SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Characterization of scorpion peptide toxins that target voltage-gated potassium channels

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1. INTRODUCTION

1.1 Ion channels

Ion channels are integral membrane proteins, made up of multiple subunits that form a hydrophilic pore, enabling specific inorganic ions to pass through the plasma membrane according to their electrochemical gradients. Ion channels are efficient transporters with a transfer rate of one million ions/second. They are essential for cellular functions such as regulating membrane potential, muscle excitation, action potential generation, maintaining cell shape and volume, sensory transduction, blood pressure control, hormone secretion, and more. Apart from plasma membrane, they also exist in intracellular organelle membranes, including mitochondria, lysosomes, the nucleus, vesicles, and the endoplasmic/sarcoplasmic reticulum. Ion channels exhibit ion selectivity, allowing certain inorganic ions to pass while restricting others and contain a selectivity filter that aids in limiting the passage of ions. The classification of ion channels is based on; their ion selectivity (K^+, Na^+, H^+, Cl^- etc.); gating mechanisms (such as voltage, signal, ligand, etc.); and the direction of ionic current (outward-, inward- or non-rectifier). From a functional point of view, ion channels can be in two states depending on whether the pore is permeable to ions or not, i.e. they are present in a conducting state (open) and a non-conducting state (closed or inactivated).

1.2 Voltage-gated potassium channels (Kv)

Kv channels is the largest, evolutionary conseved and diverse family in the potassium channel group, possesing high K^+ selectivty. Kv channels are formed by the assembling of four α-subunits which are arranged in circumference to form a VSD from the first 4 helices (S1-S4) and a central pore domain (PD) from S5-S6 helices with connecting pore loop of each α-subunit of tetramer. The PD is responsible for conduction of K^+ ions. Kv channels are encoded by 40 genes in humans and are divided into 12 subfamilies (Kv1–Kv12). Each subfamily exhibits significant diversity due to variations in subunit composition, associated beta subunits, regulatory modifiers, and post-translational modifications. Based on the conduction Kv channels are divided into two groups: (i) the conductive group which includes; Kv1.x–Kv4.x (*KCNA* to *KCND*), Kv7.x (*KCNQ*), Kv10.x (Eag), Kv11.x (Erg), and Kv12.x (Elk) subfamilies; and (ii) the non-conductive group, which functions as gating modulators. Based on their biophysical properties of conductive group, we distinguish between slow or non-inactivating channels called as delayed rectifiers and rapidly inactivating channels that create type A currents. Moreover, several other factors also contribute in the functional diversity of Kv channels; 1) expression of subsets of Kv genes in different cell types, 2) heteromultimerization of members of same subfamily or with the silent subunits of the Kv family and 3) interactions with accessory β-subunits.

The Kv1 is a Shaker-related family of K^+ channels and composed of 8 members Kv1.1–Kv1.8. The Kv1 family have characteristic low threshold voltage-gated K^+ currents which are ideal for regulating the duration of frequency of action potential, excitability of neurons, maintaining the pattern and timing of neuron spikes and synaptic and axonal transmissions. Kv1 ion channels are widely localized in brain and the diversity and complexity arises due to the formation of heterotetrametric channels which leads to distinct functional features of ion channels. Kv1.2/Kv1.6 heterotetramers present in somatodendritic region and play a key role in regulating action potential whereas, in hippocampal pyramidal neurons Kv1.1/Kv1.2 heteromeric channels exist and are involved in integrating synaptic inputs.

1.2.1 Ca^{2+}-activated potassium channels (KCa)

Ca^{2+}-activated K^+ channels (KCa) are activated by a rise in the intracellular calcium levels. They are categorized into two groups; the first group contains small- and intermediate-conductance K^+ channels also called SKCa, respectively, whereas the second group contains the large conductance channels also known as Slo (BK). The unitary potassium conductance of SKCa channels is 10-40 pS, and in contrast, Slo channels have high conductance ranges between 200-300 pS.

The SKCa channel family consists of two subfamilies KCa2.x and IKCa1. The first subfamily (KCa2) has three subtypes; KCa2.1, KCa2.2, and KCa2.3 and are encoded by encoded by *KCNN1*, *KCNN2* and *KCNN3* genes, respectively. These channels share a high degree of similarity in their transmembrane domains ranging from 80% to 90% but differ significantly in their N- and C-terminal regions. KCa2 channels are predominantly found in the nervous system and therefore, mainly contribute in the regulation of neuronal excitability. In contrast, the IKCa1 subfamily includes a single member, KCa3.1 and is encoded by *KCNN4* gene. KCa3.1 channels are majorly express in lymphocytes, red blood cells, smooth muscle and fibroblasts. In lymphocyte, in addition to Kv1.3 channels, KCa3.1 is also involved in activation and proliferation by maintaining a negative membrane potential. This channel shares only about 40% sequence similarity with the KCa2.x subtypes and is predominantly expressed in non-neuronal peripheral tissues. Both KCa2.x and KCa3.1 channels are structurally similar to voltage-gates K^+ channels, but they contain relatively fewer positive charges in S4 segment of α-helix (VSD), making them insensitive to voltage changes in the transmembrane. These channels are activated by intracellular Ca^{2+} through calmodulin (CaM) that constitutively bound to intracellular C-terminus of channel.

Slo channel family differ from Kv and KCa2 channels as they have 7TM segments, an extra segment in the TM α-helix termed as S_0, causing the N-terminus of the channel to be localized extracellularly. These channels are activated by both changes in the membrane potential or by an increase in intracellular Ca^{2+} levels. This channel has two high affinity Ca^{2+} binding sites in each subunit of tetramer and these sites contribute to the formation of a

structural component known as the gating ring, which plays a crucial role in channel activation. Slo1 is a member of Slo family and is also called as KCa1.1 (encoded by *KCNMA1* gene) and is widely expressed across various tissues including smooth and skeletal muscles, mitochondria, kidney, brain and cochlear hair cells. The functional properties vary depending on factors such as alternative splicing, phosphorylation, and interaction with auxiliary subunits. Physiologically, it is involved in processes like smooth muscle cell hyperpolarization and the triggering of neurotransmitter release.

1.3 Structural Insights into the Kv1.2 Potassium Channel

In 1998, a breakthrough in structural biology was achieved with the determination of the first high-resolution crystal structure of a potassium channel KcsA from *Streptomyces lividans*, solved by Roderick MacKinnon and his team. This structure, resolved at 3.2 Å and it provided crucial atomic-level insight into how potassium ions (K^+) are selectively conducted through the channel, advancing our understanding of ion channel function. Regarding the Kv1.2 ion channel, in 2005, a 2.9 Å crystal structure of the channel obtained by Long *et al*. that revealed the overall architecture of the channel. However, VSD domain had relatively weak densities. To improve this, a study in 2007 introduced the structure of Kv1.2–2.1 paddle chimera, in which a part of the voltage-sensor domain (residues 267-302 from S3–S4 paddle), was substituted with the equivalent sequence from Kv2.1 K^+ channel (residues 274-305). The 2.4 Å X-ray structure of Kv1.2-Kv2.1 paddle chimera provided high-resolution insight into the VSDs and became a key reference for structural and computational studies. In 2022, Reddi *et al*. reported the crystal structure of the Kv1.2 channel (by using Kv1.2-2.1 chimera) in a C-type inactivated state (details of channel inactivation below in this chapter). To determine the structure, three mutations W362F, S367T and V377T in Kv1.2-2.1 chimera have been introduced to enhance the cell surface expression of protein and to increase the rate of C-type inactivation. A very recent study in 2024 presented near-atomic-resolution structures of Kv1.2, obtained via cryoEM, in open, C-type inactivated and toxin-blocked states. These are very similar to the previously reported structures of Kv1.2-2.1 chimeric channel.

