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Study on the Binding Interaction of Three-finger Toxins From Cobras And Mangrove Catsnake Toward Nicotinic Acetylcholine Receptors: A Computational Approach

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ABSTRACT

Introduction: Snake venom is a combination of various proteins and peptides that cause diverse biological effects in multiple organ systems. Toxins from three-finger toxin family are the mains toxins in elapid venom. Although these toxins share similarities in their structure, they are known to cause a myriad of toxic actions such as neurotoxicity, cardiotoxicity, and cytotoxicity. Unfortunately, many of these toxins are not fully pharmacologically characterized, especially on their binding affinity and selectivity towards receptors and their effects to different organ systems.

Method: This work compared the binding properties of selected three-finger toxins (3FTxs) from cobras (*Naja sumatrana* and *Naja kaouthia*) and mangrove catsnake (*Boiga dendrophila*) towards human and bird nicotinic acetylcholine receptors ($\alpha_3\beta_2$, $\alpha_4\beta_2$, α_7) using computational approaches. The sequence of the selected toxins were obtained from public database e.g UniProt and NCBI. The structure of the toxins without deposited structure were modelled using homology modelling.

Results: The results show that all toxins bind to the orthosteric site, which is located outside the extracellular domain of α subunit for all receptors in both species. Interaction between receptors and toxins occurs by the formation of hydrogen bond, ionic bond, and hydrophobic contact with important residues involved in their binding pocket.

Conclusion: Based on the data, the toxins showed different binding affinities towards nicotinic acetylcholine receptors in different species. The differences could have a significant impact on the functional characterization of venom caused by these toxins and toxins with nearly similar sequences.

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Introduction

Nicotinic acetylcholine receptor are pentameric ligand-gated ion channels and consist of five of different subunits (H. Wang et al., 2019). It can be further classified into muscle nicotinic acetylcholine receptor and neuronal nicotinic acetylcholine receptor depending on the subunit configuration and location (Lagoumintzis et al., 2021). These receptors play important roles in regulating various physiological activities and important target in multiple treatments pain, neurodegenerative and psychiatric disorders (Deba et al., 2018). Futhermore, nicotinic acetylcholine receptors also an important target receptor for snake venom neurotoxins that lead to neuromuscular blockade and paralysis in snake envenoming (Ismail, 2015; Rusmili, Yee, Mustafa, Othman, & Hodgson, 2014; Tan, Tan, Sim, Fung, & Tan, 2016).

Snake venom contains a complex mixture of toxins that exert a variety of activities (Kini & Koh, 2016).These toxins are classified into several super toxin families, namely three- finger toxins (3FTx), phospholipase A2, metalloproteases, serine proteases, cysteine- rich secretory proteins, L-amino acid oxidases, kunitz peptides, c-type lectins, disingtegrins and natriuretic peptides (Tasoulis & Isbister, 2017). Venomic profilling of Naja sumatrana and Naja kaouthia showed 3FTx and PLA2 are two main components in the venoms and have significant role in exerting venom toxic effects (Tan, Tan, Fung, & Tan, 2015; M. K. K. Yap, Tan, Sim, Fung, & Tan, 2014). 3FTxs are non-enzymatic proteins with 60-74 amino acids residues, low molecular weight (<10 kDa), and exert various effects by targeting different receptors and ion channels with high specificity (Kini & Doley, 2010; Roly, Islam, & Reza, 2014). 3FTxs are classified into four main subtypes i.e. short chain neurotoxins, long chain neurotoxins, non-conventional toxins and cardiotoxins or cytotoxins (Tan et al., 2015). Short and long chain neurotoxins bind and block nicotinic acetylcholine receptor and differ in their selectivity and affinity towards different subtypes of the receptor (de la Rosa, Corrales-García, Rodriguez-Ruiz, López-Vera, & Corzo, 2018; de la Rosa, Pastor, Alagón, & Corzo, 2017). Non-conventional toxins can bind and block different nicotinic and muscarinic acetylcholine receptor subtypes (Tan, Tan, Chanhome, & Tan, 2017). Cardiotoxins causes distruption in the cell membrane, causing leakage and distrupting cellular integrity. However they do not have a known distinct receptor as their target (Shulepko et al., 2017).

The work attempt to elucidate the binding properties of selected three-finger toxins from *Naja sumatrana, Naja kaouthia* and *Boiga dendrophila* towards different nicotinic acetylcholine receptor subtypes from different species by using combination of computational techniques. These toxins were chosen based on function to serve as representative to their sub-group in 3FTxs family. This will provide insight into molecular properties of toxins such as their structure, binding affinity, and selectivity towards nicotinic acetylcholine receptor subtypes.

Methodology

Protein sequence alignment and homology modelling of target receptor

Proteins whose structures are available in Protein Data Bank were obtained: Human α_4 (PDB ID: 6CNK) and human β_2 (PDB ID: 5KXI). Amino acid sequence in FASTA for the target proteins without crystal structure were obtained from UniProt database with the following entry identifier: P32297 (human α_3), P36544 (human α_7), P09481 (chicken α_3), P09482 (chicken α_4), P22770 (chicken α_7) and P09484 (chicken β_2). The sequences were analyzed using BLAST (Basic Local Alignment Search Tool) to identify conserved residues related to target proteins. The sequences obtained were selected for further alignment using the following criteria: maximum identity >50%, model quality estimation (QMEAN) and global quality estimation score (GMQE) where higher numbers indicate higher reliability of the residues. Multiple sequences alignment was conducted using ClusterOmega at https://www.ebi.ac.uk/Tools/msa/clustalo/.

The homology models of the target proteins were modelled using Swiss Model (Biasini et al., 2014). Then hydrogen atoms were added to the models generated from Swiss Model using MolProbity. The stability of the homology models was validated by Ramachandran plot analysis in MolProbity. Then, the homology models of the receptor subunits were assembled using Pymol to form pentamer structures of $\alpha_3\beta_2$ and $\alpha_4\beta_2$. For the α_7 pentamer, it was assembled using Symmdock (Al-Refaei, Makki, & Ali, 2020). All the pentamer structures were energy-minimized using Chimera to remove bad clashes in prior to molecular docking procedure.

Preparation and homology modelling of toxin structure

A total of four 3FTxs were studied. The three-dimensional structures of α-cobratoxin (PDB ID: 1CTX), cobrotoxin-c (PDB ID: 1JE9) and denmotoxin (PDB ID: 2H5F) were retrieved from Protein Data Bank. There was no crystal structure available for cytotoxin 3, therefore the structure was homology modeled following the procedure used in preparing the target protein. Amino acid sequence in FASTA was obtained from UniProt with entry identifier P60302 from Naja sputarix which also known as Naja sumatrana. The stability of the best homology model was validated using Ramachandran plot.

Receptor- toxin docking

Extracellular pentamer of receptors were renumber before docking using WHAT IF webserver (https://swift.cmbi.umcn.nl/servers/html/renumb.html). The interface residues (active and passive) for target toxins and target pentamer nicotinic acetylcholine receptors ($\alpha_3\beta_2$, $\alpha_4\beta_2$ and α_7) were predicted by CPORT webserver (http://haddock.science.uu.nl/services/CPORT/). Molecular docking between all receptors and toxins were performed using HADDOCK webserver. Active and passive interface residues were identified and set as required in docking parameter setting. The results from HADDOCK include docked complexes, z-score, and binding score.

Binding interaction analysis

The generated model of complexes between receptor and toxin were visualized in three-dimensional (3D) using PyMOL. Hydrogen bond interaction and salt bridges interactions were analyzed using PDBePISA, while hydrophobic contact was analysed using Protein Interactions Calculator (PIC).

Results

Protein sequence alignment and homology modeling of target receptors.

Homology modelling was performed for receptor with no crystallized structure and amino acid sequence of the proteins were obtained from UniProt. The receptor structure templates were selected based on sequence identity, query coverage and QMEAN value from BLAST analysis (Table 1). In general, the protein with known structure having at least 30% sequence identity to that of the protein with known sequence is accepted as reliable template (Bienert et al., 2017; Kumar, Suresh, & Priya, 2015). For the query coverage, the higher the number, the greater the reliability of the template. As for the QMEAN values, the value around zero indicate good agreement between the template and the predicted model, while the value below than -4.00 indicate low quality between the template and predicted model (Biasini et al., 2014).

The result in Table 1 shows that the sequence identity similarities of all models to the template are ranging from 47.89% to 96.33%, while the query coverage are ranging from 54% to 68%. Both of these results indicate moderate to good reliability of the templates. For the QMEAN, the values are between -2.47 to -3.57, which suggests moderate agreement between the template and the predicted model. The best template for both species α_3 and α_7 receptors was obtained from chain A of the α subunit of human $\alpha_3\beta_2$ receptor (PDB ID: 6CNK) with resolution of 3.9 Å. For chicken receptor, the best template for α_3, α_4 and α_7 were obtained from chain A of the α subunit of the 6CNK structure while for the β_2 subunit, it was obtained from chain C of β subunit of the 6CNK structure.

