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The *Ascaris suum* nicotinic receptor, ACR-16, as a drug target: Four novel negative allosteric modulators from virtual screening

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ABSTRACT

Soil-transmitted helminth infections in humans and livestock cause significant debility, reduced productivity and economic losses globally. There are a limited number of effective anthelmintic drugs available for treating helminths infections, and their frequent use has led to the development of resistance in many parasite species. There is an urgent need for novel therapeutic drugs for treating these parasites. We have chosen the ACR-16 nicotinic acetylcholine receptor of *Ascaris suum* (Asu-ACR-16), as a drug target and have developed three-dimensional models of this transmembrane protein receptor to facilitate the search for new bioactive compounds. Using the human α7 nAChR chimeras and *Torpedo marmorata* nAChR for homology modeling, we defined orthosteric and allosteric binding sites on the Asu-ACR-16 receptor for virtual screening. We identified four ligands that bind to sites on Asu-ACR-16 and tested their activity using electrophysiological recording from Asu-ACR-16 receptors expressed in *Xenopus* oocytes. The four ligands were acetylcholine inhibitors (SB-277011-A, IC_{50}, 3.12 ± 1.29 μM; (+)-butaclamol Cl, IC_{50}, 9.85 ± 2.37 μM; fmoc-1, IC_{50}, 10.00 ± 1.38 μM; fmoc-2, IC_{50}, 16.67 ± 1.95 μM) that behaved like negative allosteric modulators. Our work illustrates a structure-based in silico screening method for seeking anthelmintic hits, which can then be tested electrophysiologically for further characterization.

1. Introduction

Soil-transmitted gastrointestinal nematodes, namely roundworms, whipworms and hookworms, infect approximately two billion people worldwide and pose a significant health challenge to humans and animals (de Silva et al., 2003; Bethony et al., 2006). The infections with the soil-transmitted helminths can cause malnutrition, iron-deficiency anemia and impaired cognitive performance (Crompton, 2000; Hotez et al., 2007). Currently, there are no effective vaccines available (Hewitson and Maizels, 2014), and sanitation is not adequate in many countries. The World Health Organization (WHO) recommends four anthelmintics for treatment and prophylaxis of soil-transmitted nematode infections: albendazole, mebendazole, levamisole and pyrantel (Keiser and Utzinger, 2008). The repeated use of a limited number of anthelmintic drugs has led to an increase in drug resistance in animals and there are similar concerns for humans. It is therefore important to identify novel therapeutic compounds that selectively target receptors of parasitic nematodes so that we maintain effective therapeutics.

The nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels that mediate synaptic transmission at neuromuscular junctions of vertebrates and invertebrates (Changeux and Edelstein, 1998). The neurotransmitter, acetylcholine, activates nAChRs by binding to orthosteric binding sites on the extracellular domain of the receptor and triggers the opening of the channel pore in the transmembrane domain. The opening of the nicotinic receptors leads to an influx of sodium and calcium depending on the receptor subtypes, as well as an output of potassium ions, followed by membrane depolarization and muscle contraction.

Nicotinic anthelmintics are selective agonists of nematode

Abbreviations: ECD, extracellular domain; TID, transmembrane and intracellular domain; (+), principal subunit; (−), complementary subunit; NAM, negative allosteric modulator; nAChR, nicotinic acetylcholine receptor; AChBP, acetylcholine-binding protein.

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muscle nAChRs which cause spastic paralysis of the parasites (Martin and Robertson, 2010; Buxton et al., 2014). There are three different pharmacological subtypes of nAChRs present on muscle of *Ascaris suum*. The anthelmintics, levamisole and pyrantel are selective agonists of L-subtypes of nAChRs in *A. suum* (Martin et al., 2012). Bephenium selectively activates B-subtypes of nAChRs. Nicotine and oxantel selectively activate N-subtypes of nAChRs in *A. suum* (Qian et al., 2006). The anthelmintic monepantel activates nAChRs which are composed of DEG-3-like subunits (*Haemonchus. contortus* MPTL-1, *Caenorhabditis. elegans* ACR-20 and *H. contortus* ACR-23 subunits (Rufener et al., 2010; Buxton et al., 2014). We have selected the N-subtype of nAChR that is composed of ACR-16 subunits (Ballivet et al., 1996; Polli et al., 2015) for a drug target, because it is pharmacologically different to the other nicotinic receptor subtypes (Raymond et al., 2000), for further study. Asu-ACR-16 transcript has been found in *A. suum* muscle and may be involved in locomotion.

The ACR-16 nicotinic acetylcholine receptor of *A. suum* (Asu-ACR-16) is a homomeric receptor made up of five identical α subunits. Homomeric nAChRs have five identical orthosteric binding sites where agonists and competitive antagonists bind at the interface of two adjacent subunits. The orthosteric site is in the extracellular domain and is formed by the loops A, B & C of the principal subunit and by the loops D, E & F on the complementary subunit (Galzi et al., 1991; Arias, 2000). In addition, three allosteric binding sites close to the orthosteric binding sites in the extracellular domain have been observed in the α7 nAChR-AChBP chimera (Spurny et al., 2015). In the transmembrane domain, an intrasubunit allosteric binding site has been found in *Rattus. norvegicus* α7 nAChR (Young et al., 2008), while an intersubunit allosteric binding site has been found in C. elegans glutamate-gated chloride channel (GluCl) (Hibbs and Gouaux, 2011). These well-studied binding sites in nAChRs or other Cys-loop receptors provided our framework for characterizing putative orthosteric and allosteric sites in Asu-ACR-16.

