Identifying Convergence of ShK Toxins in Sea Anemones

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Abstract: Toxins and other naturally derived products synthesized for predatory defense or prey capture can often be harmful to humans, but in some cases they may also present pharmaceutical potential. One toxin in particular, commonly referred to as ShK, has been isolated from the sea anemone *Stichodactyla helianthus* and exhibits potential to treat autoimmune diseases such as multiple sclerosis. The currently existing ShK toxin as well as its analogs are not ideal in their target and treatment, exhibiting some flaws regarding effective transport and binding to Kv1.3 channels. Toxins containing this domain are ubiquitous across sea anemones as they target potassium ion channels, potentially being used to immobilize prey or deter predators. We hypothesized that there may be variations of the toxin that are naturally produced by other species. Our bioinformatic approach has found 586 ShK toxin candidates in 23 sea anemone species. Our results are the first step towards identifying ShK proteins that could combat various types of autoimmune or even neurological diseases.

1. Introduction

Sea anemones are members of the phylum Cnidaria, which is characterized by the presence of stinging cells called nematocysts that serve as a vehicle for injecting venom into the predator or prey target. On a molecular level, sea anemone venoms are complex cocktails of toxic peptides and proteins (Norton, 2009, Ashwood et al. 2020). Sea anemone toxins are not very well characterized and therefore categorizing them can be difficult (Prentis et al, 2018), but they can generally be subdivided into three classes: neurotoxins that act on sodium channels, neurotoxins that act on potassium channels, and actinoporins that act on cell membranes (Honma and Shiomi, 2006). The ShK protein we are interested in is a potassium channel neurotoxin originally isolated in 1995 from the Caribbean sea anemone, *Stichodactyla helianthus* (Castaneda et al, 1995). ShK is a small, 35-residue polypeptide characterized by six

cysteines at positions 3, 12, 17, 28, 32, and 35 (Norton, 2005, Shafee et al. 2016). These cysteines form three disulfide bonds that contribute to the relatively simple structure indicative of ShK (Pohl et al, 1994).

ShK poses a pharmaceutical interest due to its ability to block the voltage-gated potassium channel Kv1.3. This channel is found in multiple different human body cells, but only in lymphocytes does it dominate the membrane potential (Kalman et al, 1998). The immune system has a variety of lymphocytes that can be categorized into natural killer cells, B-cells, and T-cells. In a quiescent state, Kv1.3 role is to prevent membrane depolarization of T-cells, if there is no presence of an antigen (Calahan and Chandy, 2009). Kv1.3 is activated when a T-cell recognizes an antigen-presenting cell (APC) and begins to differentiate. Calcium ions flood into the cell as a response to the APC, and Kv1.3 helps keep the membrane polarized to allow more calcium ions to enter the cell and propagate differentiation into specialized T-cells that will fight the specific antigen (Chandy et al, 2004).

T-cells can be further divided into naive, central memory (T_{CM}), and effector memory (T_{EM}) cells (Beeton et al, 2011). Naive cells are lymphocytes that have not yet been “trained” to seek out and destroy pathogens. T_{CM} cells signal an immune response from another part of the body such as secondary lymphoid tissue, whereas T_{EM} cells directly attack the pathogen due to their cytotoxic properties (Mahnke et al, 2013). T_{EM} cells differ structurally from other T-cells by presenting many more Kv1.3 channels than other potassium channels in their cellular membranes (Chandy et al, 2004, Norton et al, 2005). Kv1.3 is crucial for the activation of T_{EM} cells because it allows the cells to proliferate after they have recognized an antigen. When Kv1.3 is blocked, the membrane becomes depolarized and inhibits calcium signalling, preventing the ions from entering the cell and as a result, preventing T-cell differentiation

(Calahan, Wulff, & Chandy, 2001). In autoimmune diseases, the “antigen” recognized by T-cells is a body cell. Therefore, if ShK can bind to T-cells that would have targeted the body cells, then the T-cells would not recognize those cells as an “antigen”.

Not many pharmaceutical or clinical trials have been conducted on potassium channel blockers due to questions about the safety of the various treatments, though the ones that have been conducted have either revealed negligible or positive results (Judge & Bever, 2006). ShK is effective in binding to Kv1.3 channels, but the problem of lack of selectivity arises. The protein also binds to other voltage-gated potassium channels: Kv1.1, Kv1.4, and Kv1.6 (Calahan, Wulff, & Chandy, 2001). Kv1.1 is found in the heart and central nervous system and controls neuron excitability (Kalman et al, 1998). This may not be an issue if ShK could be specifically geared towards targeting T-cells, but because T-cells are in the blood, any potential ShK treatment would have to be administered in the blood, which flows throughout the body and into the heart. Additionally, an immunocompromised person who would need the treatment would have a weakened immune system to begin with, increasing the likelihood of ShK passing through the blood-brain barrier and into the central nervous system (Norton et al, 2005).