Sequence alignment and secondary structure prediction for each target receptors with their selected template were done using ClusterOmega. Most of the amino acids were found identical when protein sequence of target receptors and selected templates were aligned (Figure 1). Consistent number of residues for human α_3, human α_7, chicken α_3, chicken α_4 and chicken α_7 subunit were obtained in the α subunit sequence of 6CNK structure. The fully conserved residues covered from L38 to G493 for human α_3, L29 to G485 for human α_7 and chicken α_7, L29 to G484 for chicken α_3 and L35 to G611 for chicken α_4. The strong similarity residues were found to be located from E35 to L494 in human α_3, Q26 to M486 in human α_7 and chicken α_7, E26 to L487 in chicken α_3 and E32 to L612 in chicken α_4. On the other hand, the residues with weak similarity were found to be located from S32 until V486 in human α_3, G23 until F476 in human α_7 and chicken α_7, S23 until V477 in chicken α_3 and A29 until V604 in chicken α_4. Based on the alignment result, most of the residues in chicken β_2 which located from T19 to K491 were fully conserved and strong similarity was seen from Y28 until T489 and weak similarity from T330 to G486.

Table 1: Target receptor BLAST analysis result

Protein	Accession	Template	Sequence identity (%)	Query coverage (%)	QMEAN
Human α_3	P32297	6cnk.1. A	66.86	68	-3.45
Human α_7	P36544	6cnk.1. A	47.89	66	-3.43
Chicken α_3	P09481	6cnk.1. A	66.77	68	-3.57
Chicken α_4	P09482	6cnk.1. A	93.41	54	-3.13
Chicken α_7	P22770	6cnk.1. A	48.48	65	-3.39
Chicken β_2	P09484	6cnk.1.C	96.33	67	-2.47

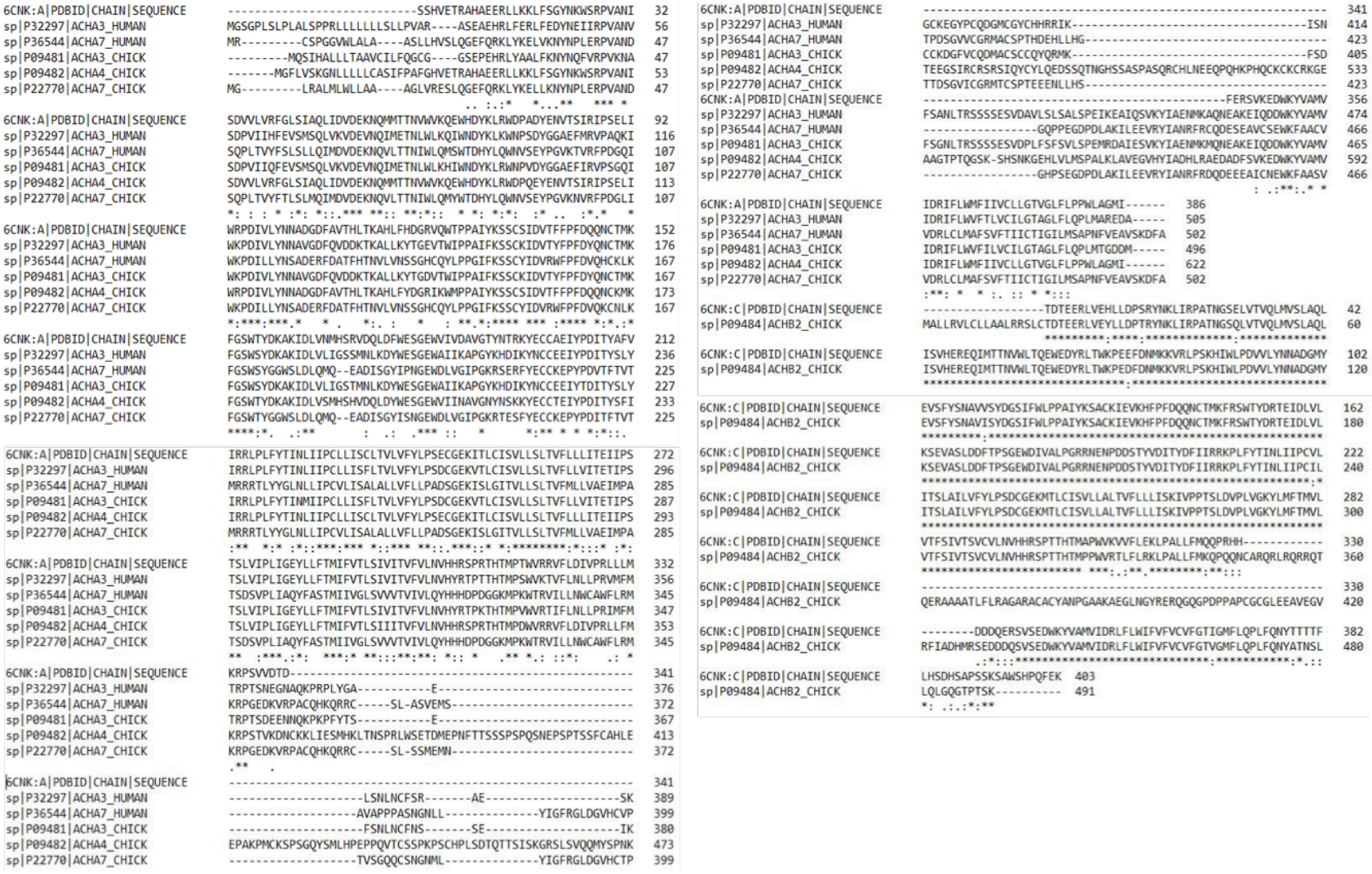

Figure 1: Sequence alignment result for human α_3, human α_7, chicken α_3, chicken α_4, chicken α_7 and chicken β_2 Asterisk (*) indicates positions which having a single and fully conserved residue. Colon (:) indicates conservation between groups of strongly similar properties. Period (.) indicates conservation between groups of weakly similar properties.

The predicted homology models indicated more than 90% of residues located in favored region and more than 97% residues were in the allowed region with range range from 95.36% to 96.45% and 99.4% to 100.0% respectively (Table 2). Predicted receptor subunit structures were assembled to form pentamer of human and chicken receptor (Figure 2). $(\alpha)_2(\beta)_3$ stoichiometry was chosen for $\alpha_3\beta_2$ and $\alpha_4\beta_2$ receptors based on its ability to bind with high affinity to acetylcholine and nicotine (Pandya & Yakel, 2011).

Table 2: Favored and allowed region from Ramanchandran plot for receptor homology models.

Protein	Ramachandran plot	
	Favored region (%)	Allowed region (%)
Human α_3	96.45	100
Human α_7	95.71	99.7
Chicken α_3	95.52	100
Chicken α_4	96.04	100
Chicken α_7	96.30	99.4
Chicken β_2	95.36	100

Cytotoxin-3 homology modelling

The best template for the homology model of cytotoxin-3 (Fig.3) was found to be the crystal structure of cardiotoxin A3 from Taiwan cobra (*Naja atra*) with 2.31Å resolution (PDB ID: 2BHI) based on BLAST analysis. The percentage of sequence identity between cytotoxin-3 and the template, cardiotoxin A3 was 100.0%, with the query coverage 68% and QMEAN value -0.19. The superimposition of cytotoxin-3 model with the template showed root mean square deviation (RMSD) value of 0.052 angstrom, based on the backbone carbon atom alignment calculation.

Toxin docking against human nicotinic acetylcholine receptors

All receptors were renumbered to ensure no repeated number in their amino acid sequence prior to docking. Active and passive interface residues responsible for the function of target proteins and located at the active site of the proteins of target toxins and target receptors were predicted using CPORT. Information on the active and passive residue was used for the docking process using HADDOCK. The four toxins were docked to the human and chicken nicotinic acetylcholine receptors ($\alpha_3\beta_2$, $\alpha_4\beta_2$ and α_7) and produced a total of 48 complex structures for each species. The complexes were ranked based on z-score, binding score and number of residues contacted (Table 3). All four toxins showed higher preference towards α subunit at the extracellular domain of $\alpha_3\beta_2$, $\alpha_4\beta_2$ receptors and α_7 receptor (Figure 4 and 5). However, the location of binding and the residues facilitating the binding of the toxin are slightly different.

