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Exploring Venom Toxins as Molecular Models for Chronic Pain Treatment

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ABSTRACT

The purpose of this project is to propose venomous animal toxins as molecular models for pain medication. Chronic pain is a prevalent health problem among the general population, and current pharmacological treatments are oftentimes ineffective or limited due to undesirable side-effects. This project explores the role of specific Voltage-Gated Sodium Channels, such as Sodium Channel 1.7 (NaV 1.7), in setting the stage for proposing analgesics with binding properties in peripheral pain-sensing neurons. Sodium Channels, notably NaV 1.7, play a major role in human pain signaling pathways that propagate action potentials in excitable cells. By inhibiting and blocking them, analgesic effects are known to be achievable. Through means of bioinformatic tools, we explore amino acid sequence alignments, Motif scans, tertiary structure modeling, and molecular docking of venomous animal toxins for in-silico new drug discovery research. Cysteine residues in toxins were reviewed as a possible link between acting upon the receptor and their analgesic effects. This led to the questioning of cysteine's role in the search for potential antagonists of NaV 1.7. Eventually, the attempt for a further investigation prompted the consideration for molecular docking between selected toxins and the receptor, aiming to seize chronic pain.

Introduction

The plethora of studies done on the possible implications of toxins to medicine undeniably suggest the world of venom to be a pharmacological gold mine. To date, there are 11 approved toxin-based molecules marketed [1]. The significance in discoveries of toxin-based molecules with pain killing properties lies in something they are not, opioids. For chronic pain conditions, meaning pain that carries on for 12 weeks despite medication or treatment prescriptions, opioids are still the standard of care [2]. Unfortunately for most conditions that would suggest long-term usage or reliance on opioids for relief, addiction as well as many other side-effects can result [3]. This boils down to the reality that chronic pain has no viable treatment options, and requires more specific and specialized medications void of such outcomes.

Chronic pain can be classified as nociceptive in which the pain results from the activation of receptors known as nociceptors that are sensitive to noxious stimuli [4, 5]. Noxious stimuli is actually, or potentially can be, damaging to tissue and liable to cause pain, making chronic nociceptive pain result from activity in neural pathways that are secondary from the actual tissue damage or even potentially tissue damaging stimuli. These nociceptors are essentially a specialized subset of sensory neurons located in any area of the body that can sense said noxious stimuli, one of these being the spine [6]. (See Figure 1)

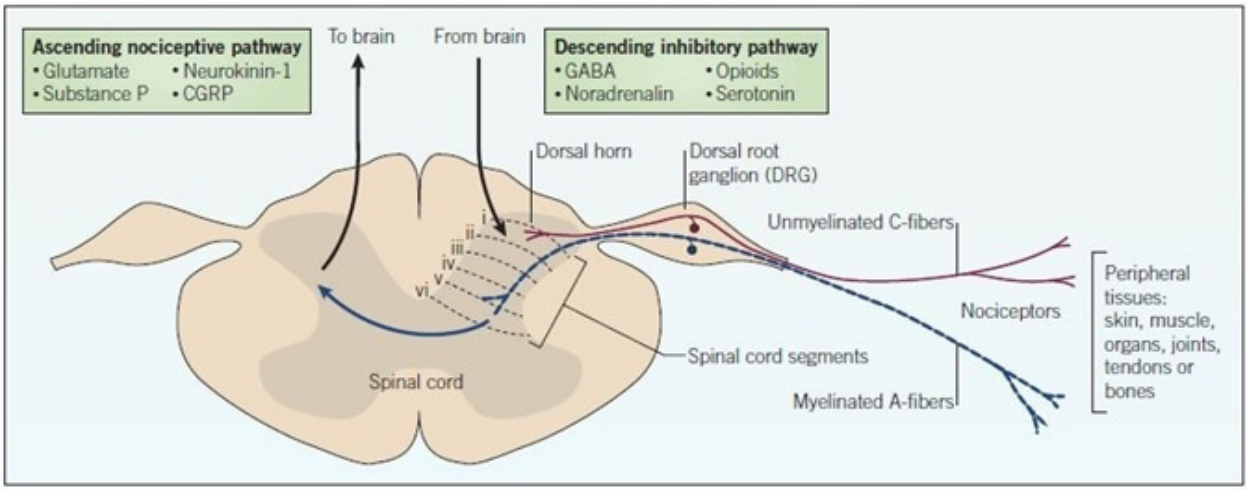


Figure 1. Nociceptive Pain Pathway (Tocris, 2019)