In order to account for these issues, research regarding synthetic analogs to ShK has been conducted. Multiple analogs have been produced to try to select specifically for Kv1.3, but where selectivity issues with some are solved, more issues arise with other channels. The most promising analog thus far has been ShK-186, or dalazatide. Though other ShK analogs have been tested against autoimmune diseases, dalazatide is the first Kv1.3 inhibitor to be tested in clinical trials for autoimmune diseases and as of 2017 had just completed its phase 1b clinical trial (Tarcha et al, 2017). No information since then regarding proceedings in clinical trials has been published. The most common model organism has been rats with experimental

autoimmune encephalomyelitis (EAE), the rodent equivalent of multiple sclerosis (MS) in humans (Schmitz et al, 2015).

Up to this point, most trials and tests have been conducted using synthetic ShK analogs if not *S. helianthus* ShK itself. Relatively no work we could find has explored the possibility of a natural ShK analog that may be more effective. We have used a variety of bioinformatic tools to search for convergent ShK toxin analogs within transcriptomesof other anemones that have currently not been identified. We specified convergent rather than homologous proteins because homologous proteins do not necessarily exhibit the same function despite having a common ancestor, whereas convergent proteins do have the same function. We hypothesized that there exists at least a few convergent ShK proteins that have not yet been identified.

2. Results

We identified 586 ShK candidates across all 24 sea anemone transcriptomes. The program CLANS recovered four different clusters based on their overall similarities across focal transcripts (Figure 1). Only two of the clusters (cluster 1 and cluster 4) assembled with the query sequences, while cluster 2 and 3 did not include any of the original query sequences. The number of sequences across sluters varied Cluster 1 was the most diverse with 311 sequences and included every sequenced species. Cluster 4 was the least diverse with only 14 sequences, all of which were from *L. danae* (Table 1). There were 26 ShK-like sequences that exhibited sequence variation beyond the 4 clusters that had formed and did not become part of a cluster.

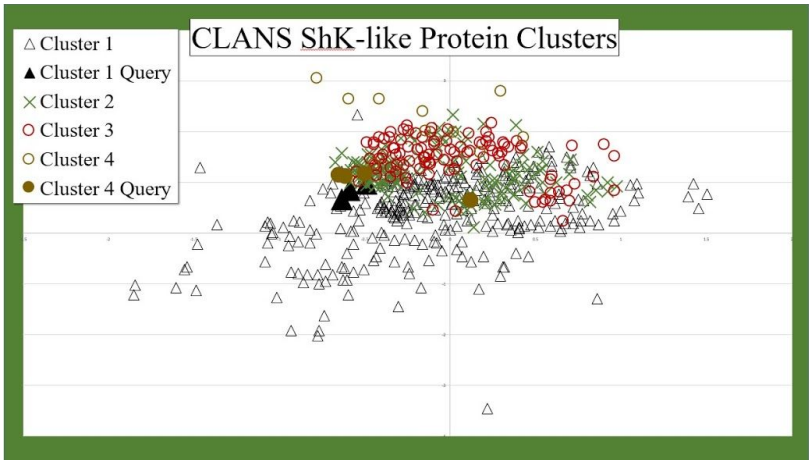

Figure 1. Qualitative representation of the clusters contents and how they relate to each other by similarity

Overall sequence similarity of the remaining 560 sequences were highly variable. The position of the six cysteines are conserved across all clusters (Figure 2), however, the number of amino acids between the 3rd and 4th cystine seemed to vary across clusters (Figure 2). Among each cluster there were conserved amino acids that may carry functionally important roles. For example, lysines between the fourth and fifth cysteine were conserved across all clusters, a region that has been characterized as functionally important for binding (Pennington et al. 1996). Additionally, several members of each cluster exhibit tryptophan, lysine, and glycine between the second and third cysteines.