Among the four toxins,denmotoxin was found to have the lowest z-score (-2.3) and binding energy (-45607.5 kcal/mol) when docked to the $\alpha_3\beta_2$ human receptor (Table 3). A total of 18 residues were involved in the denmotoxin binding interaction. The binding of denmotoxin was found to be facilitated by three types of interactions include hydrogen bond, salt bridge and hydrophobic contact (Figure 5a). The first two hydrogen bonds were observed beween carboxyl oxygen and carbonyl oxygen of Asp190 of human $\alpha_3\beta_2$ receptor with the guanidine group from Arg49 and amine group from Lys50 side chain of denmotoxin, respectively. The third hydrogen bond was formed between carbonyl oxygen from Asp134 of $\alpha_3\beta_2$ receptor and guanidine group from Arg60 side chain of denmotoxin, while the fourth hydrogen was seen between the amine from Lys132 of the receptor and backbone oxygen from Gly58 of denmotoxin. There were three hydrogen bonds observed between Ile134, Asp134 and Ile144 of the receptor and Arg60 of denmotoxin. The other hydrogen bonds were facilitated by Asp383, Tyr137 and Tyr208 of the receptor as the donor, while Asn54, Met66 and Ala106 of denmotoxin as the acceptors. A total of eight salt bridges were interaction predicted between human $\alpha_3\beta_2$ receptor and denmotoxin, which were seen involving Asp134 and Asp190 of the human $\alpha_3\beta_2$ receptor and Arg60, Arg49, His41 and Lys50 of denmotoxin. Hydrophobic contacts also occur between the aromatic ring from Tyr137 of receptor and aromatic from Pro40 of toxin.

For cytotoxin-3, it was predicted to bind to $\alpha_4\beta_2$ human with the observed z- score of -1.8 and binding energy score of -40740.9 kcal/mol. Nine hydrogen bonds, six salt bridges and three hydrophobic contacts were predicted between docked complex of $\alpha_4\beta_2$ human receptor and cytotoxin (Figure 5b). The first and second hydrogen bonds were observed between carbonyl oxygen and hydroxyl oxygen from Glu175 of the receptor and hydrogen from Lys33 side chain and backbone nitrogen from Leu27 of cytotoxin. The third one was predicted between backbone oxygen from Gly177 of the receptor and amine group from Lys26 side chain of cytotoxin. The fourth and fifth hydrogen bonds involved amine group from Arg167 of the receptor and hydroxyl oxygen from Asp61 side chain and backbone oxygen from Ala37 of cytotoxin. The rest of hydrogen bonds were assisted by Asp172, Arg210, Trp179 and Ile181 of the receptor and Lys56, Thr34, Lys26 and Cys35 of the cytotoxin. In addition, four of the salt bridges were observed between Arg167 of the receptor and Asp61 from the cytotoxin. The remaining two were predicted to occur between Glu175 and Asp172 of the receptor and Lys33 and Lys56 of the cytotoxin respectively. All three hydrophobic contacts with same distance 4.00Å occur between Leu171 side chain of

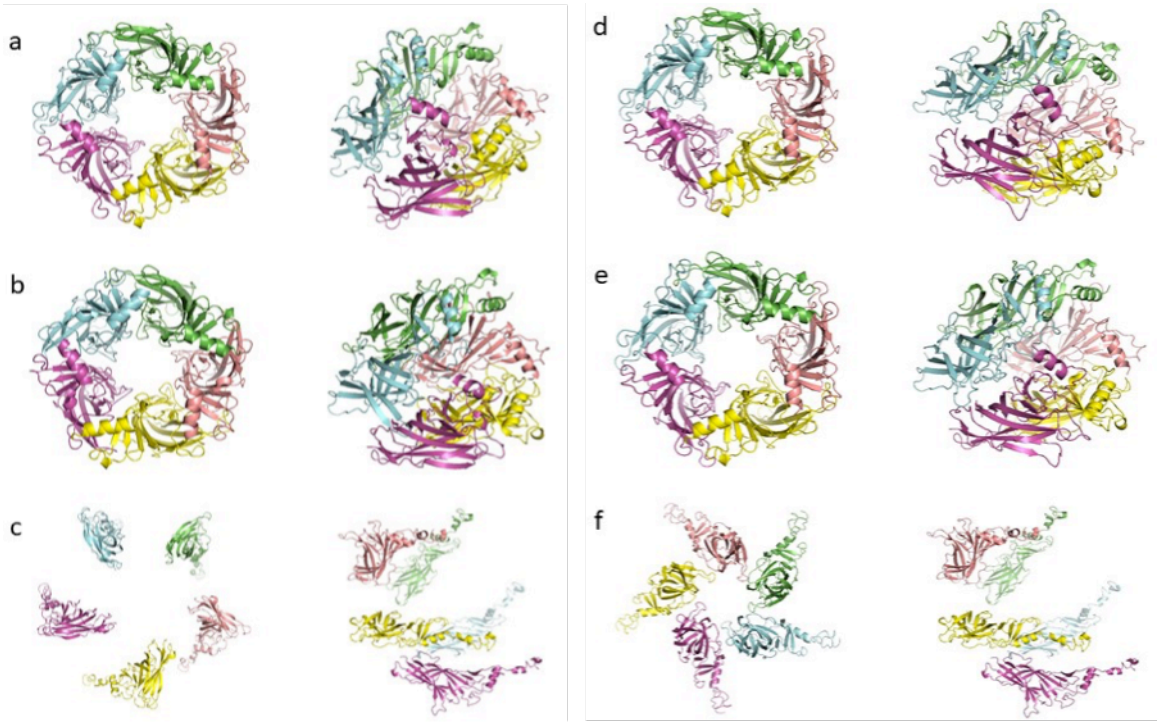

Figure 2: Homology models of human and chicken nicotinic acetylcholine receptor in pentamer after assembled. a) Human α3β2, b) Human α4β2, c) Human α7, d) Chicken α3β2, e) Chicken α4β2, f) Chicken α7, Left side: Top view, Right side: Side view

Figure 3: 3D structure of cytotoxin-3

Table 3: Docking results of toxins against human nicotinic acetylcholine receptors

Protein/Parameter	Z-score	Binding energy (kcal/mol)	Number of residues
$\alpha_3\beta_2$			
α-cobratoxin	-1.5	-38150.8	18
Cobrotoxin-c	-1.5	-35304.2	13
Cytotoxin-3	-1.9	-35309.7	16
Denmotoxin	-2.3	-45607.5	18
$\alpha_4\beta_2$			
α-cobratoxin	-1.2	-38870.0	15
Cobrotoxin-c	-1.7	-35624.1	25
Cytotoxin-3	-1.8	-40740.9	17
Denmotoxin	0.0	-30663.4	14
α_7			
α-cobratoxin	-2.2	-92615.3	15
Cobrotoxin-c	-1.8	-90307.8	17
Cytotoxin-3	-1.1	-95399.8	14
Denmotoxin	-1.1	-96465.5	16

the receptor and Ile60 side chain of the toxin, indole side chain from Trp174 of the receptor and isopropyl side chain from Leu27 of the toxin and isopropyl group from Leu212 of the receptor and benzene group from Tyr32 of the toxin. Based on Table 3, α-cobratoxin preferred to bind towards α_7 human receptor when compared to other toxins. The docked complex of α-cobratoxin and α_7 human receptor had the z-score of -2.2 and binding energy score of -92615.3 kcal/mol, in which the binding was facilitated by 15 residues. The binding interaction analysis showed that α-cobratoxin strongly bind to α_7 human receptor with formation of sixteen hydrogen bonds, two salt bridges and eight hydrophobic contacts (Figure 5c). The first three hydrogen bonds were facilitated between amine group from of receptor and carbonyl oxygen from Asp8, Cys3 and Phe4 of α-cobratoxin respectively. The fourth and fifth hydrogen bond were observed involved amine group from Arg101 of receptor and carbonyl oxygen from Asp13 of the α-cobratoxin. The next two hydrogen bonds were seen between carbonyl oxygen from Ser188 of receptor and amine group from Arg36 and Arg33 of the toxin. Four of the hydrogen bonds facilitated by carbonyl oxygen from Cys41 of the toxin and amine group from Asn129 and amine group and hydroxyl oxygen from Thr128 of the receptor. The remaining hydrogen bond involved between Ile187, Asn75, Gln61, Tyr190 and Phe126 of the receptor and Arg36, Lys49, Gly51, Ala28 and Ala43 of the toxin respectively. The carbonyl oxygen from Asp8 of toxin was observed to form salt bridge with amine group from Arg101 of the receptor. All hydrophobic contacts occur with same distance between the receptor and α-cobratoxin. For the hydrophobic contact, the amino acids involved are Leu60, Ala124, Phe126, Leu131, Ile191 and Pro192 of the receptor and Trp25, Pro46, Ala43, Ile5, Pro7 and Ala28 of the toxin.