On the molecular side of things, sodium channels within primary sensory neurons like those of the dorsal root ganglion play an important role in pain [7]. Hyperexcitability or increased baseline sensitivity of these cells can lead to abnormal bursts which produce chronic pain [8]. More specifically, voltage gated sodium channels control the flow of sodium ions that can trigger excitability of pain-sensing nociceptors. NaV 1.7 is a sodium ion channel expressed at high levels in nociceptive pain neurons at dorsal root ganglion. [9]. (See Fig. 2)

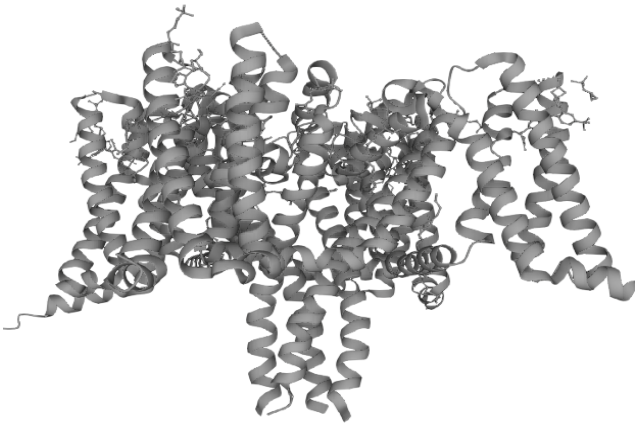


Figure 2. Human Voltage-Gated Sodium Channel: They can be found on the surface of nociceptors where they conduct Na+ currents in response to membrane depolarizations, triggering action potential firing as well as sending pain signals (Q15858, Uniprot).

Through inhibiting sodium channels, the influx of sodium ions is blocked which stops nerve conduction and prevents further signals to the brain. Conotoxins, which are the venoms of marine snails, served as a template for the pain killer Ziconotide [10]. This is a non-opioid pain reliever that is used to treat pain by blocking the nerves in the spinal cord that signal this pain.

All of the toxins outlined in the table below do indeed inhibit the NaV 1.7 sodium ion channel, therefore warranting the question whether or not toxins should be studied more seriously as models for analgesic medications and chronic pain treatments. These toxins are displayed in the table below, read from left to right and by rows of five as A0A2L0ART2, P83476, P0DL57, D2Y2D7, P01523, P58426, Q90WJ7, P45697, F8UWP3, P0DM12. (See Table. 1)

The aim of this project will be to possibly find toxins to be molecular models for drug designs through the use of bioinformatic databases and software as explored later on.

Table 1. Venomous Animals Selection: Scientific Name, Uniprot toxin code, Envenomation signs and symptoms

Scolopendra subspinipes (Vietnamese Centipede) - A0A2L0ART2	**Thrixopelma Pruriens** (Peruvian Green Velvet Tarantula) - P83476	**Phlogius sp.** (Tarantula Spider) - P0DL57	**Cyriopagopus hainanus** (Chinese bird spider) - D2Y2D7	**Conus Geographus** (Geography Cone) - P01523
Extreme pain and reddening around the bite is expected, resulting in death in extreme cases. [11]	Excruciating pain with red swelling around the bite occurs, stunning and paralyzing prey. [12]	Nausea and vomiting along with warmness and redness around the bite commonly occur. [13]	It is aggressive and induces severe nerve damage, rendering the victim paralyzed. [14]	Muscle paralysis, pain, nausea, vomiting and abdominal colic occurs. [15]
				
Heteropoda venatoria (Brown huntsman spider) - P58426	**Takifugu pardalis** (Panther puffer) - Q90WJ7	**Mesobuthus martensii** (Manchurian scorpion) - P45697	**Centruroides vittatus** (Striped bark scorpion) - F8UWP3	**Pamphobeteus Nigricolor** (Giant blue bloom tarantula) - P0DM12
Nausea, abdominal pain, breathing difficulties, as well as muscle weakness and numbness. [16]	Numbness begins around the mouth, leading to trembling, tremors and seizures. [17]	There is tingling and burning at the site, however the venom is comparatively mild. [18]	Sting is often mild to strong, causing Edema, discoloration, and pain lasting up to several days. [19]	Swollen and itchy bumps may form, and other symptoms such as breathing difficulties. [20]
				

