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

Studying interactions between peptide toxins and voltage-gated K^+ channels at the molecular level

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2023

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The PhD defence takes place at Lecture Hall F003-004, Life Science Building, University of Debrecen, at 11:30 a.m. on 20th June 2023.

1. PREFACE

Potassium ion (K^+) channels regulate various physiological functions in both excitable and non-excitable cells. In human T-cells K^+ channels maintain the negative resting membrane potential by the K^+ efflux and thus, regulate Ca^{2+} signaling required for T-cell proliferation and production of cytokines. Voltage-gated K^+ channel $K_V1.3$ is drastically upregulated in effector memory T (T_{EM}) cells that are key mediators of pathological autoinflammation. Since selective blockade of $K_V1.3$ persistently suppresses activation and proliferation of T_{EM} cells, $K_V1.3$ has become an attractive therapeutic target to treat several autoimmune diseases driven by T_{EM} cells such as rheumatoid arthritis and multiple sclerosis. Emerging evidence implicates $K_V1.3$ in activation of microglia, brain-resident macrophages. Thus, $K_V1.3$ is also a potential drug target in treatment of neuroinflammatory disorder such as Alzheimer's and Parkinson's diseases.

Scorpion venom contains a complex mixture of peptide toxins which can bind to K^+ channels with great affinity and module their function. Several peptide toxins have been derived from scorpion venom which inhibit $K_V1.3$ with pico-nanomolar affinity and high selectivity over other K^+ channels, for example, Margatoxin (K_d = 11 pM),) Vm24 (K_d = 3 pM) and HsTX1 (K_d = 11 pM). Their inherent high affinity for $K_V1.3$ makes them attractive lead compounds for drug development and designing a diagnostic tool for autoimmune diseases. These toxins consist of 23-40 amino acid residues and share a common structural motif consisting of an α-helix/β-sheet scaffold stabilized by 3-4 disulfide bridges. A typical functional dyad that consists of a lysine residue and an aromatic residue is also considered a common feature of these toxins. The critically positioned lysine of the dyad that protrudes into the selectivity filter of channel is essential for blocking the current. However, some scorpion toxins completely lacking this dyad, yet block $K_V1.3$ with nanomolar affinity, also exist. Thus, their diverse structural nature and potential application in $K_V1.3$ mediated autoimmune diseases encourages the exploration of different scorpion venoms from various geographic regions for novel toxins.

In the present work, first we describe the pharmacological and functional properties of a novel peptide toxin isolated from Colombian scorpion *Centruroides margaritatus*. Secondly, we characterize a fluorescent analogue of HsTX1[R14A], a potent $K_V1.3$ inhibitor peptide, as a new tool for selective visualization of $K_V1.3$ expression. Thirdly, to meet the demand of large quantities of peptide toxins for pharmacology and therapeutic development, we aim to optimize *Pichia pastoris* expression system for high-level production of margatoxin as an exemplary peptide.

2. SCIENTIFIC BACKGROUND

2.1 Overview of potassium channels

Potassium channels are critical in the regulation of multiple physiological processes not only in excitable cells such as neuronal excitability, axonal conduction, hormone, and neurotransmitters secretion, but also in non-excitable cells where they participate in controlling cell volume regulation, calcium signaling, proliferation and apoptosis. For these processes, the human genome contains 78 different K^+ channel genes. Among these voltage-gated potassium channels (K_V) family includes 12 subfamilies (K_V1-K_V12).

2.2 Molecular architecture of $K_V1.3$ channel

$K_V1.3$ is one of the 8 members of the mammalian K_V1 family (*Shaker*-related) of K_V channels. The basic structural organization of K_V1 channels bears a high degree of similarity. The functional K_V1 channels are assembled from four non-covalently linked α-subunits of similar structure which lie approximately in symmetrical position with respect to each other. Each α-subunit is composed of a large cytoplasmic tetramerization domain called T1, and 6 transmembrane alpha helices (TM) commonly denoted as S1-S6. The voltage sensor domain (VSD) consists of TM α helices S1-S4, with the S3 having a net negative charge and the S4 containing multiple positive residues repeating RXXR motif, thus playing a prominent role in voltage-dependent channel opening. S5-S6 TM α helices and extracellular pore loop (P) connecting S5 with S6 form the pore domain (PD). The P contributes to form the highly conserved selectivity filter, responsible for the selective permeation of K^+ ions. The four S6 TM α helices of tetrameric channel come together on the intracellular region of the cell membrane and form the internal activation gate (A-gate). When the cell is at rest this gate is closed, however, upon membrane depolarization the S4 of the VSDs move towards the extracellular space while undergoing a conformational change. This structural rearrangement is presumably transferred through the S4-S5 linker region to the S6, whose displacement opens the A-gate and allows K^+ ions efflux. Under prolonged depolarization, $K_V1.3$ channels undergo C-type inactivation, a process which is considered to involve confirmational changes in selectivity filter that interrupts the ion coordination thereby diminishing K^+ ion permeation. The functional properties of the channels are mainly determined by the selectivity filter and the VSD, however, additional subunits can also influence the gating, expression, or sensitivity of the channel to certain molecules.