Toxin docking against chicken nicotinic acetylcholine receptors

The same procedures as in the toxin docking against human nicotinic acetylcholine receptors were applied. A total of 48 complex structures were generated from the docking process. The complexes were ranked based on their z-score, binding energy score and number of residues contacted, as listed in Table 4.

From the docking result, denmotoxin was predicted to strongly bind to the $\alpha_3\beta_2$ chicken receptor, with the z-score of -1.8, binding energy score of -36602.1 kcal/mol and 20 residues facilitated the interactions. Cytotoxin-3 was also observed to exert the best binding score towards the $\alpha_4\beta_2$ chicken receptor with the z-score of -1.9 and binding energy score -29489.7 kcal/mol, in which the interactions were facilitated by 19 residues. On the other hand, cobrotoxin-c was predicted to exert the best binding score towards α_7 chicken receptor (z-score: -2.1, binding energy score: -68719.8 kcal/mol), where 18 residues facilitated the interactions. Observation through the superimposition of all the docked complexes (Figure 6) showed that all the

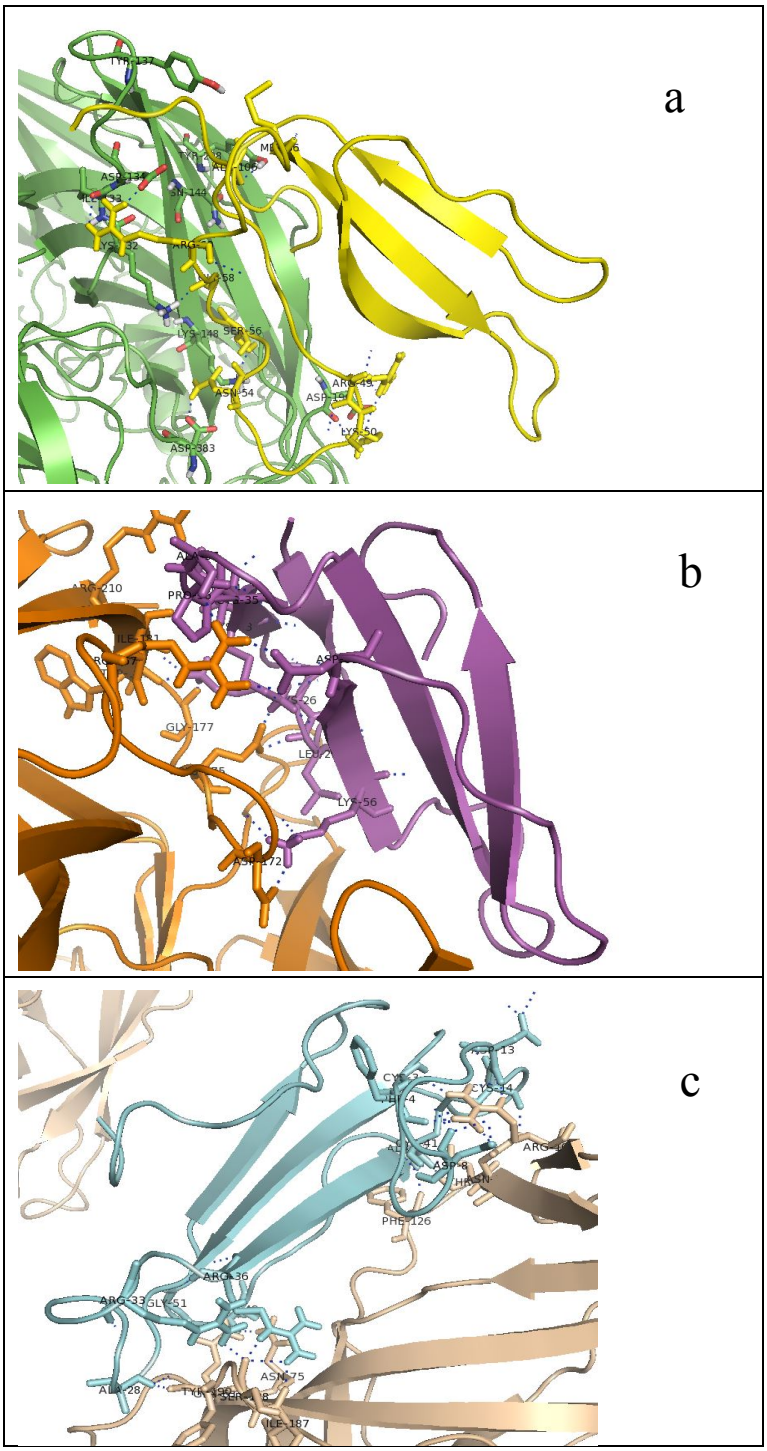

Figure 4: The complexes with the best docking score, complex structure between human receptor and toxin with hydrogen bond interaction. The dot blue line shows the hydrogen bond between receptor and toxin. a) Human $\alpha_3\beta_2$ complexes with denmotoxin, b) Human $\alpha_4\beta_2$ complexes with cytotoxin, c) Human α_7 complexes with α-cobratoxin, Green: $\alpha_3\beta_2$. Orange: $\alpha_4\beta_2$. Wheat: α_7. Cyan: α-cobratoxin. Mangeta: Cytotoxin. Yellow: Denmotoxin.

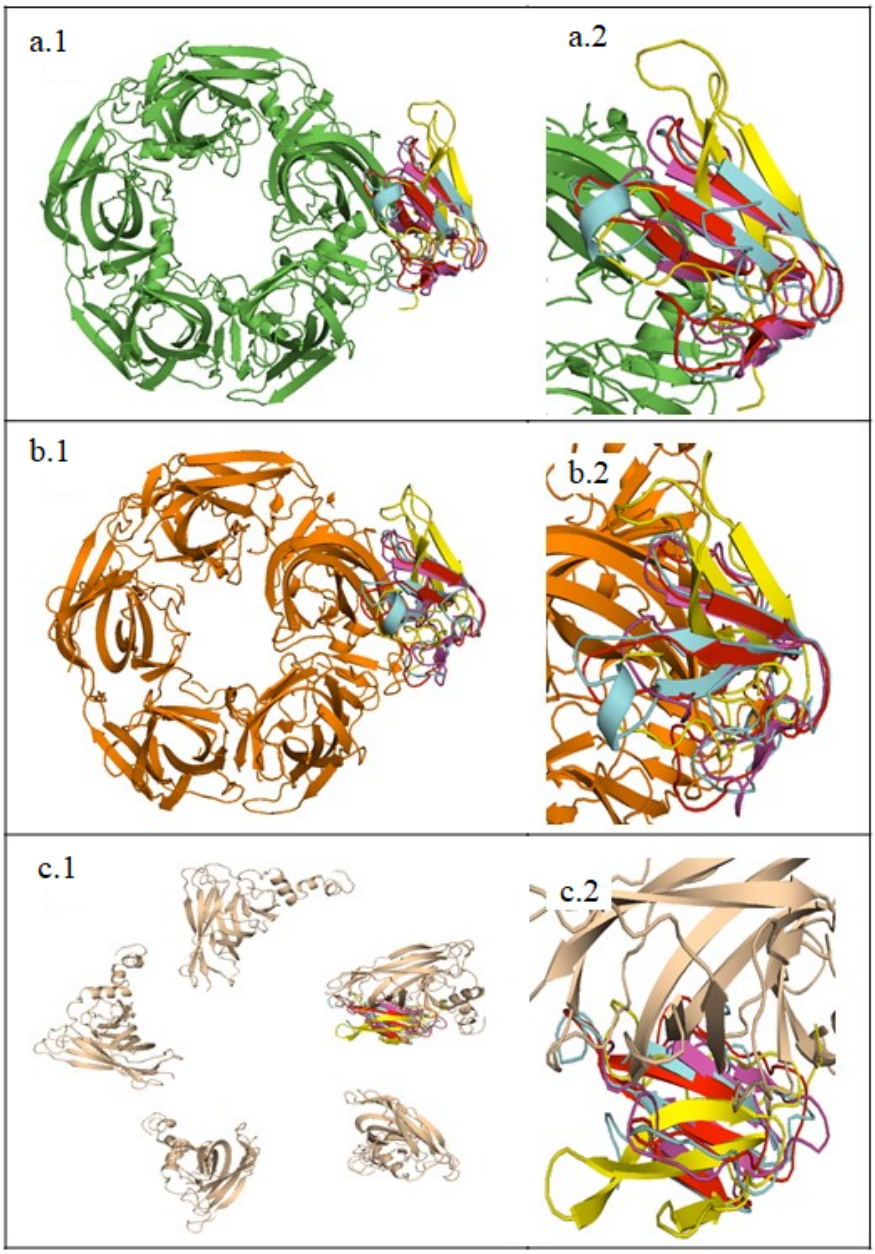

Figure 5: Top view and close up view of docked complexes of the four toxins on human nicotinic receptors. a.1 and a.2) $\alpha_3\beta_2$, b.1 and b.2) $\alpha_4\beta_2$, c.1 and c.2) α_7. α-cobratoxin is colored in cyan, cobrotoxin-c in red, cytotoxin in magenta and denmotoxin in yellow.

four toxins prefer to bind to α subunit outside the extracellular domain of $\alpha_3\beta_2$, $\alpha_4\beta_2$ receptors and α_7 receptor.