Methods and Materials

Pubmed**.** Pubmed is a resource supporting the search and retrieval of literature from biomedicine and and life sciences, connecting users with more than 32 million citations and abstracts. I initially came across Pubmed

when in search for articles that would later shape and guide research of my very own. The 10 references for inhibitory toxins as well as the publishings on cyestine's analgesic uses were located through PubMed. PubMed, https://pubmed.ncbi.nlm.nih.gov, National Center for Biotechnology Information - National Library of Medicine. U.S. National Library of Medicine. [21]

Uniprot. Uniprot is a comprehensive resource for protein sequence and annotation data. I used Uniprot for two purposes, for obtaining the Fasta sequences of the receptor and the toxins, as well as the PDB files for the receptor and a select two ligands. UniProt, https://www.uniprot.org/help/about, ELIXIR core data resource. UniProt Consortium European Bioinformatics Institute Protein Information Resource SIB Swiss Institute of Bioinformatics. [22]

Cobalt. COBALT is a multiple sequence alignment tool that finds a collection of pairwise constraints derived from a conserved domain database, protein motif database, and a sequence similarity using BLASTP. I used this tool in order to align the ten Fasta codes from the 10 toxins obtained from UniProt. Through sequence alignment, the regions of similarity that are a consequence of evolutionary relationships between the sequences are identifiable. COBALT: Multiple Alignment Tool, https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi, U.S. National Library of Medicine. U.S. National Library of Medicine. [23]

Motif Scan, MOTIF. Motif scanning means finding all known Motifs that occur in a sequence. I chose to run motif scans in order to determine whether there is any overlap in the functions of all of the toxins as they all act upon the same receptor. If similar Motifs were found across the board, it would essentially pinpoint the inhibiting agent in the toxins. Upon pasting in a protein sequence, in this case the Fasta file, collections of Motifs such as PROSITE, Pfam, and NCBI-CDD were scanned through. In order to identify similarities between the 10 toxins that are all inhibitors of the sodium ion channel Nav 1.7, the softwares Motif Scan and MOTIF were run on all of the toxins, as well as the receptor itself. Both databases serve the same purposes, therefore serving as backup/reassurance when checking for Motifs. Motif Search, GenomeNet, https://www.genome.jp/tools/motif/, Motif scan [24]. Motif Scan, Expasy, http://cgl.ucsf.edu, Swiss Bioinformatics Resource Portal [25].

SWISS-MODEL**.** Swiss-Model is an automated comparative protein modelling server. The tertiary structure modelling function allows for the colour scheme highlighting of cysteine. The reason why I ran the modelling was for the purpose of verifying the presence of cysteine even in toxins that did not present with cysteine rich profiles. These structures were generated using the Fasta sequences obtained through the Uniport database. SWISS-MODEL, https://swissmodel.expasy.org, Swiss Bioinformatics Resource Portal [26].

RCSB PDB, Uniprot**.** The Protein Data Bank is a database that allows for advanced searches for what in this case were the codes for SCN9A as well as the two toxins used later on for docking purposes. I used these databases in order to obtain the PDB codes and files for the docking process which required the receptor and the ligands. Uniprot was used as a secondary source as a means of verification, allowing me to check for the lowest resolution score to utilize in the docking process. The Protein Data Bank, https://www.rcsb.org [27].

ClusPro**.** ClusPro is a web server for protein-protein molecular docking that presents as a simple home page requiring only two files in PDB format for basic use. Docking itself is a jigsaw-like assembly where the receptor and the ligand are brought to a stable state once together. It requires the input of a receptor molecule, which in this case was the SCN9A gene PDB code: 6j8g as well as the ligand molecule which was either the 2m4z with the cysteine rich profile or the one without, being 5t4r**.** The reason I used this software rather than other was because it was the most user-friendly after having made use of other softwares. ClusPro, https://cluspro.org, Vajda Lab and ABC Group, Boston University and Stony Brook University [28].

AutoDock Vina, UCSF Chimera**.** These two programs were run in combination in order to view the solution.pdb file generated above through the ClusPro docking algorithm. Autodock Vina itself is an open-source program for doing Molecular docking, and can be run through UCSF Chimera which is a program for the interactive visualization and analysis of molecular structures and related data. Through the use of these two programs, I had the ability to open and view the docking solutions for both sets of the receptor and ligands.

Autodock Vina, http://vina.scripps.edu [29]. UCSF Chimera an Extensible Molecule Modeling System, https://www.cgl.ucsf.edu/chimera, Regents of the University of California [30].