1.3.1 Biophysical characteristics of Kv1.2

Kv1.2 belongs to the delayed-rectifiers class of ion channels. The conductance of a single Kv1.2 channel ranges between 14-18 pS. For the determination of biophysical parameters of Kv1.2 ion channels patch-clamp electrophysiology is used both in whole-cell and cell-attached patch configurations. Kv1.2, unlike other Kv1 channels, has variable activation gating kinetic. Most studies report that Kv1.2 channels activate rapidly with a half-activation voltage ($V_{1/2}$) between −15 mV and −43 mV (Half activation voltage ($V_{1/2}$) is the membrane potential where the whole-cell K^+ conductance reaches half of its maximal value). However, another study showed a much slower activation kinetics ($\tau \approx 25$ ms at +40 mV, where τ is the

activation time constant of the current, the time required to reach 67.3% of the peak current) and a more depolarized $V_{1/2}$ of +27 mV. This variability in activation kinetics was further investigated by Rezazadeh *et al*. who observed significant differences in the activation gating kinetics of Kv1.2. At +35 mV, 27% of the cells expressing Kv1.2 channels showed rapid activation time course ($\tau = 4.5 \pm 1.7$ ms), 25% of cells showed mixed (biphasic, a mix of fast and slow components) and 48% cells demonstrate slow activation kinetics ($\tau = 90 \pm 6$ ms). Analysis of the voltage-dependence of steady-state activation revealed that cells with fast kinetics exhibited activation threshold potentials at negative voltages with $V_{1/2} = -18.8 \pm 2.3$ mV, while cells with slow and mixed kinetics had more positive $V_{1/2}$ values (16.6 ± 1.1 mV; and 14.5 ± 1.6 mV, respectively). On the other hand, regarding inactivation of Kv1.2, it displays C-type inactivation however with a very slow rate: development of inactivation requires several seconds by a current trace recorded under long depolarization pulse from CHO cells expressing human Kv1.2.

1.3.2 Expression and functions of Kv1.2

Kv1.2 is predominantly present in the CNS mainly in the pons, medulla oblongata, hippocampus, spinal cord and thalamus. They are abundantly present in the axon initial segment (AIS) of human cortical pyramidal neurons. Studies showed that the expression of Kv1.2 channels varies across different brain regions such as the medial layer II neurons, cerebellar cortex, Purkinje cells and corpus callosum have high levels of Kv1.2 mRNA. Kv1.2 channels are crucial for maintaining neuronal excitability and proper synaptic function. They regulate subthreshold excitability and spike timing, particularly in dendrites and axon terminals, influencing action potential initiation and synaptic transmission. Generally, a neuronal action potential begins with depolarization, when the membrane reaches a threshold voltage, triggering voltage-gated sodium channels (Nav) to open and allow Na^+ influx, rapidly depolarizing the membrane potential. After about 1 ms, Nav channels become inactivated. Then, repolarization occurs as slower-opening Kv channels activate, allowing efflux of K^+ ions and restoring the membrane potential. Kv channels remain open slightly longer than necessary, causing the membrane potential to briefly fall below the resting level, a phase called hyperpolarizing afterpotential, before returning to the resting potential. Kv1.2 contribute to the voltage-dependent delayed K^+ current known as the D-type current. This current plays a crucial role by activating at subthreshold membrane potentials, thereby delaying action potential initiation and suppressing repetitive firing. In presynaptic terminals, Kv1.2 channels suppress hyperexcitability following action potential and preserve synaptic fidelity. In myelinated axons, Kv1.2 channels help in maintaining the conduction, nevertheless their overactivity during demyelination can suppress action potential propagation.

1.4 Kv1.3 channel: expression and function

Kv1.3 is also a member of *Shaker* related Kv channel family and bears a high degree of similarity in basic structural organization with other members of Kv1 family. It is broadly expressed in T lymphocytes and thus plays a vital role in the development of immunity. Upon antigen stimulation, T-cell activates and initiates calcium signaling via endoplasmic calcium release, which is followed by extracellular calcium entry through calcium release activated channel (CRAC). Kv1.3 restores the membrane potential that was depolarized by Ca^{2+} entry, thereby maintaining the persistent calcium signaling which regulates the T cell proliferation. Upregulation of Kv1.3 in T-cells has been proven to be a major cause of autoimmune diseases such as multiple sclerosis (MS), type 1 diabetes mellitus (T1DM) and rheumatoid arthritis (RA). Moreover, Kv1.3 are also expressed in microglia, brain immune cells, and its upregulation is a hallmark of neurodegenerative disorders like Parkinson's disease. Since Kv1.3 is involved in multiple channelopathies, specific inhibition of Kv1.3 holds a great potential to cure such diseases.

Several studies showed the applicability of Kv1.3 inhibitors to target autoimmune and neurodegenerative disorder. For example, ShK-186, a selective peptide inhibitor of Kv1.3, has demonstrated therapeutic potential in animal models of multiple sclerosis and rheumatoid arthritis. Moreover, a recent study found that inhibiting Kv1.3 with PAP-1, (small molecule based Kv1.3 inhibitor) reduced inflammation in a primary microglial *in vitro* model and ameliorated disease symptoms in mouse models of Parkinson's disease (PD).

1.5 Comparison of pore and selectivity filter of Kv1.2 with Kv1.3

The Kv1.2 and Kv1.3 ion channels possess a high similarity in selectivity filter and pore regions. The K^+ selectivity determining signature sequence TVGYGD is highly conserved but subtle differences are present in the remaining part of SF. In the outer vestibule, the turret region that consists of the extracellular loop connecting the S5 to the pore helix, significantly varies between Kv1.2 and Kv1.3. The turret region together with the tip of the S6 and the loop connecting the pore helix to S6 forms the toxin binding site where peptide toxins dock and block the channel by occluding the pore from the extracellular side. These structural differences in the PD of Kv1.2 and other closely related Kv1 channels are of great pharmacological importance, as these can contribute to its variable sensitivities towards animal toxins and provide opportunity to design a peptide blocker with high selectivity for single target.

Studies showed that in the case of Kv1.2 ion channel, the filter region predominantly plays a vital role in determining the selectivity of toxin binding. For example, the scorpion toxin Maurotoxin (MTX, α-KTx 6.2) binds Kv1.2 with high affinity (K_d = 0.7 nM), and mutations in the turret region had minimal effect. Simulation studies showed that Kv1.2 turrets bend away from MTX, suggesting that pore region residues are primarily responsible for binding. In

contrast, Kv1.3 is less sensitive to MTX (K_d = 3.3 μM) but a single mutation in the filter region (His to Thr in the "TVGYGDMH" motif) greatly increased its sensitivity (K_d = 0.6 nM). Similarly, Mesomartoxin (MMTX, α-KTx 26.4) inhibits Kv1.2 with K_d = 15.6 nM and shows no affinity for Kv1.1, however mutations in Kv1.1 filter region renders the Kv1.1 sensitivity to Mesomartoxin with similar potency as for Kv1.2 (K_d = 16.6 nM). These examples verify that the filter region of Kv1.2 is critical for toxin binding and selectivity.

1.6 Kv1.2 related channelopathies

Kv1.2, encoded by *KCNA2* gene in human, plays a role in enabling efficient neuronal repolarization following an action potential. The involvement of Kv1.2 in channelopathy was first described Brew *et al.* when *KCNA2* knockout mice display neuronal hyperexcitability and an epileptic phenotype. In another study, a *KCNA2* related missense mutation in mouse diminished Kv1.2 functional expression and caused cerebellar ataxia. Mutations in human *KCNA2* gene could result in either loss- or gain of function (LOF and GOF) of the ion channel activity. A decade ago, de novo LOF and GOF mutations in *KCNA2* were identified for the first time in patients with epileptic encephalopathy, providing clear evidence that Kv1.2 plays a significant role in neuronal channelopathies.

1.6.1 Kv1.2-related GOF mutations in Epileptic encephalopathies

Epileptic encephalopathies (EE) represent a diverse group of severe childhood-onset neurological disorders, typically marked by intractable epilepsy and progressive cognitive and neurological impairments. In 2015, for the first time, Syrbe *et al*. reported two Kv1.2-related GOF mutations in two different patients suffering from EE i.e., R297Q and L298F. Both these mutations exist in the S4 segment of the α- subunit and it has already been established that S4 segments make up the VSD of the ion channel and thus play a vital role in the activation of the channel. Both patients were presented with severe intellectual disability, moderate to severe ataxia and seizures. Upon studying biophysical characteristics, it was revealed that both R297Q and L298F mutations in Kv1.2 cause strong gain-of-function effects. R297Q increases current amplitude 9-fold and shifts the voltage dependence of steady-state activation by −40 mV, while L298F leads to a 13-fold increase with a −50 mV shift in the voltage-dependence of steady-state activation. These mutations cause the channels to remain open, resulting in resting membrane potentials approximately −40 mV more negative as compared to cells expressing the wild-type channel. In 2017, another novel GOF mutation was described by Masnada *et al*. E157K, in the N-terminus of α-subunit, causes a 5-fold increase in current amplitude and -12 mV shift in voltage-dependence of steady-state activation. H310Y is another mutation in Kv1.2 involved in a dual function by increasing channel activity and the surface expression of Kv1.2.