In chicken, denmotoxin also strongly bind to $\alpha_3\beta_2$ receptor facilitated by ten hydrogen bonds, three salt bridges and five hydrophobic contacts (Figure 7a). The first hydrogen bond involved amine group from Lys962 side chain of the receptor and carbonyl oxygen from Gly58 backbone of denmotoxin. Five hydrogen bonds were seen between amine group from Arg207 of the receptor and carbonyl oxygen from Lys85, Asn48 and Cys47 of denmotoxin. The remaining hydrogen bonds were facilitated by amine group from Lys966 and Glu175 and carbonyl oxygen from Glu172 and Leu209 of the receptor with hydroxyl oxygen from Ser107, amine group from Asn54 and Leu62 and carbonyl oxygen from Arg60 of the toxin. All salt bridges for this complex involved carbonyl and hydroxyl oxygen from Glu175 of the receptor with amine group from Arg60 of denmotoxin. Meanwhile, the hydrophobic contacts were formed between Pro136,

Table 4: Docking results of toxins against chicken nicotinic acetylcholine receptors

Protein/Parameter	Z-score	Binding energy (kcal/mol)	Number of residues
$\alpha_3\beta_2$			
α-cobratoxin	-1.1	-33801.5	15
Cobrotoxin-c	-1.4	-31540.9	18
Cytotoxin-3	-1.0	-30672.3	18
Denmotoxin	-1.8	-36602.1	20
$\alpha_4\beta_2$			
α-cobratoxin	-1.3	-32926.3	18
Cobrotoxin-c	-1.6	-29449.6	17
Cytotoxin-3	-1.9	-29489.7	19
Denmotoxin	-1.8	-34592.5	21
α_7			
α-cobratoxin	-1.5	-71024.4	19
Cobrotoxin-c	-2.1	-68719.8	18
Cytotoxin-3	-1.3	-67897.5	15
Denmotoxin	-1.2	-72674.9	18

Phe137, Leu167 and Phe968 of the receptor and Ala88, Pro92, Trp5 and Val37 of the toxin, respectively.

Cytotoxin was shown to bind strongly to $\alpha_4\beta_2$ chicken receptor, facilitated by twenty hydrogen bonds, sixteen salt bridges and three hydrophobic contacts (Figure 7b). The first two hydrogen bond involved hydroxyl and carbonyl oxygen from Glu175 of the receptor and amine group from Lys44 and Arg57 of the cytotoxin. The next three hydrogen bonds facilitated by hydroxyl and carbonyl oxygen from Asp78 of the receptor and amine group from Arg1023 and Lys984 of the toxin. This is followed by another two hydrogen bonds between hydroxyl oxygen from Asp1039 of the receptor and amine group from Lys23 of the cytotoxin. The eighth and ninth hydrogen bonds were facilitated by amine group from Lys44 of the toxin and carbonyl oxygen from Trp179 and Gly177 of the receptor. The next three hydrogen bonds were observed between carbonyl oxygen and amine group from Asn76 of the cytotoxin and amine group from Tyr173 and carbonyl oxygen from Tyr173 and Leu171 of the receptor. The remaining hydrogen bond facilitated by hydroxyl oxygen from Glu970 and Glu970, amine group from Arg210, Lys968, Gln170 and Lys968 and carbonyl oxygen from Glu178 of the receptor with amine group from Arg79, Asn76 and Lys52 and carbonyl oxygen from Val48, Cys80, Pro64 and Asn81 of the toxin, respectively. For the first four salt bridges interaction, it was predicted between carbonyl and hydroxyl oxygen from Glu175 of the receptor and amine group from Lys44 and Arg57 of the cytotoxin. Another four salt bridge bonds involving the carbonyl and hydroxyl oxygen from Glu970 of the receptor and amine group from Arg79 of the toxin were seen. Five salt bridges were detected between amine group from Asp78 of the cytotoxin and amine group from Arg1023 and Lys984 of the receptor. The remaining of the salt bridge interactions were facilitated by carbonyl and hydroxyl oxygen from Asp1039, Glu178, Glu970 of the receptor and amine group from Lys23, Lys52 and Arg79 of the toxin. As for the hydrophobic contacts, the first one was predicted between Tyr173 side chain of the receptor and Val73 side chain of the cytotoxin. The other hydrophobic contacts include Val180 and Leu212 of the receptor and Phe46 side chain of cytotoxin.

The cobrotoxin-c, which has the best score towards α_7 chicken receptor, interacts with the receptor by forming fifteen hydrogen bonds, eight salt bridges and six hydrophobic contacts (Figure 7c). The first six hydrogen bonds were seen between hydroxyl oxygen from Asp104 and Tyr30, and amine group from Lys35 and Arg10l of the receptor with amine group from Lys46, carbonyl oxygen from Gly19, Glu20 and Gln7, and hydroxyl oxygen from Tyr24 and Glu37 of the toxin. Three hydrogen bonds were observed between amine group and carbonyl oxygen from Lys15 of the toxin and carbonyl oxygen from Leu21 and

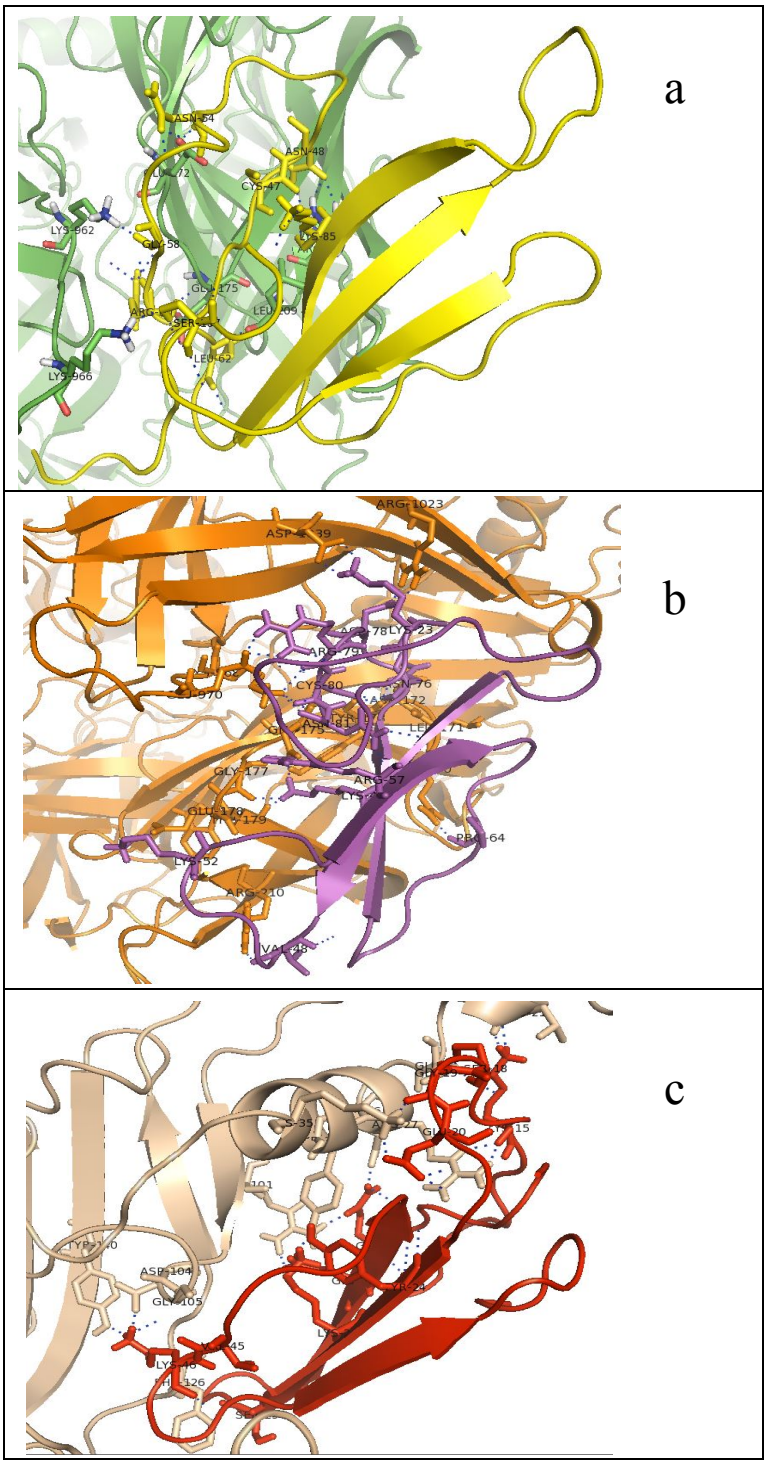

Figure 6: The complexes with the best docking score, complex structure between chicken receptor and toxin with hydrogen bond interaction. The dot blue line shows hydrogen bond between receptor and toxin. a) Chicken $\alpha_3\beta_2$ complexes with denmotoxin, b) Chicken $\alpha_4\beta_2$ complexes with cytotoxin, c) Chicken α_7 complexes with Cobrotoxin-c. Green: $\alpha_3\beta_2$. Orange: $\alpha_4\beta_2$. Wheat: α_7. Red: Cobrotoxin-c. Mangeta: Cytotoxin. Yellow: Denmotoxin.