Because of the lack of a crystal structure for Asu-ACR-16, we used homology modeling to predict the protein structure, based on the observations that proteins with similar sequences usually have similar structures (Cavasotto and Phatak, 2009). In this study, we used homology modeling to predict the three-dimensional structure of Asu-ACR-16, based on the observed experimental structures of the human α7 nAChR chimeras and the *Torpedo marmorata* nAChR as templates. Virtual screening was performed for the ACR-16 orthosteric binding sites, using the predicted structure to identify the potential candidates of agonists and competitive antagonists. Allosteric binding sites were also used to examine the binding properties of the virtual screening hits. Subsequently, we tested the pharmacological profiles of virtual screening hits on Asu-ACR-16 receptors expressed in *Xenopus laevis* oocytes, using a two-electrode voltage clamp to test the activity of the hits on the receptors.

2. Materials and methods

2.1. Identification of template structures

We selected the extracellular domain of Asu-ACR-16 (ECD-Asu-ACR-16) because it forms a homologomer that allows homology modeling. In addition, many of the agonists that activate Asu-ACR-16, acetylcholine, nicotine, cytisine, epibatidine (Abongwa et al., 2016), are also known to bind to the orthosteric binding sites of extracellular domain of *Lymnaea stagnalis* AChBP or *A. californica* AChBP (Celie et al., 2004; Li et al., 2011; Rucktooa et al., 2012; Olsen et al., 2014a). In addition to the orthosteric binding site, three separate allosteric binding sites in the extracellular domain of α7 nAChR are now recognized (Bertrand et al., 2008; Pan et al., 2012; Spurny et al., 2015), increasing the possibility of identifying allosteric modulators.

The amino acid sequence of Asu-ACR-16 (Fig. 1) was obtained from the UniProtKB/SwissProt database with the accession number F1KYJ9 (Wang et al., 2011). Structural templates were identified by using BLASTP on NCBI network service (Altschul et al., 1997) and PSI-BLAST on the ProtMod server (Rychlewski et al., 2000) by searching in the Protein Data Bank (Berman et al., 2000). Three crystal structures of human α7 nAChR chimeras with different co−crystal ligands in orthosteric binding site were used: epibatidine bound (PDB code: 3SQ6; Li et al., 2011), no ligand (PDB code: 3SQ9; Li et al., 2011), and α-bungarotoxin bound (PDB code: 4HQP; Huang et al., 2013). These structures were selected as the templates for three different bound-forms of the ECD-Asu-ACR-16. The three models were: the agonist-bound form ECD-Asu-ACR-16; the apo form ECD-Asu-ACR-16 and; the antagonist-bound form ECD-Asu-ACR-16 (Fig. 2A).

We modeled the transmembrane and intracellular domains of Asu-ACR-16 (TID-Asu-ACR-16, Fig. 2B) because of the presence of an intrasubunit allosteric binding site that is found in α7 nAChR and an intersubunit allosteric binding site that is demonstrated in a Cys-loop receptor, GluCl crystal structure in complex with ivermectin (Young et al., 2008; Bertrand et al., 2008; Hibbs and Gouaux, 2011). Ivermectin is a known allosteric modulator of α7 nAChRs (Krause et al., 1998). The *T. marmorata* nAChR (PDB code: 2BG9 chain A; Unwin, 2005) is the only pentameric nAChR structure with the transmembrane domains and partial intracellular domains determined. Therefore, the transmembrane and intracellular domains of *T. marmorata* nAChR (TID-Tma-nAChR) were selected as the template for our TID-Asu-ACR-16 model.

The sequence of the ECD-Asu-ACR-16 and the human α7 nAChR chimera (SwissProt ID: P36544; Peng et al., 1994) were aligned using CLUSTALW multiple alignment (Thompson et al., 1994). The sequence of the TID-Asu-ACR-16 and TID-Tma-nAChR (SwissProt ID: P02711; Devillers-Thiery et al., 1983, 1984) were aligned using CLUSTALW.

2.2. Homology modeling of Asu-ACR-16

We used Modeller (Eswar et al., 2007) to build a three-dimensional model of ECD-Asu-ACR-16 and used JACKAL (http://wiki.c2b2.columbia.edu/honiglab_public/index.php/Software:Jackal) to build the model of TID-Asu-ACR-16 for each of the five subunits. These five subunits were then assembled to generate the pentamer using COOT software (Emsley and Cowtan, 2004). The model geometry was first refined manually, and then optimized by PHENIX software (Adams et al., 2010). Each of the TID-Asu-ACR-16 subunits were then merged into the ECD-Asu-ACR-16 model by using COOT to edit and alter the C_α coordinates of residues around the outer membrane regions. The final optimized pentameric model was then visualized using the program PyMol (The PyMOL Molecular Graphics System, Version 1.7.4, Schrödinger, and LLC., Figs. 2C & S1).

2.3. Structure-based virtual screening

Smiles strings of ligands were downloaded from the lead-like subset of commercially available compounds in the ZINC Database (Irwin et al., 2012) and were converted initially to PDB formats using the PHENIX-eLBOW program (Moriarty et al., 2009). The ligand and receptor input files were then prepared in PDBQT format for AutoDock Vina by using the AutoDock Tools package (Morris et al., 2009). For initial screening, a docking area was defined visually around the orthosteric binding site of ECD-Asu-ACR-16