeply embedded into the binding pocket of acetylcholine (ACh) rather than to the discrete binding site above Loop C. The discrepancy between the computationally predicted binding mode and the X-ray crystallography determined binding mode was originated from the inappropriate conformation of the loop C of the homology model, as well as the incapacity of molecular docking for management of the flexibility of the proteins. Indeed, Pérez *et al*. failed to dock α-conotoxin RgIA into the Ach binding pocket of their homology model, as a consequence of not considering the flexibility of the proteins (Pérez *et al*., 2009). Therefore, instead of using molecular docking they directly built the complex model of α9α10 nAChR/RgIA using the crystal structure of AChBP/ImI as the template, and then refined their model using molecular dynamics simulations. In MD simulations, Arg7 of RgIA formed three interactions, an intra-residue salt-bridge with Asp5, an inter-residue salt-bridge with α9(+) Asp198, and a hydrogen bond with the carbonyl group of Pro197 in the α9(+) subunit.

Unwin and colleagues determined the electron cryo microscopy structure of the *Torpedo* nAChR in unliganded closed state (4 Å resolution), and first gave people the whole picture of the structure of nAChR (Unwin *et al*., 2005; Miyazawa *et al*., 1999). Afterwards, the crystal structures of the extracellular domain of the α1 and the α9 subunits appeared, respectively (Dellisanti *et al*., 2007; Zouridakis *et al*., 2014). These crystal structures showed more details of the configuration of nAChR possessing higher sequence identity to nAChR than to AChBP, and therefore they were used in combination with AChBP as templates for homology modeling of the nAChR pentamers. Yu and colleagues rebuilt the homology model of the α7 nAChR/ImI using both the crystal structure of AChBP/ImI (PDB ID: 2c9t) and crystal structure of α1 subunit (PDB ID: 2qc1). Their model was further refined using MD simulations for incorporation of the flexibility of the proteins and the peptide (Yu *et al*., 2011). Their model not only qualitatively explained pairwise interactions from the mutagenesis experiment, but also gave excellent correlation between mutational energies calculated from their model and mutational energies derived from the experiment. In molecular dynamics simulations, the Loop C of the α7-nAChR in complex with ImI was stabilized in an open conformation, and the side chains between α7-Y195 and ImI-R7 were tightly packed, which well explained the cation-pi interactions observed in previous experimental study (Quiram *et al*., 1999).

The α9α10 nAChR was first identified in the auditory system where it mediates synaptic transmission between efferent olivocochlear cholinergic fibers and cochlea hair cells (Elgoyhen *et al*., 2009). This receptor gained further attention for its potential role in chronic pain and breast and lung cancers (Quiram *et al*., 1999). The α-CTX RgIA, one of a few α9α10 selective ligands identified to date, is 300-fold less potent on human versus rat α9α10 nAChR (Azam *et al*., 2015). This species difference was conferred by only one residue in the complementary subunit (-), rather than at the principal (+) subunit. In light of this unexpected discovery, other residues that interact with α-CTX RgIA were determined through computational modeling approaches. Based on the crystal structures of one subunit of the extracellular domain (ECD) of human α9 nAChR and AChBP homopentamer in complex with ImI (PDB ID: 2c9t), the ECD of rat α9α10 nAChR and its complexes with α-CTX RgIA and acetylcholine were rebuilt (Azam et al., 2015). The computational modeling data supported the interaction of α-CTX RgIA at the α10(+)/α9(-) rather than the α9(+)/α10(-) nAChR subunit interface, and the results are expected to facilitate the