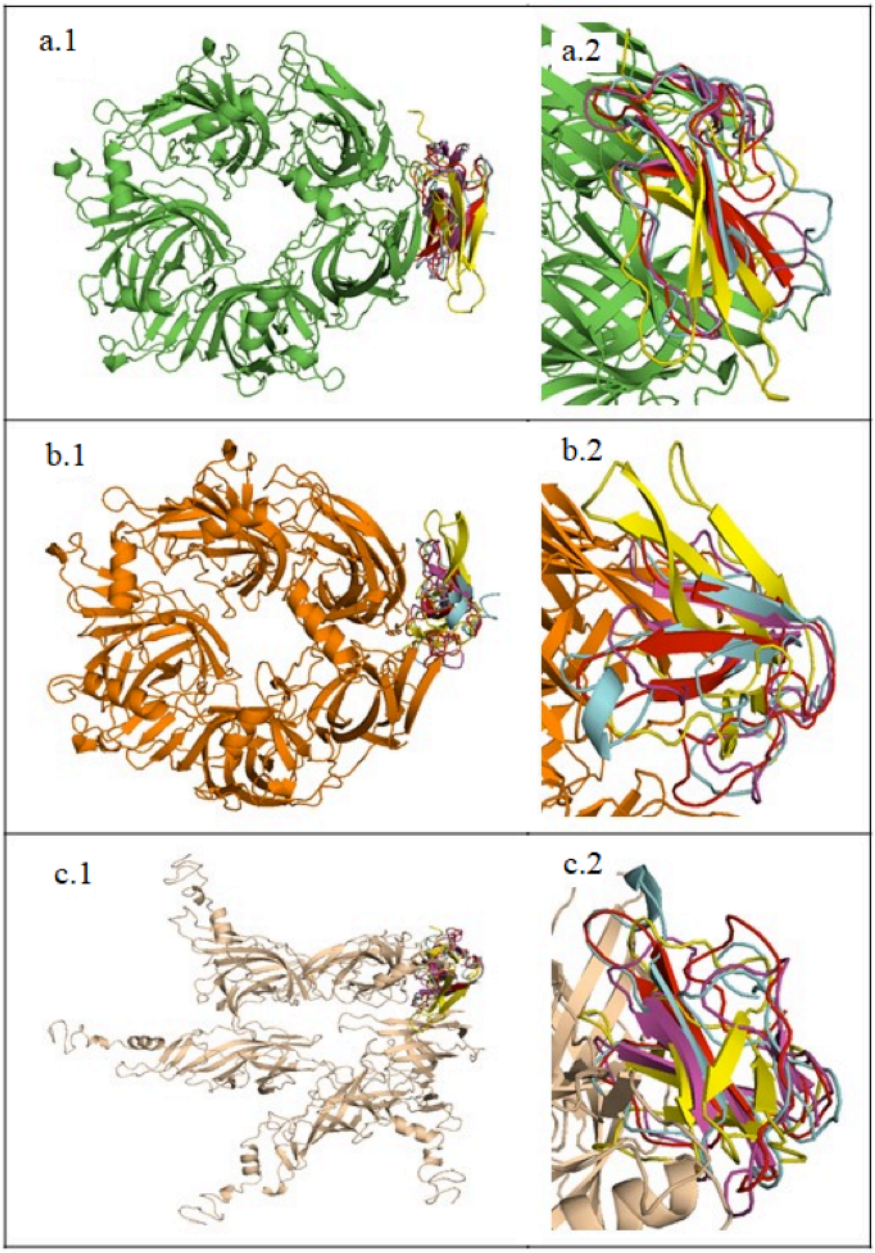

Figure 7: Top view and close up view of docked complexes of the four toxins on chicken nicotinic receptors. a.1 and a.2) $\alpha_3\beta_2$, b.1 and b.2) $\alpha_4\beta_2$, c.1 and c.2) α_7. Both receptors and toxins were represented in cartoon. α-cobratoxin is colored in cyan, cobrotoxin-c in red, cytotoxin in magenta and denmotoxin in yellow.

amine group from Arg27 of the receptor. The remaining hydrogen bond involved hydroxyl oxygen from Tyr140, Tyr30 and Glu24, carbonyl oxygen from Gln26 and Gly105, and amine group from Phe126 of the receptor with amine group from Lys46, Lys26, Gln7, Ser18 and Val45, and carbonyl oxygen from Ser29 of cobrotoxin-c. Most of the salt bridge bonds in this complex were formed between amine group from Arg101 of the receptor and hydroxyl and carbonyl oxygen from Glu37 of the toxin. In addition, another two salt bridges involved hydroxyl and carbonyl oxygen from Asp123 of the receptor and amine group from His13 of the toxin. The remaining salt bridges were seen between carbonyl oxygen from Asp104 and amine group from Lys35 of the receptor with amine group from Lys46 and hydroxyl oxygen from Glu20 of the toxin. For the

hydrophobic contacts, the contacts were seen between amino acids such as Tyr30, Leu34, Leu106, Phe126 and Phe1125 of the receptors, and Tyr24, Pro43, Pro47, Trp28 and Val49 of the cobrotoxin-c.

Discussion

3FTxs family is one of the most abundant toxins family in snake venom (Roly et al., 2014; Tasoulis & Isbister, 2017). There are many studies on 3FTx, mostly focusing on isolation and identification of the toxin, as well as the biological effects it exerts. However, there are limited information on the 3FTxs binding affinity and selectivity towards receptors. Molecular docking is a computational tool that predicts binding affinities and binding interactions between target molecules and target receptors. It is performed to explain and complement the experimental results (Lim, Rahman, & Tejo, 2011; Teo et al., 2012). This computational approach has been proven as relatively cost-efficient and fast experiment method (Murgueitio, Bermudez, Mortier, & Wolber, 2012).

Isolation and characterization of pharmacological activities for cobra toxins from cobra venom originated from various geographical localities have been previously described (Pawlak & Kini, 2008; Tan et al., 2015; Tan et al., 2016; Torres-Bonilla et al., 2016; M. Yap, Tan, & Fung, 2011; M. K. K. Yap et al., 2014). However, the information on receptor-binding for these toxins is limited. In this molecular docking study, four toxins, namely α-cobratoxin (PDB ID: 1CTX), cobrotoxin-c (PDB ID: 1JE9), denmotoxin (PDB ID: 2H5F) and cytotoxin 3 (UniProt no: P60302) from 3FTXs family were chosen as the toxins, while three types of nicotinic acetylcholine receptors ($\alpha_3\beta_2$, $\alpha_4\beta_2$ and α_7) from human and bird were chosen as the receptors. These 3FTXs were chosen because they contribute to different levels of toxicity effects and exhibit a wide variety of biological effects (Girish et al., 2012; Kini & Doley, 2010; Roly et al., 2014; Utkin, 2013). The target receptors and subunit. i.e. the $\alpha_3\beta_2$, $\alpha_4\beta_2$ and α_7 subunits were chosen due to its important role as neurotransmitter receptors in vertebrates and invertebrates (Millar & Denholm, 2007).