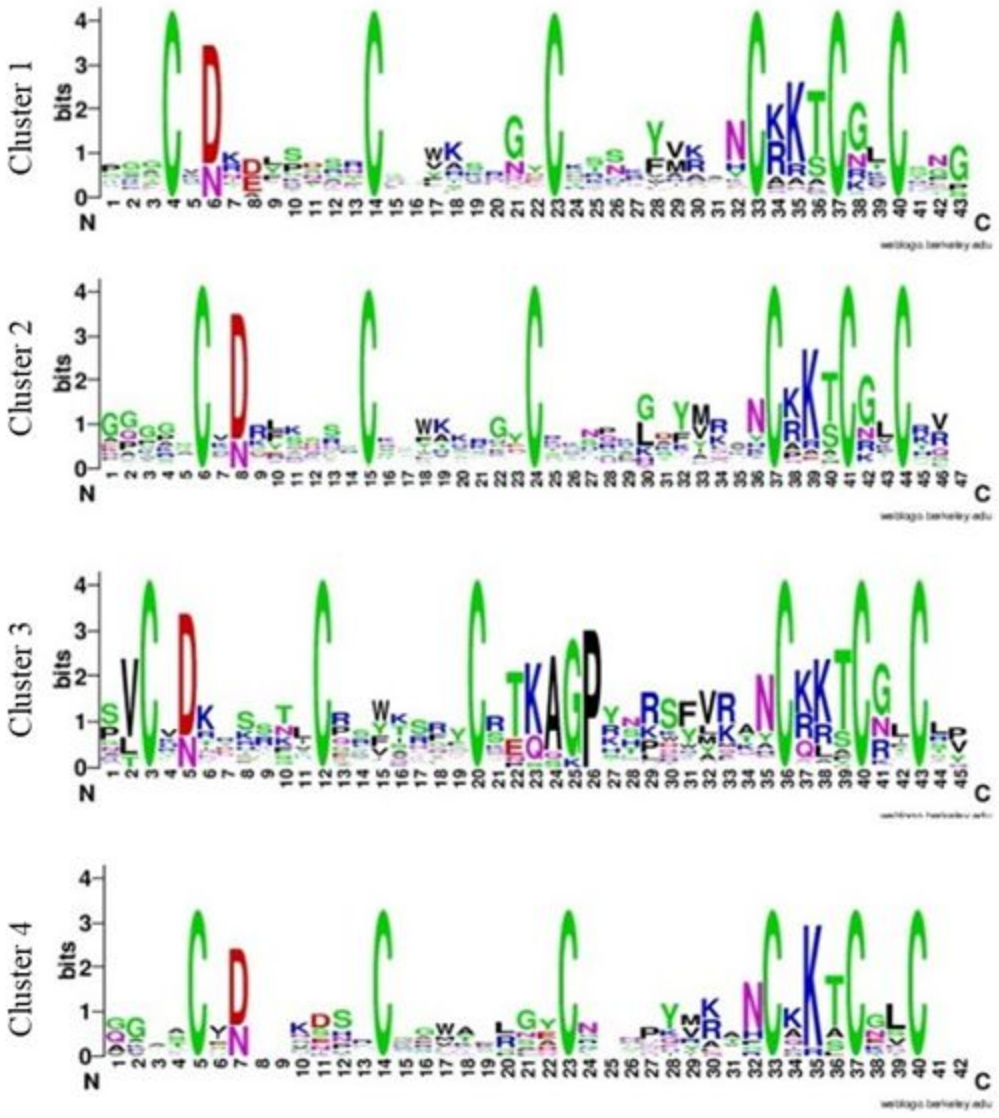

Figure 2. WebLogo visual representations of cluster 1-4

3. Discussion

The ShK toxin domain remains largely uncharacterized despite it being ubiquitous across sea anemone species. The presence of so many ShK candidates provides a substantial chance of there being an efficient pharmaceutical to combat autoimmune disorders and other neurological diseases. The number of functionally characterized proteins is low, however, our study highlights the necessity for further characterization into the protein function as there is

tremendous sequence diversity across all anemone species examined. Because toxin protein sequences such as those belonging to families like ShK are so small and variable, the resulting candidates may look structurally similar while having diverse functions.

ShK domains are prevalent across venomous and non-venomous animals (Shivers et al. 2008), therefore, it is highly likely our 586 are not all venom components. In order to screen functionality, these candidates require further analysis to determine whether further exploration is warranted. Shafee et al. (2019) attempted to sort this highly ubiquitous protein domain by characterizing details about each individual protein such as hydrophobicity, charge, and molecular mass. We could use a similar approach along with the currently characterized ShK members to help narrow down toxin candidates to a compressed list for more in-depth investigation. Ultimately, the best way to learn if a protein will serve as a good autoimmune disease treatment is to test its function in a laboratory setting. Once candidates are more thoroughly filtered, those that still prove to be possible candidates would be worth testing.

Potential errors include inconsistencies of protein sequences between databases. For example, upon BLASTing the query sequence thought to be from *S. helianthus* according to its UNIPROT ID, BLAST yielded the result to be from *S. mertensii* instead. Better documentation of can only be accomplished with further work being done to sequence these highly variable proteins.

4. Materials and Methods

We used Geneious Prime software to conduct a BLAST search using the tblastn program (Altschul et al, 1990) against 24 unpublished sea anemone transcriptomes representing broad diverse sea anemone taxa (Table 1). The sea anemone transcriptomes

were previously sequenced using Illumina Paired End Sequencing (100 bp PE) and assembled in Trinity version 3.14 (Grabherr et al, 2011).