1.7 Kv1.2: a drug target to cure GOF-related channelopathies

Kv1.2-related GOF-mutations lead to hyperactivity and alteration of neuronal functions, thus causing *KCNA2*-related epileptic encephalopathy, characterized by severe seizures, ataxia and ID/GDD. Upon discovery of these mutations Kv1.2 emerged as a potential target to cure GOF-related epileptic encephalopathy. It was hypothesized that the inhibition of GOF-Kv1.2 currents by a blocker would be able to restore normal electrophysiological activity of Kv1.2 in neurons. Hedrich *et al*. conducted the first study and used 4-aminopyridine (4-AP, a small molecule) as a potential therapeutic agent to counteract the GOF effects in Kv1.2 activity. 4-AP is a well-studied nonselective Kv channel blocker which inhibits the currents by binding to the pore region of channels and it is already in clinical trials for downbeat nystagmus syndrome and episodic ataxia type 2. The effect of 4-AP was tested on three GOF-Kv1.2 mutants (E157K, R297Q and L298F) expressed in oocytes. 1 mM 4-AP inhibited the mutant channels by decreasing current amplitudes and shifting the voltage dependence of steady-state activation toward more depolarized potentials. Moreover, R297Q mutants expressed in hippocampal neurons showed a significant decrease in the firing frequency upon application of 0.1 mM 4-AP. 4-AP was also given to eleven patients and improvement in seizure control, cognition, or ataxia in nine out of eleven cases was observed. The most significant outcomes were observed when treatment began in early childhood, highlighting the importance of early intervention.

1.8 Ion channel blockers

1.8.1 Venom derived K^+ channel blockers

Over the past four decades, several peptide toxins targeting potassium (K^+) channels have been identified. The Kalium database, a collection of peptides toxin that affect K^+ channels, currently includes more than 430 entries of natural toxins with 218 specifically isolated from the venom of scorpions. Potassium channel inhibitor toxins (KTxs) derived from scorpions are classified into seven different families based on their structural and functional characteristics: α-KTx, β-KTx, γ-KTx, δ-KTx, ε-KTx, κ-KTx, and λ-KTx. These toxins exhibit diverse structural features and block various K^+ channels with affinities ranging from picomolar (pM) to micromolar (μM) concentrations. Two types of mechanisms are known by which peptide toxins modulate ion channel activity. (i) By blocking the pore domain; such peptides are referred as pore blockers that occlude the pore domain of the ion channel thereby by hindering the flow on ions leading to current reduction. (ii) By modifying the voltage-dependence of steady-state activation; such peptides are called as gating modifiers. Peptides that bind to the VSD of the ion channels lead to a prominent shift in the voltage-dependence of steady-state activation towards more positive voltages. Binding of the toxins therefore reduces the open probability of the channels at physiological voltages. One such example of gating

modifier is Hanatoxin, isolated from spider *Grammostola spatulata* and inhibits Kv2.1 currents by binding to the VSD of the channel.

The toxins of α-KTx family, in general, inhibit Kv and KCa channels and are divided into 32 subfamilies based on the sequence similarity. α-KTx peptides contain 23-42 amino acid residues and share a cysteine-stabilized α/β scaffold as a common structural motif which comprises of α-helix and β-sheets stabilized by 3-4 disulfide bridges. The presence of a "functional dyad" is a major feature of majority of potassium channel blocking peptides. This dyad is composed of a critically positioned lysine (Lys27 in ChTx) residue and an aromatic residue, typically tyrosine (Tyr), nine positions downstream. The dyad lysine is on the beta sheet surface facing the channel pore upon binding, the 9-position difference in the primary sequence separates the Cα of the lysine by 6.6 ±1.0 Å from the center of the benzene ring of the tyrosine (Tyr36 in ChTx) in the 3D structure of ChTx. This functional dyad is considered essential for the interaction of peptides with K^+ channels. The Lys side chain penetrates into the selectivity filter of the channel and plugs the pore, preventing K^+ efflux. This interaction of Lys residue with SF of Kv1.2 channel has been shown in a crystal structure of ChTx bound Kv1.2-Kv2.1 chimera by Banerjee *et al*. More recently, Selvakumar *et al*. have described the Lys penetration into SF of Kv1.3 in cryroEM structure of K1.3 bounded with ShK, a peptide toxin from Sea anemone *Stichodactyla helianthus*.

Kv1.2 has emerged as a potential drug target recently, after the discovery of GOF mutations in *KCNA2* that play a key role in the development of epileptic encephalopathy and relevant neuronal disorders (see 2.5.1 chapter). So far only a few peptide toxins are known that inhibit Kv1.2 with high affinity but with very low specificity. For example, MgTx (K_d=6.4 pM), HgTx-1 (K_d = 170 pM) and Pi1 (K_d = 440 pM) block Kv1.2 with picomolar affinities, however, these toxins also inhibit Kv1.3 with similar potencies of 11 pM, 86 pM and 9.7 nM, respectively. There are very few known toxins that target Kv1.2 with high specificity and potency. Pi4 (α-KTx 6.2, isolated from scorpion *Pandinus imperator*) is the only known potent and specific inhibitor of Kv1.2. It inhibits Kv1.2 with K_d value of 8 pM and has >million-fold selectivity over Kv1.3. However, Pi4 also blocks KCa2 ion channels with K_d of 500 nM. High affinity and specificity peptides from venoms are attractive entities that could be exploited for the drug discovery of ion channels, therefore, identifying a specific inhibitor of Kv1.2 with minimum off-target effects would serve as a valuable therapeutic advancement in managing the GOF-related Kv1.2 channelopathies.

2. AIMS OF THE STUDY

2.1 Pharmacological characterization of seven novel peptide toxins isolated from *Centruroides bonito*

Scorpion venom represents a prolific source of peptide toxins that modulate voltage-gated potassium (Kv) channels. In the pursuit of potent and selective Kv1.2 ion channel blocking peptide toxins, seven novel peptides were recently isolated from the venom of *Centruroides bonito* by our collaborators and named as CboK1-7. My aim was

- to comprehensively characterize the pharmacological profile of the CboK peptides, with a particular focus on their specificity and affinity towards Kv1.1, Kv1.2 and Kv1.3 ion channels.

2.2 Pharmacological characterization of two new peptide toxins from venom of *Centruroides villegasi*

Two novel peptide toxins were identified and isolated from the venom of the scorpion *Centruroides villegasi* and named Cvill6 and Cvill7. My aims were

- to investigate the pharmacological properties of Cvill6 and Cvill7 peptides on a range of physiologically significant Kv channels.
- to study the concentration-dependent effect of Cvill peptides on Kv1.2 and Kv1.3 as well as characterize their kinetic parameters of binding to these channels.

2.3 Pharmacological characterization of synthetic peptide sCm39 against Kv1.2 and Kv1.3

Cm39 was first identified from the venom of *Centruroides margaritatus* and subsequently, produced synthetically (sCm39) for functional studies. My aims were

- to characterize the activity of the synthetic Cm39 against Kv1 channels.
- to understand the mechanism of block of Kv1.2 by sCm39 and determine the interaction kinetics.

3. MATERIALS AND METHODS

3.1 Chemicals and reagents

All chemicals and reagents utilized in this study were sourced from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise.

3.2 Toxins

3.2.1 CboK peptide toxins

Seven novel peptide toxins were isolated from the venom of Mexican scorpion *Centruroides bonito* in Prof. Lourival Possani's laboratory (our collaborator from UNAM, Mexico). The Scorpions were captured, and venom was extracted by electrical stimulation. The three-step purification scheme was utilized to isolate the peptides from crude venom. Initially, the crude venom was fractionated using size exclusion chromatography. The main fraction (FII) obtained was then subjected to ion-exchange chromatography (IEC) generating 14 subfractions. Subsequently, all IEC fractions were further purified by reverse-phase high-performance liquid chromatography (HPLC) using an analytical C_{18} column. All seven CboK peptides were obtained from subfraction 13. The amino acid sequence of the CboK peptide was determined through automated Edman degradation, performed with a Biotech PPSQ-31A/33A Protein Sequencer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA).

3.2.2 Cvill peptide toxins

Two novel peptide toxins named Cvill6 and Cvill7 were isolated in Prof. Lourival Possani's laboratory from the venom of scorpion *Centruroides villegasi* in section 3.2.1.