Despite having many similarities and highly conserved sequences in the selectivity filter and pore regions of K_v1 family members, subtle differences may exist among the topology of extracellular vestibules. Among these the so called “turret region” that forms the extracellular vestibule of the channel, is a part of the extracellular loop connecting the S5 to the pore region differs among K_V1 channels. This turret region along with the tip of the S6 and the loop connecting the pore helix to S6 forms the receptor site for animal toxins that plug the pore by docking into the extracellular vestibule of the channels. This structural difference among the K_V1 channel types is of great pharmacological importance as it may contribute to the different sensitivity for animal toxins. For example, $K_V1.2$ ion channel is less sensitive to kaliotoxin

(KTX, a scorpion derived peptide toxin), which inhibits the $K_V1.3$ ion channel in picomolar concentrations (K_d = 100 – 400 pM).

2.3 Role of $K_V1.3$ in T lymphocyte activation

Upon antigen presentation, activation of T lymphocyte requires a Ca^{2+} signal that depends critically on Ca^{2+} entry into the cytoplasm through the calcium-release-activated Ca^{2+} channels (CRAC), formed by the pore-forming Orai1 subunits in the plasma membrane and the STIM proteins embedded in the endoplasmic reticulum membrane. As a consequence of Ca^{2+} influx through CRAC channels or other channels involved in Ca^{2+} signal following the T cell receptor (TCR) mediated stimulation, depolarization of membrane reduces electrochemical driving force for sustained Ca^{2+} influx. This effect is significantly counterbalanced by efflux of K^+ ions through two K^+ channels; voltage-gated $K_V1.3$ channel and Ca^{2+}-activated K^+ channel $K_{Ca}3.1$ (IK_{Ca}). The $K_V1.3$ channels are activated by membrane depolarization induced by Ca^{2+} influx, whereas the elevated intracellular Ca^{2+} mediates the opening of $K_{Ca}3.1$ channels by binding to the calmodulin located in the C-terminus region of the channel. Following activation of K^+ channels, an outward K^+ flow reinstates and sustains hyperpolarized membrane potential, thereby maintaining the driving force required for Ca^{2+} entry. This crucial role of K^+ channels in persistent Ca^{2+} signaling, makes the T lymphocyte activation sensitive to K^+ channel inhibitors. Therefore, pharmacological manipulation of T cell function through drugs targeting these K^+ channels bear a great potential in manipulating the immune responses.

2.4 $K_V1.3$ as an attractive therapeutic target

The expression pattern of $K_V1.3$ and $K_{Ca}3.1$ channels in T cell depends on the T cell subset, activation, and differentiation status. In the resting state, 200-300 $K_V1.3$ and 5-35 $K_{Ca}3.1$ ion channels are present on the surface of human naïve T, central memory T_{CM} and effector memory T_{EM} cells. However, antigen induced stimulation of T cells leads to differential expression pattern of these K^+ channels in T cell subsets. $CCR7^+$ effector T cells and T_{CM} cells up-regulate the $K_{Ca}3.1$ channel to 500 channels per cell with little or no change in $K_V1.3$ expression. In contrast, following several rounds of antigen-stimulation chronically activated $CCR7^-$ T_{EM} cell drastically up-regulate the $K_V1.3$ expression to 1500 channels per cell (~5-fold increase compared to resting state) with largely unchanged number of $K_{Ca}3.1$ channels. This T cell subtype-dependent expression of the $K_V1.3$ or $K_{Ca}3.1$ ion channels confers differential sensitivity of the proliferation of these T cell subsets to selective K^+ channel inhibitors. Specific blockers of $K_{Ca}3.1$ target the function of $CCR7^+$ naïve and T_{CM} cells however, $K_V1.3$-selective blockers preferentially suppress the proliferation of $CCR7^-$ T_{EM} cells.

There are >80 different types of autoimmune diseases have been identified and together these affect about 5-8% of global population. For several autoimmune diseases, the overexpression of $K_V1.3$ in autoreactive T_{EM} cells has been confirmed such as multiple sclerosis, rheumatoid arthritis, type 1 diabetes mellitus and psoriasis/psoriatic arthritis. The upregulation of $K_V1.3$ channels in autoreactive T_{EM} cells has emerged as an exciting therapeutic target to remit the autoimmune diseases, as the selective inhibition of $K_V1.3$ impairs only terminally activated T_{EM} cell responses leaving the protective immune responses of naïve and T_{CM} cells unharmed. Emerging evidence implicates $K_V1.3$ in activation of microglia, brain-resident macrophages.

Thus, $K_V1.3$ is also a potential drug target in treatment of neuroinflammatory disorder such as Alzheimer's and Parkinson's diseases.