Discussion

My proposal suggests looking into toxins being studied more seriously as models for analgesic medications and chronic pain treatments. The set of toxins used in this work are those of 10 from various kinds of animals: 1 Centipede, 2 Tarantulas, 1 Cone snail, 3 Spiders, 1 Pufferfish, and 2 scorpions that were selected for means of diversity [31-40]. This said diversity was an important factor in deciding which animals were included to eliminate as much overlap as possible, assuming that too many of the same species or kind of animal would have made it all the more difficult to pinpoint what exactly the inhibiting factors of the venoms were. By including this diversity, the similarities that did indeed show up would be more accurate indications, and be of greater relevance to the aim of the study.

Multiple alignment was performed using Cobalt for amino acid sequences from venomous animal toxins. Alignment of the complete toxins indicating cysteine enriched domains indicated by the red box. Conserved sites such as hydrophobic or positively charged amino acid residues involved in are highlighted in blue. The regions implicated in the secondary structure were predicted helices and beta sheets are shown in yellow or red motifs on Table 3 and docking experiments as it follows. (See Table 3.)

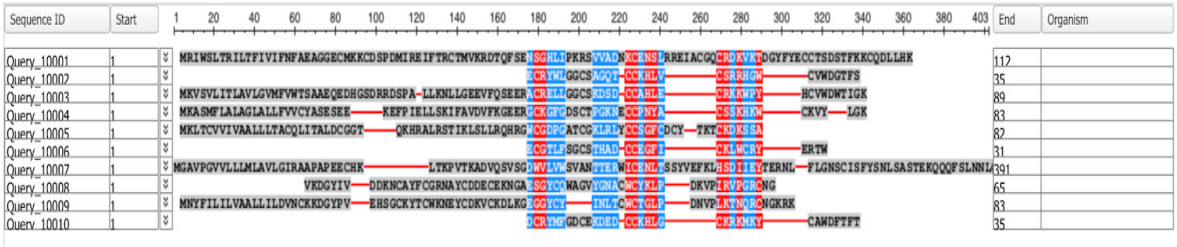


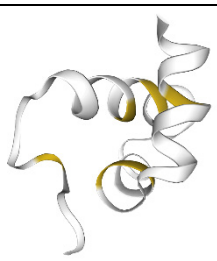
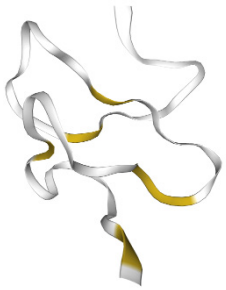
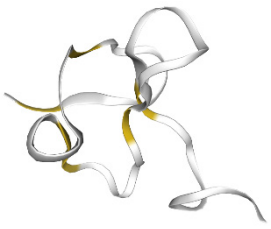
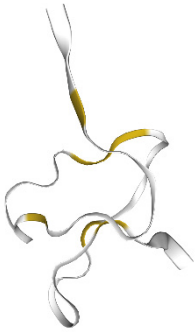
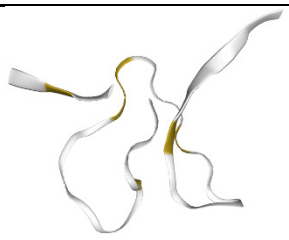
Figure 3. Cobalt Multiple Alignment

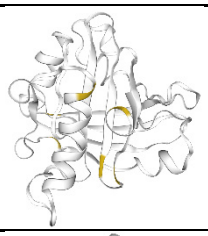
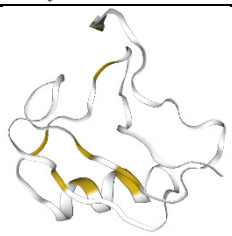
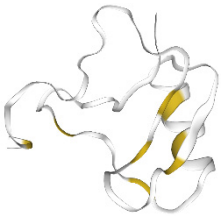
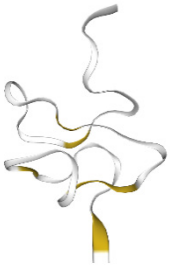
Additional preliminary findings include information gathered on Motifs, specifically ones regarding cysteine. As clearly mentioned above, Motif scans were run in order to determine any overlap in the functions of all of the toxins as they all act upon the same receptor. If similar Motifs were found across the board, it would ideally pinpoint the inhibiting agent of the toxins which could then prove helpful in future modeling endeavors for pain medication.