3.2.3 sCm39

Cm39 peptide was initially identified from the venom of *Centruroides margaritatus*. To obtain a sufficient quantity of the Cm39 peptide for electrophysiology assays, it was synthesized synthetically in Prof. Possani's lab using Merrifield's solid-phase method and after refolding, peptide was purified using HPLC.

3.3 Multiple sequence alignment

To find the phylogenetically related toxins with, CboK peptides, Cvill peptides and Cm39, BLASTP was performed. Multiple sequence alignment of mature chains was done by using MAFTT version 7.

3.4 Modeling of Cvill peptides

The tertiary structural prediction of peptides was performed using the AlphaFold3, an AI-based tool designed for predicting protein structures. Based on the amino acid sequences, peptides Alphafold3 generated multiple folding models. The model with the highest reliability scores was chosen, which had a pTM of 0.72 and a pLDDT score (the per-atom confidence measure) exceeding 90.

3.5 Cell culture

3.5.1 Isolation and activation of PBMCs

Human venous blood from anonymized healthy donors was obtained from the blood bank, with sample collection approved by the Ethical Committee of the Hungarian Medical Research Council (approval number: 36255-6/2017/EKU). Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque 1077 density gradient centrifugation. The isolated cells were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum (Sigma-Aldrich), 100 µg/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Cells were seeded in 24-well culture plates at a density of 5×10^5 cells/ml and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 3 to 6 days. To activate the PBMCs and enhance Kv1.3 channel expression, phytohemagglutinin A (PHA) was added to the culture medium at concentrations of 5, 7, and 10 µg/ml.

3.5.2 CHO cells

Chinese hamster ovary (CHO) cells were maintained under standard culture conditions in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA; Cat. #11965084) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin-G (Sigma-Aldrich). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Subculturing was performed three times per week using a 2–5-minute incubation with 0.05% trypsin-EDTA solution.

3.5.3 Heterologous expression of ion channels

CHO cells were used for the transient expression of ion channels as they do not have any endogenous Kv currents to cause contamination of the current records. CHO cells were transfected transiently with ion channel-encoding vectors i.e. hKv1.1, hKv1.2, hKv1.5, hKCa2.2 and hhKCa3.1 using the Lipofectamine 2000 kit (Invitrogen, Waltham, MA, USA), following the manufacturer's protocol. The "h" indicates human origin whereas "m" in front of the gene/ion channel name indicates mouse origin. In cases where the ion channel construct lacked a fluorescent tag, cells were co-transfected with a plasmid encoding GFP to facilitate identification. Cells expressing either GFP or YFP were visualized using a Nikon TS-100

fluorescence microscope (Nikon, Tokyo, Japan), equipped with excitation and emission bandpass filters of 455–495 nm and 515–555 nm, respectively. Whole-cell electrophysiological recordings were conducted 20–30 hours post-transfection.

Human embryonic kidney 293 cell line (HEK) that expresses hKv11.1 (hERG1, *hKCNH2* gene) in a stable manner was a kind gift from Heike Wulff (University of California, Davis, CA, USA). Th HEK293 cell line expressing mKCa1.1 (*mKCNMA1* gene) in a stable manner was generously provided by Christine Beeton (Baylor College of Medicine, Houston, TX, USA).

3.6 Patch-clamp electrophysiology

Whole-cell currents were recorded using the patch-clamp technique in voltage-clamp mode, following standard protocols. Recordings were performed at room temperature (20–25 °C) using either a Axopatch 200B or Multiclamp 700B amplifiers (Molecular Devices, Sunnyvale, CA, USA), connected to a personal computer via an Axon Digidata 1440 digitizer. Data acquisition was carried out using Clampex 10.7 software. Current traces were low-pass filtered using the amplifier's built-in 4-pole Bessel filters and sampled at 4–50 kHz, ensuring a sampling rate at least twice the filter cutoff frequency. Micropipettes were fabricated from GC150F-7.5 borosilicate glass capillaries (Harvard Apparatus, Holliston, MA, USA or Kent, UK) using a Sutter P2000 laser puller, yielding a tip resistance of 3–6 MΩ in the bath solution. Only recordings with leak currents at the holding potential (V_h) of less than 10% of the peak current at the test potential were included in the analysis. Solutions were applied to cells using a gravity-driven microperfusion system equipped with an AutoMate Perfusion Pencil Multi-Barrel Manifold Tip (AutoMate Scientific, Berkeley, CA, USA) at a flow rate of approximately 200 µl/min. Excess bath solution was continuously removed from the recording chamber using vacuum suction.

3.6.1 Solutions

For patch-clamp measurements, extracellular solution (bath solution) for both Kv and KCa channels consisted of 145 mM NaCl, 5mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 5.5 mM glucose, pH 7.35 and osmolarity between 302 and 308 mOsM/L. For positive control solutions containing various concentrations of tetraethylammoniums (TEA+), Na+ was substituted with TEA-Cl in equimolar concentration in bath solution and all other ingredients remained unchanged. To avoid toxin adsorption onto the plastic surfaces of the perfusion system, 0.1 mg/mL of bovine serum albumin (BSA, Sigma-Aldrich, Budapest, Hungary) was also added into all bath solutions prior to the patch-clamp assay. The internal solution (pipette filling solution) for Kv1.1, Kv1.2, Kv1.3, Kv1.5, and mKCa1.1 composed of 140 mM KF, 2 mM MgCl2, 1 mM CaCl2, 11 mM EGTA and 10 mM HEPES having a pH of 7.22 and for Kv11.1 consisted of 140 mM KCl, 2 mM MgCl2, 10 mM EGTA and 10 mM

HEPES, pH of 7.22. For KCa2.2 and KCa3.1 the composition of the internal solution was 150 mM K-Aspartate, 5 mM HEPES, 8.5 mM CaCl2, 2 mM MgCl2 and 10 mM EGTA, pH 7.22, with an estimated free Ca2+ of ∼1−2 μM based on the MaxChelator program WEBMAX-C software (C. Patton, Stanford University, retrieved here: https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/webmaxc/webmaxcE.htm). The osmolarity of the internal solutions was ~ 295 mOsm/L. Apamin and Charybdotoxin was acquired from Smartox Biotechnology (Saint Egrève, France) and Cm39 was chemically synthesized in-house.

3.6.2 Voltage protocols

To evoke the K^+ currents from Kv1.x channels, depolarization pulses to +50 mV from a holding potential (V_h) of −120 mV were applied every 15 s. The duration of depolarization pluses was 15 ms for Kv1.3, 50 ms for Kv1.1 and Kv1.5. However, for Kv1.2, 15-500 ms long pulses were used to achieve the saturated peak current due to the variable activation kinetics of this channel as discussed previously. For Kv11.1 channels, currents were elicited by applying a voltage step to +20 mV for 1.25 s from a V_h of –80 mV followed by a step to –40 mV for 2 s, during which peak currents were measured, with pulses delivered every 30 s. mKCa1.1 currents were recorded by applying 600-ms-long voltage steps to +100 mV from a V_h of –100 mV. KCa2.2 and KCa3.1 currents were recorded by applying a 150-ms-long voltage ramps to +50 mV from -120 mV every 10 s, the V_h was set to –85 mV.

3.7 Data analysis and statistics

The Clampfit 10.7 software package (Molecular Devices, Sunnyvale, CA, USA) was used to analyze current recordings. Before analysis, all current traces were digitally filtered using three-point boxcar smoothing and were corrected for ohmic leakage, only if required. The blocking effect of the toxin at a given concentration was calculated as remaining current fraction (RCF = I/I_0), where I represent the peak current at equilibrium block at a given toxin concentration or peak current recorded after 3 min perfusion of toxin solution in the absence of measurable block and I_0 is the peak current in the absence of the toxin. Data points in the concentration-response curve represent the mean RCF values of three to six individual cells, with error bars denoting the standard error of the mean (SEM). The Hill equation was fitted to the datapoints obtained at each concentration

$$\mathrm{RCF} = \frac{K_d^H}{K_d^H + [\mathrm{Toxin}]^H} \qquad (1)$$

where K_d is the dissociation constant, H represents the Hill coefficient and [Toxin] is the concentration of the toxin.