In the present work, 96 toxin-receptor complexes were obtained from the molecular docking experiment. These complexes were ranked based on their docking score (or z-score) and binding affinities toward nicotinic acetylcholine receptors. The complex with the lowest values of z-score and binding affinity is considered as the best docked complex. Several toxins were identified to bind with strong binding affinities towards the nicotinic acetylcholine receptors. These toxins were further investigated to understand their binding interactions. In this study, all toxins were observed to bind at a consensus binding site, which is located outside the extracellular domain of α subunit, similar to the reported ligands binding domain (Luttmann et al., 2009; Pavlovicz et al., 2011). Previous works on acetylcholine receptor have shown that ligand-binding site is located at the extracellular domain of the receptor and interface between α subunit and β subunit (Changeux, 2018; Mohamed, Jayakar, & Hamouda, 2015; Spurny et al., 2015; J. Wang & Lindstrom, 2018). Comparison of the toxins binding sites and the ligand binding site showed the same amino acid residues involved in the interactions for human and chicken receptors. Common amino acids involved in the interaction were Leu60, Asn75, Asp104, Ala124, Phe126, Leu171, Ser187, Ser188, Asp190, Tyr190, Ile191, Pro192 and Arg210 (Kalamida et al., 2007; Marotta, Lester, & Dougherty, 2015; Spurny et al., 2015; Walsh et al., 2018).

Denmotoxin (PDB ID: 2H5F) from *Boiga dendrophila* is a non-conventional toxin and reported as a bird-specific toxin (Blanchet, 2017; Saviola, Peichoto, & Mackessy, 2014; Weinstein, White, Keyler, & Warrell, 2013). It was observed to exert postsynaptic neuromuscular blockade activity ~100 times more potent on birds when compared to mammals via 'pseudo-irreversible' mechanism (Heyborne & Mackessy, 2013; Pawlak et al., 2006; Pawlak et al., 2009). Among the four toxins, denmotoxin has the lowest z-score i.e. highest binding affinities, towards the $\alpha_3\beta_2$ receptor for both species. However, the predicted z-score is lower in the human $\alpha_3\beta_2$ receptor than that of the chicken $\alpha_3\beta_2$ receptor, suggesting the preference of denmotoxin to bind to the human $\alpha_3\beta_2$ receptor. It formed the same number of hydrogen bond in both species but formed different number of salt bridge and hydrophobic contact in human receptor, which cause higher binding affinity in human receptor than the bird receptor (Figure 5a).

On the other hand, cytotoxin-3 has highest binding affinities towards the $\alpha_4\beta_2$ receptor for both species. Based on binding interactions between both species, the cytotoxin-3 from *Naja sumatrana* interacted with the $\alpha_4\beta_2$ human and bird receptor interacts via the same number of hydrophobic contacts but exerted different numbers of hydrogen bond and salt bridge. These differences in the binding interactions cause cytotoxin-3 binds with high affinity in the bird receptor than the human receptor (Figure 7b). Cytotoxin-3 (Accession no: P60302) was isolated from *Naja sumatrana* venom and it works by penetrated the membrane, triggers pharmacological activity in cells and causes necrosis (Ebrahim, Shirazi, Mirakabadi, & Vatanpour, 2015; Kalam, Isbister, Mirtschin, Hodgson, & Konstantakopoulos, 2011; M. Yap et al., 2011). Information regarding the cytotoxin- 3 from *Naja sumatrana* was very limited. Recent research had shown that this toxin was a concentration-dependent process and can induce caspase-dependent mitochondrial-mediated apoptosis without transforming the death cell pattern to primary necrosis(Teoh & Yap, 2020).

α-cobratoxin and cobratoxin-c isolated from *Naja kaouthia* were shown to have the highest binding affinities toward α_7 human and bird receptor, respectively. Although

they are from the same species, these toxins formed different numbers of hydrogen bond, salt bridge and hydrophobic contact with the human and bird α_7 receptor. α-cobratoxin (PDB ID: 1CTX) is one of the main neurotoxins in *Naja kaouthia* venom and classified under long chain neurotoxin of 3FTXs. This toxin also has been proven as an antagonist with high affinity on α_7 nicotinic acetylcholine receptors in peripheral and central nervous systems (Gong et al., 2015; Utkin et al., 2017; Zhang et al., 2012). It blocks nerve transmission on postsynaptic membranes by binding to the receptor and causes paralysis (Silva, Cristofori-Armstrong, Rash, Hodgson, & Isbister, 2018).α-cobratoxin had been reported to have analgesic potency which was postulated due to binding with mice α_7 receptor and it also has been illegally used in race horses because this effect (Bailly-Chouriberry et al., 2013; Shi et al., 2011). On the other hand, cobrotoxin-c (PDB ID: 1JE9) is classified as a novel short chain neurotoxin with long-lasting effects of analgesic activity (Guo et al., 2013; Meng et al., 2002). Replacement of residues in loop II of cobrotoxin-c with neutral residues and different structure of shoulder and outside of loop III make this toxin has higher toxicity level than cobrotoxin-b (Meng et al., 2002).

Majority of the salt bridges formed in both species are complex salt bridges, which involve several salt bridges in a residue. Salt bridge is an ionic bond and known as a stable and strongest non- covalent bond with distance below 4.00Å (Gvritishvili, Gribenko, & Makhatadze, 2008; Yang et al., 2012). The strength of salt bridge was influenced with the distance between residue from the toxin and the receptor to undergo electrostatic attraction (Bosshard, Marti, & Jelesarov, 2004; Laha & Wagner, 2011). In salt bridge interaction, a proton migrated from carboxylic acid group to primary amine or guanidine group (Yang et al., 2012). Based on the results, amino acid residues from the $\alpha_3\beta_2$ human receptor and chicken receptor serve as the donor, while residues on the denmotoxin serve as acceptor. Upon protonation, Arg60 on Loop I of the denmotoxin, which has two nitrogen atoms formed a salt bridge with Asp134 on the $\alpha_3\beta_2$ human receptor and Glu175 on the $\alpha_3\beta_2$ chicken receptor. Arginine, which is a basic amino acid residue has higher possibility of forming salt bridge because of its highest pairing frequency with other acidic residues (Nayek, Gupta, Banerjee, Mondal, & Bandyopadhyay, 2014). The Arg60 of the denmotoxin contributed to salt bridge interaction for both species. Further analysis of interaction between Arg60 and human and chicken receptors showed the amino acid had 143.21Å^2 and 168.54 Å^2 of buried area, respectively. Other amino acids residues of toxin involved in salt bridge interaction from the $\alpha_3\beta_2$ human receptor were His41, Arg49 and Lys50 with lower buried area numbers of 39.43Å^2, 56.95Å^2 and 64.50Å^2, respectively. Arginine residues with larger buried areas form stronger salt bridges compared with exposed residues (Donald, Kulp, & DeGrado, 2011). Buried surface area used in the process of estimation the interface size between two macromolecules and determine the stability of the complex by calculated atomic coordinates of the complex (Chakravarty, Guharoy, Robert, Chakrabarti, & Janin, 2013; Rashin, 1984). In fact, had been observed buried surface area is directly proportional with affinity which means buried surface area increases, binding affinity increases (J. Chen, Sawyer, & Regan, 2013). Lone pairs of Lys33 on loop I and Lys44 on loop II from the cytotoxin formed a salt bridge with Glu175 during the protonation. Similar case also occurs in Lys46 on loop I of the cobrotoxin-c. Lys has a buried area which plays as intermediate residues in salt bridge formation between Arg and His (Donald et al., 2011). Asp8 on loop I of the α-cobratoxin protonated and form salt bridge with Arg101. ASP is known as basic residues and has small buried area in the protein surface (Nayek et al., 2014). Based on Laha et al, the amino acids includes Asp134, Asp190, Glu175, Arg167, Asp172, Arg1023, Lys984, Asp1039, Glu178, Glu970, Arg101, Lys35, Asp104 and Asp123 on α subunit of each receptors might undergo rearrangements as a response of receptor activation or also known as state-dependence interaction (Laha & Wagner, 2011). Hence, the predicted interaction based on our result may occur directly or indirectly from secondary interaction from residue side chains or structural rearrangement.

Apart from the salt bridges, hydrophobic contact and hydrogen bond also play important in the binding of toxin towards the receptors. Thera are few amino acids involved in each interaction for each complex. In human receptor, Asp190 of the $\alpha_3\beta_2$ receptor facilitated about 20% and 50% in hydrogen bond and salt bridge interaction with the denmotoxin respectively. Besides that, Asp134 also facilitated about 50% in salt bridge interaction with the denmotoxin. Arg101 of the α_7 receptor facilitated the most in hydrogen bond interaction with the α-cobratoxin about 31%. Furthermore, Phe126 and Leu131 of α_7 receptor facilitated 25% in hydrophobic contact with the α-cobratoxin. In chicken receptor, few residues of $\alpha_4\beta_2$ receptor facilitated 11% in hydrogen bond with the cytotoxin which Glu175, Glu970, Asp1039, Lys968 and Tyr173. Other than that, Glu175, Glu970 and Asp1023 of $\alpha_4\beta_2$ receptor facilitated about 25% in salt bridge interaction with the cytotoxin. Lys35, Tyr30, Arg101 and Arg27 of α_7 receptor facilitated 13% of hydrogen bond with cobrotoxin-c. Moreover, Arg101 of α_7 receptor facilitated half of the salt bridge interaction with cobrotaxin-c. Phe126 of α_7 receptor facilitated about 33% in hydrophobic contact with cobrotoxin-c.