A similar Motif identified in two of the toxins were cysteine-rich profiles. Upon running the Swiss-model program, the appearance of cysteine in all of the toxins with only 2 showing actual Motifs prompted a question for what exactly it was that was similar about all the toxins that indeed inhibited the VGSC NaV 1.7 (See Table. 3.). Surprisingly, the amino acid cysteine was indeed present in all of the toxins although not all of them housed a cysteine-enriched profile in their Motif results, urging the need for a means of comparison between ligands with and those lacking cysteines, in their bonding to the receptor. For this very reason, scans on Scolopendra subspinipes that present with the profile (See Fig. 5.), and Pamphobeteus Nigricolor that presents without it, (See Fig. 4.) were run.

Table 3. Swiss Model Tertiary Structures and Cysteine Amino Acids

Organism	Toxin - Protein	Swiss Model: Cysteine	Motif for Cysteine- rich profile

Scolopendra subspinipes (Vietnamese Centipede)	Mu scoloptoxin (03)-Ssm2a Structure: Mu-SLPTX-Ssm6a		prf:GLG1_C_RICH *Cysteine-rich GLG1 repeated profile.*
Thrixopelma Pruriens (Peruvian Green Velvet Tarantula)	Beta/omega-theraphotoxin-Tp1a		N/A
Phlogius sp. (Tarantula Spider)	Mu-theraphotoxoin-Phlo1a		N/A
Cyriopagopus hainanus (Chinese bird spider)	Hainantoxin-111 9		N/A
Conus Geographus (Geography Cone)	Mu-conotoxin GVIIJ		prf:CYS_RICH *Cysteine-rich region profile.*
Heteropoda venatoria (Brown huntsman spider)	Kappa-sparatoxin-Hv1c		N/A

Pakifugu pardalis (Panther puffer)	psbp1		N/A
Mesobuthus martensii (Manchurian scorpion)	Neurotoxin BmK AGAP-SYPU2		N/A
Centruroides vittatus (Striped bark scorpion)	Alpha-toxin Cv1V4		N/A
Pamphobeteus Nigricolor (Giant blue bloom tarantula)	Mu-theraphotoxin-Pn3a		N/A