To study the binding kinetics of Cvill toxins, peak current during the application of toxin at time point t (I_t) were normalized to the peak current (I_0) before the toxin exposure ($I_{norm} = I_t/I_0$) and plotted as a function of time. The association time constant (τ_{on}) were determined by the fitting a single exponential function to the data points during the toxin wash-in procedure (equation 2, one-phase decay to RCF) and for the dissociation time constant (τ_{off}), a single exponential function rising to maximum were fitted to data points during the wash-out procedure (equation 3, RCF followed by one-phase association) for individual cells.

$$I_{norm}(t) = RCF + \left((1 - RCF) \times e^{-\frac{t}{\tau_{on}}}\right) \quad (2)$$

$$I_{norm}(t) = RCF + \left((1 - RCF) \times \left(1 - e^{-\frac{t}{\tau_{off}}}\right)\right) \quad (3)$$

The time constants (τ_{on} and τ_{off}) were used to calculate the association rate constant (k_{on}) and dissociation rate constant (k_{off}) based on a simple bimolecular interaction between the channel and the toxin, and using equation 4 and equation 5, respectively

$$k_{on} = \frac{1 - (\tau_{on} \times k_{off})}{\tau_{on} \times [toxin]} \quad (4)$$

$$k_{off} = \frac{1}{\tau_{off}} \quad (5)$$

To construct the voltage-dependance of steady-state activation of Kv1.2, peak conductance (G) at different test potential (−60 to +100 mV in 10 mV steps) was calculated from peak current (I) at a test potential (E_m) and the K^+ reversal potential (E_K) using chord-conductance equation $G = I/(E_m - E_K)$. The G values were normalized (G_{norm}) to the maximum value and plotted as a function of test potential and data points were fitted with Boltzmann sigmoidal equation:

$$G_{norm} = \frac{1}{1 + e^{\left(\frac{V_{50} - E_m}{k}\right)}} \quad (6)$$

where V_{50} is the midpoint voltage, E_m is the test potential, and k represents slope factor of the function.

Representative graph plotting and statistical analysis were conducted using Graph pad prism software package (version 8.0.1, La Jolla, CA, USA). All the data was presented with standard errors of mean (SEM). For pairwise comparison, Student's *t*-test was used and for multiple comparisons, one-way ANOVA with post-hoc Dunnet's test was performed. Statistical significance is indicated in terms of P values.

4. RESULTS

4.1 Pharmacological characterization of seven novel peptide toxins isolated from *Centruroides bonito*

The city of Acapulco, in the state of Guerrero, Mexico, has a newly described scorpion species that belongs to family *Buthidae*, named *Centruroides bonito*. This venom of *C. bontio* is toxic with a LD_{50} value of 16.7 μg/20g mouse body. This species is known to cause envenomation in human; however, its venom has not yet been fully characterized. Accordingly, we have reported the proteomic analysis of the venom of *C. Bonito* leading to the isolation of seven novel peptide toxins named CboK1 to CboK7 by the laboratory of Prof. Possani, at in UNAM, Mexico.

4.1.1 Pharmacological characterization of CboK Peptides

The sequence analysis shows that the seven CboK peptide toxins possess a great deal of resemblance to the α-KTxs family toxins that are known to block voltage-gated K^+ channels. Based on the percentage identity of CboK peptides with other α-KTxs and the literature available for the activity of closely related peptides, Kv1.1, Kv1.2, and Kv1.3 K^+ channels were considered as the potential target of CboK peptides. Macroscopic Kv1.1 and Kv1.2 currents were measured in transiently transfected CHO cells. To record Kv1.3 currents, human peripheral T lymphocytes were activated with Phytohemagglutinin A (PHA) to boost Kv1.3 expression. Ca^{2+}-free intracellular solution was used to avoid the KCa3.1 channel opening. Thus, whole-cell currents were measured exclusively from Kv1.3 ion channels. Currents were evoked by applying 15-ms-long de-polarization pulses to +50 mV from a holding potential (V_h) of −120 mV except for Kv1.2, as it has highly variable activation kinetics; therefore, 15–500 ms-long pulses were applied to maximize the open probability of Kv1.2 channel. All peptide toxins were dissolved in a freshly prepared bath solution supplemented with 0.1 mg/ml BSA and applied to the cells in whole cell patch configuration using a gravity-driven micro perfusion system at a flow rate of 200 μl/min. The complete exchange of solutions in the bath chamber and the proper functioning of the perfusion system were regularly verified using fully reversible inhibitors as positive controls at a concentration equivalent to their K_d values, i.e., 0.3 mM tetraethylammonium (TEA) for Kv1.1, 14 nM Charybdotoxin (ChTx) for Kv1.2, and 10 mM TEA for Kv1.3. Approximately 50% reduction in K^+ current at equilibrium block in the presence of positive controls served as an indication of the expression of the proper ion channel and a complete solution exchange in recording chamber.

We found that CboK3 and CboK4 block less than 10% the Kv1.1 currents at 100 nM concentration; however, CboK7 showed ~42% block of Kv1.1 at this concentration. The low blocking potency, combined with the limited availability of CboK peptides from the native source, precluded further investigation of their concentration-dependent inhibitory effects on

the Kv1.1 channel. CboK2 to CboK6 peptides reduce 60-92% Kv1.2 currents at 1 nM concentration. CboK7 showed outstanding potency for Kv1.2 by inhibiting ~96% of the current at 1 nM concentration. In the case of Kv1.3, CboK2–CboK7 peptides demonstrated moderate inhibitory effects with 41–81% block at 100 nM, whereas CboK1 showed less than 4% inhibition.

4.1.2 Potent inhibition of Kv1.2 by CboK peptides at picomolar concentrations

All CboK peptides except CboK1 exhibited significant inhibiting effects on Kv1.2 currents when tested at 1 nM concentration. The initial screening experiments motivated us to conduct a more detailed study to characterize the inhibitory activities of CboK peptides on the Kv1.2 channel. Normalized peak currents ($I_{norm} = I_t/I_0$, where I_t is the peak current in the presence of the toxin at time t and I_0 is the peak current in the control solution at t = 0), were plotted as a function of time. The block was completely reversed by applying a toxin-free solution to the cell. The kinetics of both association and dissociation of all the CboK peptides were very slow. It took several minutes to achieve equilibrium block and to recover fully the peak current that was measured prior to the application of toxin. Consequently, slow toxin association and dissociation kinetics led us to generate the concentration–response curves for CboK peptides in a cumulative manner.

The concentration–response relationships of CboK2 to CboK6 for Kv1.2 channel inhibition. Different concentrations of each peptide were applied to the cells for a sufficient period to achieve the equilibrium block, considering the slow blocking kinetics, especially at low toxin concentrations. The RCF values were calculated, and the Hill equation was fitted to the data points to obtain the characteristic of the concentration–response curves. The resulting dissociation constants (K_d) and the Hill coefficients were K_d = 763 pM and H = 1.5 for CboK2, K_d = 106 pM, and H = 1.2 for CboK3, K_d = 125 pM and H = 1.0 for CboK4, K_d = 376 pM and H = 1.2 for CboK5, K_d = 585 pM and H=1.3 for CboK6, and K_d = 24 pM and H = 1.3 for CboK7, respectively. Out of all seven CboK peptides, CboK7 demonstrates outstanding with a high affinity for Kv1.2 with a K_d value of 24 pM.

4.1.3 Inhibition of Kv1.3 by CboK peptides

Kv1.3 ion channel currents were also sensitive to CboK peptides. Therefore, we aimed to investigate the effect of these peptides in a concertation-dependent manner on Kv1.3. The affinity of CboK1 for Kv1.3 was quite low in the screening and due to limited supply of native toxins from venom, the full concentration–response relationship was not possible to obtain. Data were not obtained for CboK5 either, as it is 100% identical to a previously known α-KTx 2.10 (Toxin Ce3), which was already characterized for Kv1.3. RCF data points obtained at different toxin concentrations and were fitted with the Hill equation to obtain concentration–response curves of CboK peptides. The resulting K_d values and the Hill coefficients were K_d =

171 nM (H = 0.8) for CboK2, K_d = 34.3 nM (H = 0.96) for CboK3, K_d = 21.7 nM (H = 0.94) for CboK4, K_d = 160 nM (H = 0.77) for CboK6 and, K_d = 20.4 nM (H = 0.8) for CboK7, respectively.

4.2 Pharmacological characterization of two new peptide toxins from venom of *Centruroides villegasi*

A new species of scorpion from the genus *Centruroides*, named *C. villegasi* was described in the town of Chilapa, in state of Guerrero, Mexico. The venom of *C. villegasi* is toxic with a LD_{50} value of 12.2 μg/20g of mouse body. The venom extraction of scorpion *C. villegasi*, purification and primary amino acid sequence determination of Cvill6 and Cvill7 peptides from the venom was carried out in UNAM, Mexico, in Prof. Possani's Laboratory.