Table 1. Taxonomic diversity of sea anemones surveyed in this study

Superfamily	Species	N	Cluster
Edwardsioidea	*Edwardsiella elegans*	52	1,3
Actinostoloidea	*Stomphia coccinea*	29	1,2
Actinioidea	*Epiactis prolifera*	16	1,2
	Actinia equina	41	1
	Anemonia sulcata	46	1
	Condylactics gigantea	11	1
	Bunodosoma cavernata	21	1,3
	Anthopleura elegantissima	55	1
	Heteractis crispa	16	1,2
	Entacmaea quadricolor	11	1,2
	Macrodactlya doreensis	14	1,2
Metridioidea	*Bartholomea annulata*	18	1
	Exaiptasia pallida	24	1,2,3
	Calliactis polypus	11	1
	Andvakia discuplorum	33	1
	Haloclava producta	19	1,2
	Metridium senile	22	1,2
	Diadumene leucolena	32	1
	Diadumene lineata	31	1,3
	Bunodeopsis globulifera	13	1
	Lebrunia danae	39	1,2,4
	Sagartia elegans	19	1
	Traictis producta	13	1

Previously characterizedShK-like proteins from from *Bunodosoma granuliferum, Stichodactyla helianthus, Aurelia aurita, Anemonia sulcata, Actinia equina,* and two from *Anemonia viridis* were used as BLAST search queries (Figure 3). These sequences were BgK (Cotton et al, 1997), aurelin (Ovchinnikova et al, 2006), AsK132958 (Krishnarjuna et al, 2018), AeK (Minagawa et al, 1999), avtx-6 (Sabourault et al, 2009), and avtx-10 (Sabourault et al,

2009).Two species, *Exaiptasia pallida* and *Lebrunia danae/neglecta* were BLASTed twice because different tissues of those species were sequenced. The query sequences were selected based on highly variable amino acid composition, the presence of three disulfide bonds, and Kv1.3-blocking properties. BLAST hits were visually inspected and removed if they did not include six cysteines that were within 35 amino acids of each other or a stop codon within 3-4 amino acids of the last cysteine.

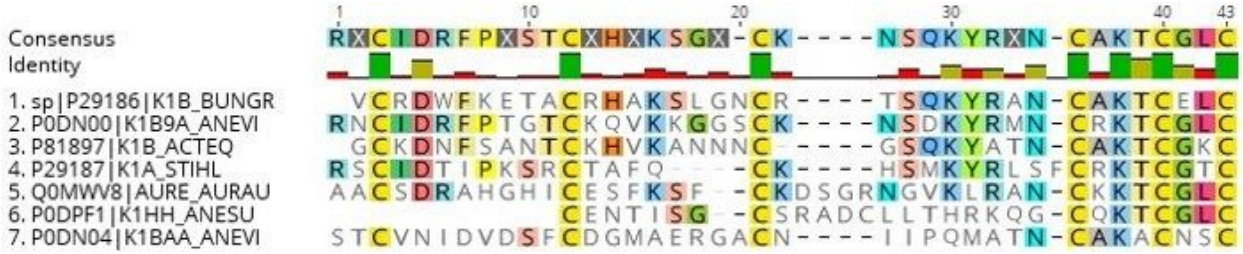

Figure 3. Query sequence alignment

BLAST hits were considered candidate ShK toxins if the hit included the six cysteines but another 6-cysteine sequence further down the transcriptome included the stop codon. Similarly, if the nearest start codon occurred before an ShK-like 6-cysteine sequence that BLAST did not identify as the hit, both the hit and the additional sequence were included so as to also include the start codon. All BLAST results were translated to protein sequences. Transcripts lacking these regions were incomplete, but were still considered if the result did not include a start codon due to the presence of stop codon before a start codon or if the first structurally important cysteine appeared out of alignment excluding any stop or start codon. All translated sequences had to include the BLAST search hit even if the portion with the stop codon was different. Any duplicate sequences were moved to a separate folder.

The candidate ShK-like sequences were further analyzed in the application CLuster ANalysis of Sequences (CLANS), a program built in Java and used to group and sequence

toxins by their similarities to each other (Frickey and Lupas, 2004). The resulting clusters were aligned using MAFFT alignment software (Katoh and Standley, 2013) in Geneious Prime. The algorithm was set to auto, the scoring matrix was BLOSUM62, the gap open penalty was 1.53, and the offset value was 0.123. The resulting alignments were screened again for the alignment of structurally important residues and visualed in WebLogo (Crooks et al, 2004)

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