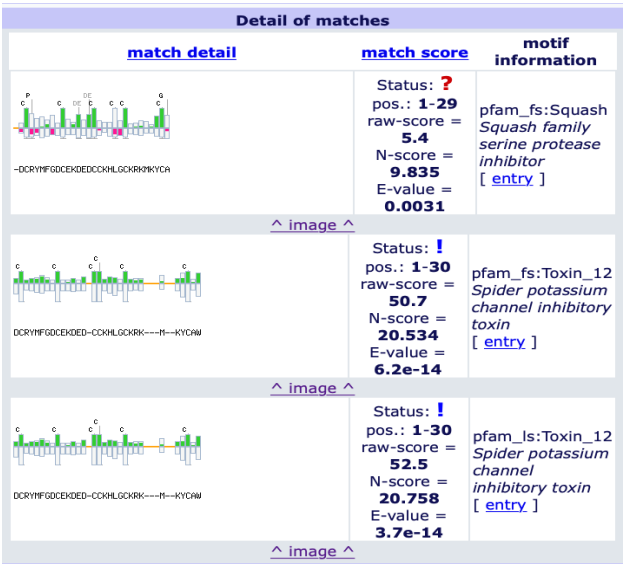


Figure 4. 5t4r Motif Scan

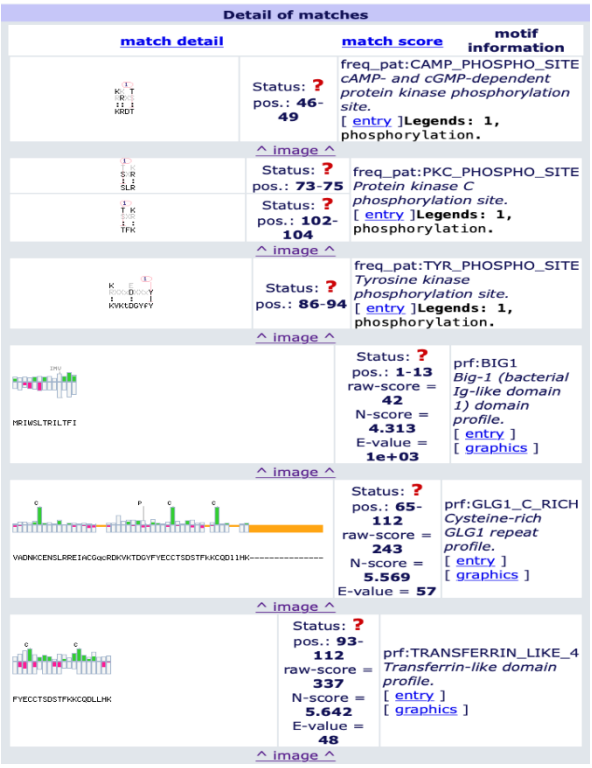
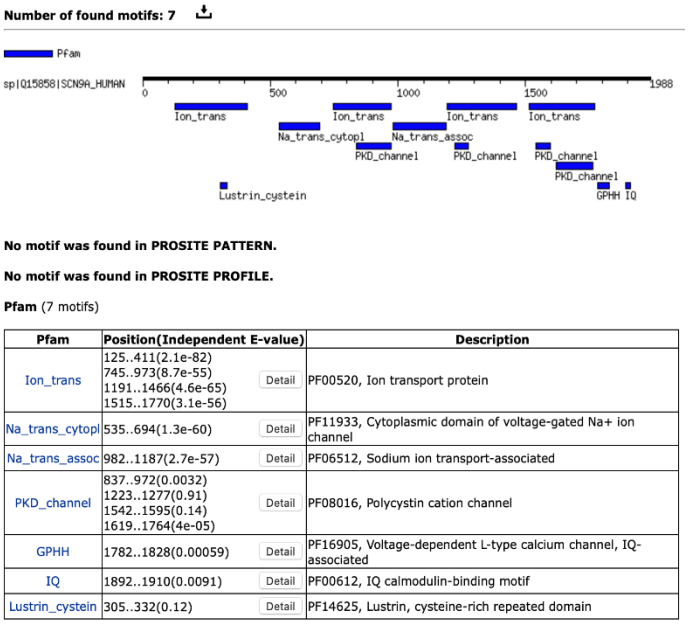


Figure 5. 2mz4 Motif Scan

A scan was also run on the voltage gated sodium ion channel located on the SCN9A gene. The results from this scan were a cysteine-rich repeat domain, making cysteine residues a valid area for investigation. (See Fig. 6.) In addition to this, the detailing on this very domain's amino acid sequence is revealed in the following figure. (See Fig. 7.)



Pfam	Position(Independent E-value)		Description
Ion_trans	125..411(2.1e-82) 745..973(8.7e-55) 1191..1466(4.6e-65) 1515..1770(3.1e-56)	Detail	PF00520, Ion transport protein
Na_trans_cytopl	535..694(1.3e-60)	Detail	PF11933, Cytoplasmic domain of voltage-gated Na+ ion channel
Na_trans_assoc	982..1187(2.7e-57)	Detail	PF06512, Sodium ion transport-associated
PKD_channel	837..972(0.0032) 1223..1277(0.91) 1542..1595(0.14) 1619..1764(4e-05)	Detail	PF08016, Polycystin cation channel
GPHH	1782..1828(0.00059)	Detail	PF16905, Voltage-dependent L-type calcium channel, IQ-associated
IQ	1892..1910(0.0091)	Detail	PF00612, IQ calmodulin-binding motif
Lustrin_cystein	305..332(0.12)	Detail	PF14625, Lustrin, cysteine-rich repeated domain

Figure 6. NaV 1.7 Motif Scan

Figure 7. Cysteine Domain

For instance, peptide toxins that hold considerable promise as novel therapeutics are often cysteine-rich and contain intricate disulfide bond patterns that are crucial for their biological activity [41]. According to papers written regarding the analgesic effects of N-Acetyl-cysteine, I became encouraged to determine whether or not the cysteine-rich profiles were exercising the same effect in the same manner [42, 43]. However, I then discovered that N-Acetyl-cysteine as it is derived from one single cysteine, is a free small molecule that would have been acting upon the receptors. Yet in the case of the cysteines within the toxins structure, or within the receptors/channels, they are a part of the amino acid sequences making peptide bonds, therefore un-free.

As mentioned above, the sodium ion receptors include numbers from 1.1-1.9, with all serving as different receptors to target for the analgesic effects of medications. The Motif scans were run on all of the other receptors as well, however only the NaV 1.7 receptor presented with the cysteine. The reality is that not all inhibitors of the NaV 1.7 VGSC have the same cysteine Motif, exposing other Motifs to be at work as analgesics. (See Table. 2.)