4.2.1 Effect of Cvill peptides on voltage-gated potassium channels

The primary amino acid sequences of Cvill6 and Cvill7 exhibit a high percentage identity to members of the α-KTx 2 subfamily, which are generally known as inhibitors of Kv ion channels. Therefore, to pursue the quest of identifying a potent and selective Kv1.2 inhibitor we aimed to investigate the effects of Cvill6 and Cvill7 on Kv ion channels. Current records show that Cvill6 blocked ~28% of Kv1.2 current (1 nM concentration) but only ~13% of the Kv1.3 current is inhibited (100 nM concentration). However, no change was observed in currents amplitudes for hKv1.1, hKv1.5, and hKv11.1 at 100 nM of Cvill6. On the other hand, Cvill7 blocked >97% of the Kv1.2 current at 1 nM concentration indicating an extraordinary potency. In addition, Cvill7 also inhibited the Kv1.1 and Kv1.3 currents by reducing ~32% and ~86% of the peak amplitudes at 100 nM concentration, respectively. Nevertheless, Cvill7 did not show any effect on Kv1.5 and Kv11.1 currents. The summary of the inhibition of the Kv channels by Cvill toxins indicates that Cvill6 affects only Kv1.2 and Kv1.3 while Cvill7 affects Kv1.1, Kv1.2 and Kv1.3. The estimated K_d values of Cvill6 from a single concentration, based on bimolecular interaction of channel and toxin (1:1), yielded ~0.84 μM for Kv1.3. Similar estimates for Cvill7 yielded ~192 nM for Kv1.1. The quantity of native toxins was insufficient to conduct the full concentration-response analysis when the affinity of the toxin for a channel was low, estimated to be above 50 nM.

4.2.2 Cvill7 selectively inhibits Kv1.2 over Kv1.3 with low-picomolar affinity

Our initial screening against Kv channels showed high affinity for Kv1.2, thus, we further extended our experiments to determine the concentration-dependent inhibition of Kv1.2 and Kv1.3 currents by Cvill7 and its binding kinetics. The development and recovery of the block of Kv1.2 at 100 pM concentration of Cvill7. Normalized peak currents ($I_{norm} = I_t/I_0$) for individual cells (n = 4) were averaged and plotted against the time. The loss of Kv1.2 current apparently saturated to 96 ± 1.4% of the initial (t = 0) peak current in 7-8 min upon application

of 100 pM of Cvill7. Fitting the individual block kinetics (cell-by-cell) using a single-exponential decay function gave the time constant (τ_{on}) of 104 ± 15 s (n = 4) for the development of block. The dissociation kinetics of Cvill7 were also extremely slow. Equilibrium block was followed by application of toxin-free solution for ~15 min, during which just one-fifth of the blocked current recovered with extremely slow kinetics, suggesting that Cvill7 is a virtually irreversible inhibitor of Kv1.2. The dissociation constant (τ_{off}) of Cvill7 for Kv1.2 channel was 5978 ± 538 s (n = 4), obtained by fitting a single-exponential rising function to the normalized peak currents during the wash-out procedure. On the other hand, unlike Kv1.2 the block of Kv1.3 by Cvill7 was fully reversible with rapid association and dissociation kinetics. The onset of equilibrium block to 67 ± 3% occurred with a τ_{on} of 20 ± 1.8 s (n = 3) upon 15 nM of toxin exposure and it fully recovered to initial normalized peak current with a τ_{off} of 34 ± 6 s (n = 3) upon perfusing the cell with toxin-free solution.

To determine the concentration-dependence of current inhibition of Kv1.2 and Kv1.3 ion channels, different concentrations of Cvill7 peptide were applied to the cells for a sufficient amount of time, taking into account the slow blocking kinetics specifically at low picomolar concentrations. At low-picomolar concentrations the concentration-response curve was determined in a cumulative manner. The remaining current fractions (RCF) were determined using the ratio I/I_0 (for details see Materials and Methods section) and plotted as a function of toxin concentration. The concentration-response curves were obtained by fitting the data points with the Hill equation (refer to Materials and Methods for details). The best fit resulted in the K_d value of 16 ± 1.56 pM with a Hill coefficient (H) of 1.03 for Kv1.2 and K_d value of 7.2 ± 0.64 nM with an H coefficient of 0.73 for Kv1.3. Thus, Cvill7 displays ~450-fold selectivity for Kv1.2 over Kv1.3.

4.2.3 Cvill6 inhibits Kv1.2 with nanomolar affinity

Cvill6 peptide has 74% identity with Cvill7. However, during the screening experiments for Kv channels, it moderately inhibited hKv1.2 currents (~16% at a 1 nM concentration) and displayed slight inhibition of Kv1.3 (~11% at 100 nM). The concentration-dependence of the block showed that Cvill6 blocked Kv1.2 with a K_d of 3.9 ± 0.27 nM and Hill coefficient of 1.4.

4.2.4 Activity of Cvill6 and Cvill7 toxins on Ca^{2+}-activated potassium channels

Although scorpion toxins from α-KTx 2 family also modulate the function of Ca^{2+}-activated potassium channels neither Cvill6 nor Cvill7 showed any effect on KCa1.1 or KCa2.2. Cvill6 reduced ~40% of KCa3.1 current, the RCF value was 0.73 ± 0.05 (n = 5) at 100 nM. On the other hand, Cvill7 inhibited ~25% of the current at the same concentration, with RCF value 0.84 ± 0.03 (n = 5). On the other hand, Cvill6 and Cvill7 inhibited the KCa3.1 channel reversibly with quick association and dissociation kinetics. The estimated K_d values for KCa3.1 from a single concentration yielded ~268 nM and ~527 nM of Cvill6 and Cvill7, respectively.

Thus, Cvill6 has a 2-fold higher affinity for KCa3.1 than Cvill7. The quantity of native toxins was insufficient to conduct the full dose-response curves at this high concentration range.

4.3 Pharmacological characterization of synthetic peptide sCm39 against Kv1.x channels

Centruroides margaritatus, a scorpion species from the Buthidae family, possessing relatively low venom toxicity, with an LD_{50} of 59.9 mg/kg. The venom of *C. margaritatus* has not been extensively studied, a peptide with a molecular weight of 3980.2 Da and named as Cm39 was identified and synthesized in our collaborator's lab (Prof. Lourival Possani) in UNAM, Mexico for the detailed study and electrophysiological characterization in our lab.

4.3.1 Effect of sCm39 on Kv1.x ion channels

My contribution in this project was to study the effect of sCm39 against Kv1.1, Kv1.2 and Kv1.3 ions channels. Both Kv1.1 and Kv1.3 displayed no sensitivity, whereas 1μM sCm39 blocked 95% of Kv1.2 currents.

4.3.2 Mechanism of Kv1.2 block by sCm39

Kv1.2 currents were recorded in CHO cells. sCm39 inhibited Kv1.2 in a concentration-dependent manner, i.e., 5-500 nM concentrations inhibited approximately 5-87% of current at equilibrium block. The remaining current fractions ($RCF = I/I_0$) were calculated for different concentrations of sCm39 and plotted as a function of concentration. The Hill equation was fit to the data points to characterize the concentration–response relationship which resulted in a K_d value of 65 nM with a Hill coefficient of 0.96.

To study the blocking mechanism, conductance–voltage (G–V) relationship for Kv1.2 in the absence and presence of the of sCm39 was determined. Whole-cell currents of Kv1.2 expressing CHO cells were recorded by applying the 300 ms long depolarization pulses from −70 to +80 mV in 10 mV steps from holding potential of −120 mV. Due to the highly variable activation properties of Kv1.2 we constrained the analysis to the records which displayed similar gating mode. The normalized conductance values were calculated for each voltage step and plotted as a function of membrane potential (E_m). The Boltzmann sigmoidal function (eq.6, see section 4.7 for details) fitted to the average data points which resulted in the superimposed lines suggesting that the presence of sCm39 (65 nM) did not affect the voltage dependence of steady-state activation of Kv1.2. The midpoint voltage ($V_{1/2}$) of the G–V relationship in the control solution (-1.0 ± 4.8, $n = 4$) was statistically similar to the $V_{1/2}$ value at equilibrium block with 65 nM sCm39 (1.6 ± 4.0, $n = 4$). These results indicate that sCm39 is not a gating modifier rather it is a pore blocker.