Conclusion

α-cobratoxin, cobrotoxin-c, cytotoxin and denmotoxin bind at the orthosteric site which outside the extracellular domain of α subunit for both species by creating hydrogen bond, ionic bond and hydrophobic contact with important residues involved in their binding

pocket. There are significant differences in affinity of the toxins towards different receptor subtypes. Denmotoxin has higher affinity towards $\alpha_3\beta_2$ human receptor, cytotoxin has higher affinity towards $\alpha_4\beta_2$ bird receptor, α-cobratoxin has higher affinity towards to α_7 human receptor and cobrotoxin-c has higher affinity towards α_7 chicken receptor. Characterization of structure, binding affinity, and selectivity for 3FTx towards different receptor subtypes are crucial in the process to understand their effect to organ system and clinical symptoms of snake bite envenoming. More works using protein simulation and wet lab assays are required to confirm the findings in this study.

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Conflict of Interest

The authors Nor Asyikin Zukifli, Muhamad Rusdi Ahmad Rusmili, Iekshan Othman, Ahmad Khaldun Ismail, Janeyuth Chaisakul and Zalikha Ibrahim declare that they have no conflict of interests or financial conflicts to disclose. This article does not contain any studies with human or animal subjects performed by any of the authors.

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SUPPLEMENTARY DATA

Supplementary Table 1: The intermolecular interactions between human receptors and toxins.

Type of complex	Type of interaction	Receptor residue	Toxin residue	Distance (Å)
$\alpha_3\beta_2$ + Denmotoxin	Hydrogen bond	Asp190	Arg49	1.73
		Asp134	Arg60	1.74
		Lys132	Gly58	1.75
		Lys148	Ser56	1.78
		Asp190	Lys50	1.79
		Asp383	Asn54	1.84
		Tyr137	Met66	2.03
		Tyr208	Ala106	2.10
		Ile133	Arg60	2.17
		Ile144	Arg60	2.49
	Salt bridge	Asp134	Arg60	2.66
		Asp190	Arg49	2.73
		Asp134	His41	2.97
		Asp190	Lys50	3.32
		Asp134	His41	3.37
		Asp190	Lys50	3.38
		Asp190	Arg49	3.76
		Asp134	Arg60	3.98
	Hydrophobic contact	Tyr137	Pro40	4.00
$\alpha_4\beta_2$ + Cytotoxin	Hydrogen bond	Glu175	Lys33	1.55
		Gly177	Lys26	1.63
		Arg167	Asp61	1.64
		Arg167	Ala37	1.74
		Asp172	Lys56	1.76
		Arg210	Thr34	1.77
		Trp179	Lys26	1.82
		Glu175	Leu27	2.92
		Ile181	Cys35	3.86
	Salt bridge	Glu175	Lys33	2.58
		Arg167	Asp61	2.63
		Asp172	Lys56	2.72
		Arg167	Asp61	2.88
		Arg167	Asp61	2.97
		Arg167	Asp61	3.70
	Hydrophobic contact	Leu171	Ile60	4.00
		Trp174	Leu27	4.00
		Leu212	Tyr32	4.00

SUPPLEMENTARY DATA

Supplementary Table 1: The intermolecular interactions between human receptors and toxins (cont.).

Type of complex	Type of interaction	Receptor residue	Toxin residue	Distance (Å)
α_7+ α-cobratoxin	Hydrogen bond	Arg101	Asp8	1.70
		Arg101	Cys3	1.83
		Arg101	Phe4	1.84
		Arg101	Asp13	1.86
		Ser188	Arg36	1.89
		Ile187	Arg36	1.92
		Asn129	Cys14	2.14
		Arg101	Asp13	2.32
		Ser188	Arg33	2.42
		Asn75	Lys49	2.45
		Gln61	Gly51	2.75
		Tyr190	Ala28	2.97
		Thr128	Cys41	3.13
		Phe126	Ala43	3.14
		Asn129	Cys41	3.57
		Thr128	Cys41	3.66
	Salt bridge	Arg101	Asp8	2.69
		Arg101	Asp8	3.81
	Hydrophobic contact	Leu60	Trp25	4.00
		Ala124	Pro46	4.00
		Phe126	Ala43	4.00
		Phe126	Pro46	4.00
		Leu131	Ile5	4.00
		Leu131	Pro7	4.00
		Ile191	Trp25	4.00
		Pro192	Ala28	4.00

Supplementary Table 2: The intermolecular interactions between chicken receptors and toxins.

Type of complex	Type of interaction	Receptor residue	Toxin residue	Distance (Å)
$\alpha_3\beta_2$ + Denmotoxin	Hydrogen bond	Lys962	Gly58	1.66
		Arg207	Lys85	1.86
		Arg207	Asn48	1.95
		Lys966	Ser107	1.96
		Arg207	Asn48	2.24
		Arg207	Lys85	2.25
		Arg207	Cys47	2.41
		Glu172	Asn54	2.78
		Leu209	Leu62	2.80
		Glu175	Arg60	3.40
	Salt bridge	Glu175	Arg60	3.22
		Glu175	Arg60	3.69
		Glu175	Arg60	3.69
	Hydrophobic contact	Pro136	Ala88	4.00
		Phe137	Ala88	4.00
		Phe137	Pro92	4.00
		Leu167	Trp52	4.00
		Phe968	Val37	4.00
$\alpha_4\beta_2$ + Cytotoxin	Hydrogen bond	Glu175	Lys44	1.58
		Glu175	Arg57	1.62
		Glu970	Arg79	1.62
		Arg1023	Asp78	1.63
		Lys984	Asp78	1.64
		Arg1023	Asp78	1.74
		Arg210	Val48	1.90
		Asp1039	Lys23	1.90
		Asp1039	Lys23	1.90
		Asp172	Asn76	1.91
		Lys968	Cys80	1.98
		Gln170	Pro64	2.04
		Glu970	Arg79	2.26
		Trp179	Lys44	2.26
		Gly177	Lys44	2.33
		Glu178	Lys52	2.39
		Lys968	Asn81	2.41
		Tyr173	Asn76	3.02
		Tyr173	Asn76	3.30
		Leu171	Asn76	3.64

Supplementary Table 2: The intermolecular interactions between chicken receptors and toxins (cont.).

Type of complex	Type of interaction	Receptor residue	Toxin residue	Distance (Å)
$\alpha_4\beta_2$ + Cytotoxin	Salt bridge	Glu175	Lys44	2.61
		Glu970	Arg79	2.63
		Glu175	Arg57	2.64
		Asp1039	Lys23	2.65
		Arg1023	Asp78	2.66
		Lys984	Asp78	2.66
		Asp1039	Lys23	2.69
		Arg1023	Asp78	2.75
		Glu970	Arg79	3.06
		Glu970	Arg79	3.17
		Arg1023	Asp78	3.25
		Glu178	Lys52	3.41
		Arg1023	Asp78	3.73
		Glu175	Arg57	3.86
		Glu970	Arg79	3.88
		Glu175	Lys44	3.98
	Hydrophobic contact	Tyr173	Val73	4.00
		Val180	Phe46	4.00
		Leu212	Phe46	4.00
α_7 + Cobrotoxin-c	Hydrogen bond	Asp104	Lys46	1.59
		Lys35	Gly19	1.69
		Tyr30	Tyr24	1.71
		Lys35	Glu20	1.72
		Arg101	Glu37	1.72
		Arg101	Gln7	1.77
		Tyr140	Lys46	1.78
		Tyr30	Lys26	1.79
		Leu21	Lys15	1.79
		Arg27	Lys15	1.91
		Gln26	Gln7	1.99
		Arg27	Lys15	2.16
		Glu24	Ser18	2.83
		Gly105	Val45	3.58
		Phe126	Ser29	3.65
	Salt bridge	Asp104	Lys46	2.64
		Arg101	Glu37	2.66
		Lys35	Glu20	2.69
		Asp123	His13	2.80

Supplementary Table 2: The intermolecular interactions between chicken receptors and toxins (cont.).

Type of complex	Type of interaction	Receptor residue	Toxin residue	Distance (Å)
α_7 + Cobrotoxin-c	Salt bridge	Asp123	His13	2.81
		Arg101	Glu37	3.26
		Arg101	Glu37	3.83
		Arg101	Glu37	3.86
	Hydrophobic contact	Tyr30	Tyr24	4.00
		Leu34	Pro43	4.00
		Leu106	Pro43	4.00
		Phe126	Trp28	4.00
		Phe126	Val49	4.00
		Phe1125	Pro47	4.00