Table 2. Cysteine Domain Motif Scan of Sodium Channels

Isoform-Sodium Receptor	Gene	Cysteine
NaV 1.7	SCN9A	PF14625, Lustrin, cysteine-rich repeated domain
NaV 1.8	SCN10A	N/A
NaV 1.9	SCN11A	N/A
NaV 1.3	SCN3A	N/A
NaV 1.6	SCN8A	N/A
NaV 1.1	SCN1A	N/A
NaV 1.5	SCN5A	N/A

The program ClusPro was run for the purpose of docking the ligands and receptors. For the receptor, the PDB code input was 6J8G. As for the toxins, in this case known as ligands, 2mz4 and 5t4r were used. These two ligands are different in that the 2mz4 does have the cysteine-rich repeated profile Motif whereas the 5t4r does not. This difference was important as running the comparative docking would allow for the determination of cysteine's true relevance to the process of inhibition.

Upon inputting the PDB files into ClusPro, 70,000 rotations exhausted all docking possibilities for each input in order to crank out the 10 best fits as shown in the model display section. The 5t4r docking is shown on the left (see Fig. 8.) and the 2mz4 docking is shown on the right (See Fig. 9). Essentially this was done to find the best free energy state in which the receptor and ligand binding was the most stable. Therefore, the figure labeled “zero” is the closest to perfection -so to speak- that we were able to get the molecular docking. Furthermore, in the Model Score tables, the “members” categorization is there to once again point out the most ideal imaging, with #0 for 5t4r being 190 (See Table 3.) and the #0 for 2mz4 being 156 (See Table 4.). As for the environment in which they are docked, a balanced one was selected as sufficient for the purposes of this project.