5. DISCUSSION

5.1 Pharmacological characterization of seven novel peptide toxins isolated from *Centruroides bonito*

CboK1-CboK6 peptides blocked Kv1.1, Kv1.2 and Kv1.3 with high picomolar or nanomolar affinity. On the other hand, CboK7 (α-KTx 2.24) demonstrates a remarkable affinity for Kv1.2 (K_d = 24 pM) and displays moderate affinity for Kv1.1 and Kv1.3 with 141 nM and 20.4 nM K_d values. CboK7 also shares a high percentage identity with CboK3 and CboK4. CboK7 differs from CboK3 and CboK4 by five amino acids but at different positions. CboK7 has Phe2, Pro15, Glu19, Ile at positions 20 and 23 and Ala27. In comparison with the previously described peptides, CboK7 is 92% identical to toxins Ce4 (α-KTx 2.11) isolated from scorpion *Centruroides elegans.* The activity of Ce4 was only tested against Kv1.3 and was shown to inhibit with 0.98 nM K_d value. The high degree of similarity in the primary structures of Ce4 and CboK7 argues for experimental determination of the affinity of Ce4 for Kv1.2. This would shed light on the role of Lys15 vs. Pro15 and Val39 vs Ile39 in the determination of Kv1.2 affinity. Overall, among the CboK toxins reported in this study, CboK7 showed remarkable affinity for Kv1.2 (K_d = 24 pM) with considerable selectivity of ~6000-fold over Kv1.1 and 850-fold over Kv1.3.

5.2 Pharmacological characterization of two new peptide toxins from venom of *Centruroides villegasi*

Cvill6 revealed that it inhibits Kv1.2 (K_d = 3.9 nM) with moderate affinity and displays relatively less affinity for Kv1.3 (K_d = ~0.84 μM),and at 100 nM concertation inhibited 40% of KCa3.1 currents. However, other Kv1 channels (Kv1.1, Kv1.5 and Kv11.1) and Ca^{2+}-activated channels (mKCa1.1, KCa2.2) remained insensitive to higher concentration of Cvill6 (100 nM).

On the other hand, Cvill7 inhibited Kv1.2 with low picomolar affinity (K_d = 16 pM) and Kv1.3 with low nanomolar affinity (K_d = 7.2 nM), having a 450-fold selectivity for Kv1.2 over Kv1.3. The closely related toxins of Cvill7, namely Ce1, Ce2, and Ct28 (>94% identical), have not been evaluated for Kv1.2 inhibition according to available literature. However, for Kv1.3, Ce1 and Ce2 have 0.7 nM and 0.25 nM K_d values. CboK4 inhibits Kv1.2 (K_d = 125 pM) and Kv1.3 (K_d = 22 nM) with ~176-fold selectivity for Kv1.2 over Kv1.3. CboK4 differs by 3 residues (~92% similarity) from Cvill7 which resulted in only 8-fold decrease in the affinity of CboK4 for Kv1.2. Comparison of amino acid sequences revealed that Cvill6 shares 74% identity with Cvill7, and both have Lys28-Tyr37 dyad however, Cvill6 has 244 times less potency for Kv1.2 than Cvill7 with the K_d values in low nanomolar and picomolar ranges, respectively. Based on these we conclude that the other differences in amnio acids between Cvill6 and Cvill7 may correspond to the determinants of high affinity for Kv1.2 which can be further explored through molecular dynamics studies of toxin and channel interaction.

5.2.1 CboK7 and Cvill7 offer a new insight into Kv1.2 blockade

Several venom derived peptide toxins are identified till date that inhibit Kv1.2 channel currents with high affinity for example MgTx (α-KTx 2.2), HgTx-1 (α-KTx 2.5) and Pi1 (α-KTx 6.1) having K_d values of 6 pM, 170 pM, 440 pM nevertheless, these toxins also block Kv1.3 currents with similar potencies (11 pM, 86 pM and 9.7 nM, respectively). In the group of high affinity and moderately selective blockers of Kv1.2 only a few toxins are known for examples, Maurotoxin (α-KTx 6.2) inhibits Kv1.2 (K_d = 0.8 nM) with ~56-fold selectivity over Kv1.1 and 225-fold over Kv1.3. CoTx1 (α-KTx 10.1) has moderate affinity for Kv1.2 (27 nM) but exhibits ~900-fold selectivity over Kv1.1 and ~200-fold over Kv1.3. It also inhibits KCa2.x and KCa3.1 channels.

This study aimed to identify a novel high-affinity peptide blocker of Kv1.2. Fortunately, two distinct candidates (CboK7 and Cvill7) were found from the venom of two different scorpions and both exhibit potent Kv1.2 inhibition. Cvill7 exhibits a negligibly higher affinity for Kv1.2 (K_d = 16 pM) as compared to CboK7 (K_d = 24 pM), however this small difference in the affinity is not very significant as both fall in the picomolar range indicating the potent inhibition of Kv1.2. CboK7 showed potentially high selectively over Kv1.3 (K_d = 20.4 nM, 850-fold) as compared to Cvill7 (K_d = 7 nM, 450-fold). On the other hand, both CboK7 and Cvill7 demonstrate high selectivity over Kv1.1, 6000-fold and 12000-fold respectively. Due to the inadequate amount of native CboK7 it was not possible to conduct selectivity profiling assays to determine its activity against another physiologically significant Kv1 and KCa ion channels. However, the selectivity profiling of Cvill7 revealed that it also has 33000-fold selectivity for Kv1.2 over KCa3.1 (K_d = 527 nM).

Studies have shown that the affinity of toxins for Kv1.2 and Kv1.3 is largely influenced by the presence of the "functional dyad" consisting of a conserved Lys residue and an aromatic or polar residue ~6–7 Å apart. This latter residue of the dyad is Tyr for high affinity blockers of Kv1.2 and Thr or Asn for high affinity blockers of Kv1.3. Both CboK7 and Cvill7 possess the functional dyad Lys28 and Tyr37. The primary amino acid sequence comparison showed 82% sequence identity in both peptides, having amino acids differences at seven distinct positions. A tertiary structure alignment is also conducted based on AlphaFold3 predicted 3-D models of CboK7 and Cvill7 peptides. Considering the potency and selectivity of both CboK7 and Cvill7 we may conclude that the differences in the primary sequences do not cause significant alteration in the predicted structures.

In the era of CryoEM a trivial solution for finding the interaction partners between the peptides and the target channel would be the analysis of the toxin-bound channel complexes. These are already available e.g. for Kv1.3 and bound ShK. Unfortunately, the “turret region” of the channels, where the toxins make multiple interactions with the channels were poorly resolved due to the very flexible nature of this region. Thus, to get more insight into the binding

of these high affinity peptides, systematic mutagenesis studies and/or novel techniques (e.g. NMR analysis of toxin-channel interaction) are required.

5.3 Pharmacological characterization of synthetic peptide Cm39 against Kv1.2 and Kv1.3

In electrophysiological evaluation against Kv1.x (Kv1.1 - Kv1.3), sCm39 demonstrated moderate inhibitory activity against Kv1.2 (K_d = 65 nM) and has no effect on Kv1.1 and Kv1.3 at the concentration of 1 μM. TsTXα (α-KTx 4.1), TdK1 (α-KTx 4.3), Tc30 (α-KTx 4.4) and Tst26 (α-KTx 4.6) shared 64.9% sequence identity with Cm39. However, as per literature only TsTXα and Tst26 were tested against Kv1.2, the reported K_d values were of 0.2 nM and 1.9 nM respectively. The interesting factor of sCm39 is that it did not show any inhibitory effects on Kv1.3. Further upon determining the binding kinetics and mechanism of block, it was found that the voltage dependence of steady-state-activation of Kv1.2 channel was completely insensitive to the presence of sCm39. Thereby clarifying that it does not interfere with the VSD, we proposed that Cm39 binds to the pore region of the ion channel and Lys27 is responsible for occluding the pore.