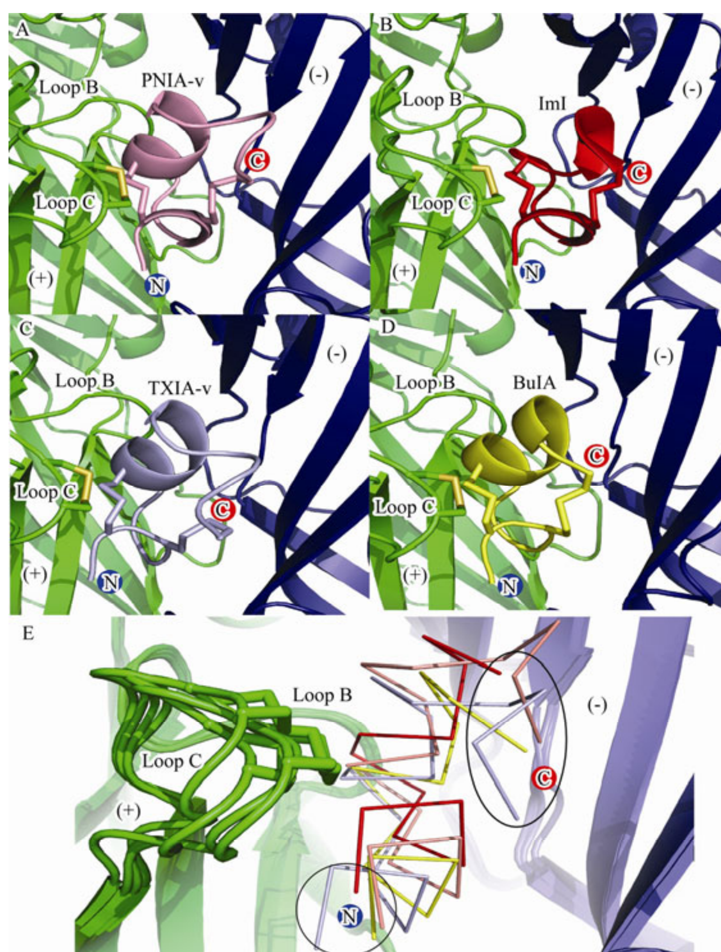

Fig.3 Crystal structures of the AChBP bound with (A) PNIA variant in pink (PDB ID: 2br8), (B) ImI in red (PDB ID: 2c9t), (C) TXIA variant in light blue (PDB ID: 2uz6), and (D) BuIA in yellow (PDB ID: 4ez1). (E), overlay of the crystal structures of AChBP bound with PNIA variant, ImI, TXIA variant, and BuIA. The N-termini and C-termini of the α-conotoxins are labeled in 'N' and 'C', respectively. The principal subunit '(+)' and the complementary subunit '(-)' of the binding pocket are shown in green and light blue color, respectively.

Table 1 Summary of computational modeling studies on the binding modes of the α-CTXs

Protocol	System	Key point	Reference
Homology modeling, MD, and MM-PBSA	Vc1.1 with α9α10 nAChR	Explained mutational data and designed more potent Vc1.1 mutants	Yu *et al.*, 2011
Homology modeling and MD	LvIA with α9α10 nAChR	Explained the selectivity of α-CTX LvIA to α3β2 vs α6/α3β2β3nAChR	Luo *et al.*, 2014
Homology modeling, docking, and energy minimization	AuIB with $(\alpha3)_3(\beta4)_2$ nAChR	Explained the binding modes of two isomers (ribbon and globular)	Grishin *et al.*, 2010
Homology modeling and docking	LtIA and MII with α3β2n AChR	Explained the inhibition mechanism of α-CTX LtIA to α3β2 nAChR	Luo *et al.*, 2010
Homology modeling and MD	AuIB, [F9A]AuIB and [F9Y] AuIB with $(\alpha3)_2(\beta4)_3$ nAChR	Identified critical residues that mediate interactions between AuIB and its cognate nAChR subtype	Grishin *et al.*, 2013
Homology Modeling, MD and docking	TxIA with α4β2 nAChR	Identified nAChR residues that control access of the α-conotoxins to its binding site	Beissner *et al.*, 2012
Homology modeling, energy minimization, and docking	PnIA with α7 and α3β2 nAChRs	Determined molecular determinants that conferred the selectivity of PnIA to α7 vs α3β2 nAChR	Hopping *et al.*, 2014
Homology modeling and docking	MII with α3β2 nAChR	Explained the antagonist properties of the α-CTX MIIs on nAChRs.	Somisetti *et al.*, 2014
Homology modeling, MD, and docking	GIC with α3β2 and α3β4 nAChRs	Explained discrimination ratio of α-CTX GIC to α3β2 vs α3β4	Lee *et al.*, 2012
Homology modeling and MD	MII and PIA/$(\alpha6)_2(\beta2)_3$ nAChR	Identified E11 as a key residue of α-CTX MII that confers specificity to α6β2 vs α3β2	Pucci *et al.*, 2011

development of selective ligands with therapeutic potential. Luo and colleagues built the homology model of LvIA/α9α10nAChR using the model of Vc1.1/α9α10 nAChR and the NMR solution structure of LvIA. Based

on this model, they proposed the molecular basis that conferred the specificity of LvIA and other CTXs with high sequence similarity to LvIA. Docking study has shown that Arg7 in loop I and Tyr10 in loop II of Vc1.1 are deeply buried at the binding interface of α9α10 nAChR (Yu *et al*., 2013), and in LvIA both of these positions are occupied by hydrophobic residues, Ala7 and Val10, respectively. Experimental result has shown that Arg7 is essential for Vc1.1 activity at the α9α10 nAChR (Halai and Craik, 2009). In the Vc1.1/α9α10 nAChR model, Yu *et al*. (2013) has shown that the hydrogen bond network formed by the guanidinium group of Arg7 is essential for the binding affinity of Vc1.1, whereas this network cannot be established by the corresponding Ala7 of LvIA. The LvIA/α3β2n AChR model shows that the negatively charged Asp11 is buried by Asp151, Lys154, and Glu194 of the α3 subunit, as well as Lys78 and Arg80 of the β2 subunit. Globally electropositive environment was formed due to this cluster of residues, which is favorable for an interaction with Asp11 (Nevin *et al*., 2007). Overall, computational modeling has become a necessary step for the experimentalists not only for the explanation of their experimental data, but also for the design of novel α-CTX analogues (Table 1).