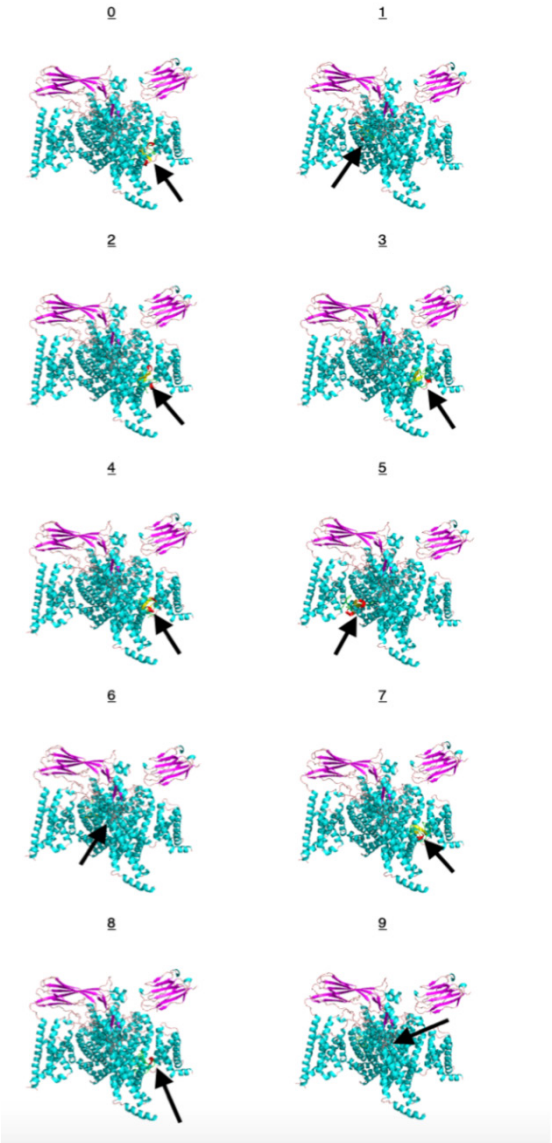


Figure. 8. 5t4r Docking Results

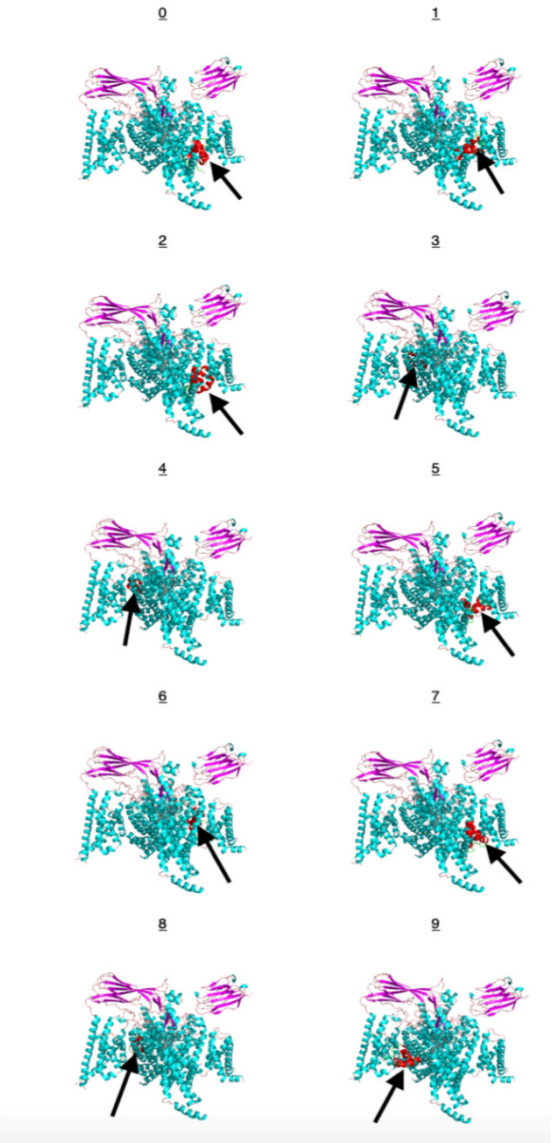


Figure. 9 2mz4 Docking Results

Table 3. 5t4r Model Score Table

Cluster	Members	Center Weighted Score
0	190	-1275.2
1	149	-1285.6
2	116	-1263.7
3	114	1265.2
4	93	1263.9
5	76	1274.0
6	69	-1290.7
7	51	1293.3
8	44	1304.5

9	35	1284.6

Table 4. 2mz4 Model Score Table

Cluster	Members	Center Weighted Score
0	156	-1047.8
1	131	-1354.2
2	83	-1044.5
3	80	-1070.3
4	68	-1223.7
5	63	-1051.7
6	59	-1279.6
7	43	-1046.4
8	35	-1115.8
9	35	-1218.8

From the ClusPro generations, it was apparent that the two toxins binded to the receptor in nevertheless the same location. Therefore, it remains inevitable that there are other biochemical properties at work that lead to the inhibiting outcomes of the toxins besides disulfide bonds to say the least. Cysteine is more profoundly known for stabilizing the tridimensional structure of proteins by way of forming disulfide bonds that then act as safety pins keeping parts of the polypeptide firmly attached to one another [44]. Therefore, it is imperative to recognize the roles of things like hydrophilic or hydrophobic properties as well as polar and nonpolar ends of the toxins [45]. These are other biochemical properties that could potentially point to what is acting as the major inhibiting factor of the Voltage-Gated Sodium Channel NaV 1.7.

Conclusion

Through the utilization of multiple bioinformatics databases and software, the general findings outlined in this paper regarding the generation of in-silico models prompts need for validation through in-vivo/in-vitro methods. The molecular docking of the receptor and ligands proved the necessity for research into the existence of other Motifs and factors at play that are truly accomplishing the analgesic effects that inhibition has on nociceptive pain.

As initially mentioned, chronic nociceptive pain is in need of newer and updated treatment options void of the side effects that come with opioid-dependency. However, the plethora of resources is not limited to the use of in-silico molecules, and includes promising development in toxin-based drug development.

We have explored a small set of in silico techniques to run pilot experiments to select toxins of interest. Along with bioinformatic analysis, the next step to investigate the potential of these molecules on pain receptors and ion channels modulation is to perform in vitro and in vivo models. A thorough experimental pipeline using derived or similar peptides based on the simulations is critical to prospect real candidates to modelling synthetic drugs.

To test toxins activities in biological systems is the ultimate step aiming to evaluate their applications in analgesia and possible desirable modifications for better specificity, biosafety, and pharmacological efficiency. Conclusively, this study has worked to prove the significance of proposing toxins as molecular models for pain medication ultimately targeting chronic nociceptive pain conditions. We encourage others to believe in the reality of a promising future with toxins in the battle against chronic pain.

Acknowledgments

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