As Kv1.2 is a typical ion channel in the central nervous system, a peptide such as sCm39, that has dual targets, is not an ideal candidate to manage Kv1.2-related channelopathies. In this regard CboK7 and Cvill7 are promising candidates treat channelopathies associated with gain-of-function mutations of Kv1.2 such as severe epileptic encephalopathy (EE). The selective inhibition of mutated Kv1.2 channels on neuron in central nervous system (CNS) using Cvill7 as potential drugs will have minimum risk of off-target effects on other K^+ channels (Kv1.1, Kv1.3, Kv1.4 and K1.6) which are also widely expressed in CNS. However, the therapeutic application of these peptide toxins is challenged by the restrictive nature of the blood–brain barrier (BBB), which limits their delivery to brain tissues. This limitation can be effectively addressed by conjugating Cvill7 or CboK7 with BBB shuttle peptides, which have been shown to significantly enhance CNS penetration and improve peptide bioavailability.

5.4 Potential of peptide-based drugs in the management of the diseases of the CNS

Peptides-based drugs have emerged as a unique class of therapeutics due to their distinctive biochemical properties and strong therapeutic potential. However, poor membrane permeability and low in vivo stability hinder their broader application in drug development. Their inability to efficiently cross cell membranes limit their access to intracellular targets, while their linear structure and susceptibility to enzymatic degradation result in short half-lives and rapid clearance from the body. In the past decade significant progresses to overcome the poor CNS availability of peptide-based drugs have been made by the development of various invasive and non-invasive approaches. For the targeted delivery of peptides an intracerebroventricular (ICV) delivery system can be utilized. For example, in case of Dravet

syndrome (an epileptic disease caused due to haploinsufficiency of Nav1.1 ion channels), Hm1a peptide derived from spider venom, has been infused via ICV to Dravet Syndrome mice and significant reduction in whole brain hyperexcitability has been observed. This proof-of-concept study confirms the practical application of the ICV for drug administration to CNS. Ziconotide is the synthetic version of ω-conotoxin MVIIA peptide isolated from the venom of fish-hunting marine snail Conus magus. Ziconotide is an FDA approved drug for the treatment of severe chronic pain by blocking N-type calcium channels. Ziconotide has limited capacity to cross the blood–brain barrier; therefore, to maximize its analgesic effect it must be administered intrathecally. For continuous delivery of the drug in the intrathecal space programmable surgically implanted variable rate infusion device such as the Medtronic SynchroMed has been utilized.

Apart from the invasive strategies discussed earlier, noninvasive methods for delivering peptide-based drugs are gaining increasing attention due to their clinical practicality and reduced risk. One widely explored approach involves the encapsulation of peptide drugs into nanoparticles or liposomes, enabling targeted delivery, protection from enzymatic degradation, and controlled release at the desired site. Another promising technique is the transient disruption of the blood-brain barrier (BBB) using focused ultrasound (FUS) or osmotic agents like mannitol, which can temporarily increase BBB permeability to facilitate drug passage into the brain. A particularly effective and extensively studied noninvasive strategy is the use of cell-penetrating peptides (CPPs) or BBB shuttle peptides for example angiopep-2, TAT (HIV protein), melanotransferrin fragment (MTFP) etc. These small peptides, typically composed of 10–30 amino acids, can cross the BBB either through direct translocation or via receptor-mediated transcytosis. By conjugating therapeutic peptides to CPPs or shuttle peptides, researchers have significantly enhanced CNS delivery while avoiding the need for invasive administration techniques. Conjugation of Ziconotide with TAT enables effective peripheral and intranasal delivery while preserving its analgesic efficacy, offering a less invasive alternative with fewer side effects. Similarly, N-terminal linkage of Vm24, a high-affinity Kv1.3 blocker, to BBB shuttle peptides retains its target affinity and is under investigation for microglial Kv1.3 targeting (unpublished data, Shakeel et al.). These strategies show strong potential to enhance CNS delivery and expand the therapeutic utility of peptide-based drugs in neurological disorders.

6. ACKNOWLEDGEMENT

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7. SUMMARY

The Kv1.2 ion channels play a vital role in the regulation of membrane potential and excitability of neurons and are widely distributed in CNS (hippocampus, cerebral cortex, cerebellum, spinal cord etc.). In 2016, Kv1.2 related GOF mutations and their role in the progression of epileptic encephalopathy (EE) were described. It has been experimentally verified that the inhibition of the Kv1.2 current can be a potential approach to cure EE. The aim of this research was to identify and characterize a potent and selective inhibitor of Kv1.2 ion channel from the venom of scorpions. With the help of our collaborators, we isolated seven new peptides (CboK1-CboK7, 32-39 amino acid residues) from venom of *C. bonito*, two peptides from the venom of *C. villegasi* (Cvill6 and Cvill7, 38 and 39 amino acid residues) and a synthetic peptide, sCm39, which was identified from the venom of a known scorpion *C. margaritatus.*

Electrophysiological characterization of seven peptides showed that among all except CboK1 all six peptides inhibit Kv1.2 with high affinity (K_d values between 24-763 pM) and have reasonable selectivity over Kv1.3 (K_d values between 20.4-171 nM). Of these seven, CboK7 emerged as a high affinity and selective blocker of Kv1.2 having 850-fold and 6000-fold selectivity over Kv1.3 and Kv1.1.

Pharmacological analysis of Cvill peptides shows that Cvill6 has moderate affinity for Kv1.2 (K_d = 3.9 nM) whereas Cvill7 possesses high Kv1.2 affinity (K_d = 16 pM). Cvill6 has 215-fold and 59-fold selectivity over Kv1.3 and KCa3.1, respectively. In contrast Cvill7 has high selectivity for Kv1.2 over Kv1.3 and KCa3.1 (450-fold and 33000-fold, respectively). Binding kinetics analysis suggests that Cvill6 and Cvill7 follow the binding pattern of classical pore blockers.

sCm39 inhibited Kv1.2 currents with K_d value of 65 nM and does not show any effect on Kv1.1 and Kv1.3 at 1 at 1 μM. The investigation for the mechanism of block and binding kinetics revealed that Cm39 binds to the pore of the Kv1.2 ion channel thus it is not a gating-modifier.

In summary, this dissertation presents the electrophysiological characterization of ten peptide toxins, among which two CboK7 and Cvill7 emerged as potent, high-affinity blockers of the Kv1.2 ion channel. These findings lay the groundwork for identifying key amino acid residues critical for high-affinity Kv1.2 blockade. Moreover, they provide a valuable foundation for the potential therapeutic application of Kv1.2-targeting peptides in the treatment of epileptic encephalopathy (EE) and other related neurological disorders.

8. APPENDIX

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Registry number: DEENK/306/2025.PL
Subject: PhD Publication List

Candidate: Kashmala Shakeel
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. Naseem, M. U., Gurrola-Briones, G., Romero-Imbachi, M. R., Borrego, J., Carcamo-Noriega, E., Beltrán-Vidal, J., Zamudio, F. Z., **Shakeel, K.**, Possani, L. D., Panyi, G.: Characterization and Chemical Synthesis of Cm39 (α-KTx 4.8): a Scorpion Toxin That Inhibits Voltage-Gated K+ Channel KV1.2 and Small- and Intermediate-Conductance Ca2+-Activated K+ Channels KCa2.2 and KCa3.1.
   Toxins. 15 (1), 1-21, 2023.
   DOI: http://dx.doi.org/10.3390/toxins15010041
   IF: 3.9
2. **Shakeel, K.**, Olamendi-Portugal, T., Naseem, M. U., Becerril, B., Zamudio, F. Z., Delgado-Prudencio, G., Possani, L. D., Panyi, G.: Of Seven New K^+ Channel Inhibitor Peptides of Centruroides bonito, α-KTx 2.24 Has a Picomolar Affinity for Kv1.2.
   Toxins. 15 (8), 1-20, 2023.
   DOI: http://dx.doi.org/10.3390/toxins15080506
   IF: 3.9

List of other publications

3. Sanches, K., Ashwood, L. M., Olushola-Siedoks, A. A. M., Wai, D. C. C., Rahman, A., **Shakeel, K.**, Naseem, M. U., Panyi, G., Prentis, P., Norton, R. S.: Structure-function relationships in domain peptides: from the sea anemone.
   Proteins. 92 (2), 192-205, 2024.
   DOI: http://dx.doi.org/10.1002/prot.26594
   IF: 3.2 (2023)

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4. Borrego, J., Naseem, M. U., Sehgal, A. N. A., Panda, L. R., **Shakeel, K.**, Gáspár, A., Nagy, C., Varga, Z., Panyi, G.: Recombinant Expression in Pichia pastoris System of Three Potent Kv1.3 Channel Blockers: Vm24, Anuroctoxin, and Ts6.
J. Fungi. 8 (11), 1-15, 2022.
DOI: http://dx.doi.org/10.3390/jof8111215
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