4 Prospects

Accurate determination of the binding modes of α-conotoxins to nAChR is essential for computer aided drug design, as well as for understanding the interacting mechanism between α-conotoxin and nAChR. Crystal structures of the nAChR in complex with α-conotoxin are still out of the reach of the current technology. Herein, computational modeling in combination of mutagenesis data is the major technology to obtain reliable models of the nAChR bound with α-conotoxin (Fig.4).

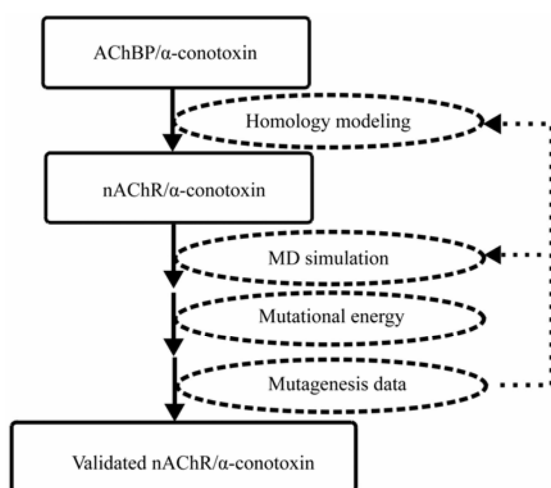

Fig.4 Proposed flow chart for determination of the binding modes of α-conotoxin to nAChR based on computational methods.

With the high resolution templates possessing higher sequence identity to the target protein, building more accurate homology models of the nAChR has become possible. In addition, the number of crystal structures of the AChBP bound with different types of α-conotoxins in the protein data bank is increasing, which gives rise to directly modeling the binding modes of these toxins to nAChRs. Interestingly, the crystal structures of AChBP in complex with α-conotoxins reveal that the binding modes of different types of α-conotoxins are quite similar, with the N-termini and the C-termini oriented at the base and the top of the binding pocket, respectively, and with the helix fragment located at the inner of the pocket, despite of their different disulfide framework (Fig.3). This binding modes similarity adds the confidence to model the binding modes of α-conotoxins to nAChR using AChBP/α-conotoxins as templates. After obtaining the initial models from homology modeling, they are further subject to MD refinement for considering the flexibility of the proteins, and the removal of the van der Waals clashes between side chains of the toxin and the nAChR. The accuracy of these MD refined models can be evaluated by analyzing the correlation between mutational energies calculated from computational modeling and mutational energies derived from mutagenesis data, considering a reliable model often gives good correlation. Here, MM/PBSA methodology is suggested for the calculation of the mutational energies due to their economical computational costs and relatively high accuracy (Yu *et al*., 2011). The accuracy of the MM/PBSA method is usually discounted by the insufficient sampling of the conformation of the binding site in plain MD. For rigorous calculation of the binding free energies of α-conotoxin to nAChR, more robust computational sampling methods, like umbrella sampling and steered MD simulations, are suggested for the binding energies calculation in future studies (Northrup *et al*., 1982; Klimov *et al*., 2000). It has to be noted that the backbone of either the N-termini or the C-termini of different α-conotoxins significantly deviate from each other (Fig.3E), highlighting the importance of performing MD or even more powerful conformation sampling methods, such as metadynamics simulations and Monte Carlo simulations, to refine the binding modes of the α-conotoxins.

5 Conclusion

α-Conotoxins are a major class of conotoxins that specifically and potently inhibit the nAChR. They can be used to develop drugs for pain inhibition, or as neurochemical tools for nAChRs study. Up to now, the crystal structure of the nAChR in complex with α-conotoxin is still unknown. Computational modeling in combination of mutagenesis data might fill this gap. The number of the crystal structures of AChBP bound with different α-conotoxins is increasing in the protein data bank, and these structures reveal that different α-conotoxins can have similar binding modes, which forms the basis for modeling the binding modes of α-conotoxins using homology modeling methods. However, it has to be noted that for α-conotoxins with distinctly different binding orientations from the known α-conotoxins, more com-

prehensive computational methods, including docking, MD simulations, and free energy calculations are required to determine their binding modes to the nAChRs. Accurate determination of the binding modes of the α-conotoxins is a prerequisite for rational modification of the α-conotoxins for therapeutic purposes.

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