Furthermore, there are many other chronic inflammatory diseases in which overexpression of $K_V1.3$ in T_{EM} cells has been observed such as asthma, inflammatory bowel diseases, allergic contact dermatitis and atherosclerosis. Abnormal/ectopic expression pattern of $K_V1.3$ has been reported across several cancer cell types and stages. Among these, elevated expression of $K_V1.3$ in breast adenocarcinoma, chronic B lymphocytic leukemia, alveolar rhabdomyosarcoma, and certain prostate cancers was reported. $K_V1.3$ inhibition exhibited useful effects *in vitro* by reducing the proliferation and metastasis formation.

2.5 $K_V1.3$ channel inhibitors

There are two large groups of molecules that block $K_V1.3$ channel (i) Small organic molecules and (ii) venom-derived peptide toxins. Small organic molecules typically have less than 800 Da molecular weight (MW) and bind to the central cavity of $K_V1.3$ channel with less interaction points, causing low affinity and poor selectivity. For Example, PAP-1 is the most potent small-molecule inhibitor of $K_V1.3$ reported to date with IC_{50} of 2 nM and at least 23-fold selectivity over other members of K_V1 family. In contrast, peptide toxins isolated from animal venom typically have a MW of 3-4 kDa and interact with extracellular pore region of channel with a fairly large interacting surface. Therefore, $K_V1.3$ inhibiting peptide toxins have high potency (ranging from pico- to nanomolar) and selectivity (>1000-fold over other K^+ channels).

2.5.1 Venom-derived K^+ channel blocking peptide toxins

In the last three decades, several peptide toxins targeting K^+ channels have been described. Currently, the Kalium database, a collection of natural peptides affecting K^+ channels enlists 350 entries. Based on the structural and functional characteristics, potassium channel inhibitor toxins (KTxs) derived from scorpion are classified into 7 different families: α-KTx, β-KTx, γ-KTx, δ-KTx, ε-KTx, κ-KTx, and λ-KTx.

The α-KTx family contains peptides with 23–42 amino acid residues and shares a common structural motif known as the cysteine-stabilized α/β scaffold, in which the α-helix and β-sheets are held together by 3–4 disulfide bridges. Based on the sequence similarity among α-KTx family members, 31 subfamilies of α-KTx have been described previously (https://kaliumdb.org). A common feature of K^+ channel blocking peptides isolated from various animal venoms is the presence of a typical "functional dyad" which consists of a critically positioned Lys residue and an aromatic residue (usually Tyr). The side chain of the critical Lys protrudes into the selectivity filter of the channel and plugs the pore, thereby preventing the K^+ ion efflux. The interaction of an aromatic residue with external vestibule of K_V1 channels is considered responsible for selectivity among $K_V1.x$ channel subtypes. Some α-KTxs bear only the critically positioned Lys residue of functional dyad but lack the aromatic residue. More interestingly, scorpion toxins lacking this signature dyad have been identified but are still active on K_V1 channels. For example, BmP02 (α-KTx 9.1), a short peptide toxin from Chinese scorpion lacks the typical functional dyad however, it still inhibits $K_V1.3$ (K_d = 7nM). This suggests that for stable toxin–channel interaction, other influential residues of toxins may interact pairwise with the channel residues contributing to their high affinity and selective

binding to different K_V1.x channels. The other mechanism through which toxins inhibit K^+ channel function is the "gating modifier" effect in which toxins bind to the voltage sensor domains (VSD) and cause a prominent shift in the voltage dependence of steady-state activation towards more depolarized potentials and consequently, reduce the K^+ current. For example, a spider venom peptide Hanatoxin is a gating modifier and inhibits K^+ current by interaction with VSD of K_V2.1 channel.

Most potent peptide toxins affect multiple ion channel subtypes with comparable affinities due to the topological similarity of the outer pore region among K^+ channels. For example, in addition to K_V1.3 inhibition with pM affinity anuroctoxin (AnTx) blocks K_V1.2, while HsTX1 blocks K_V1.1. Vm24, described by our group, is the only known natural peptide from scorpion with highest affinity (K_d= 2.9 pM) and selectivity for K_V1.3. It has more than 1500-fold selectivity for K_V1.3 over ten other channels tested including K_V1.1, K_V1.2, K_{Ca}3.1. High affinity toxins are attractive lead peptides for the development into therapeutically usable drugs. Therefore, to improve the selectivity of natural toxins for K_V1.3 over other channels multiple protein engineering strategies are employed to minimize the off-target effects. For example, HsTX1[R14A] mutant retained high affinity for K_V1.3 and showed 2,000-fold selectivity over K_V1.1. Similarly, by using sequence analysis our group engineered an analog of Anuroctoxin (α-KTx-6.12, AnTx) with double substitution (N17A/F32T) which preserved its natural potency for K_V1.3, while gaining 16,000-fold selectivity over K_V1.1, K_V1.2 and K_{Ca}3.1 Shk-186, an engineered toxin analogue from Sea anemone, is a potent and selective inhibitor of K_V1.3. ShK-186, now known as Dalazatide, has been approved as the first toxin-based drug to enter clinical trial for autoimmune diseases.

The ongoing discovery of K^+ channel blocker peptides suggests that scorpion venoms are remarkably rich sources of attractive peptides. The diverse nature of their primary sequence, robust interaction pattern with their receptors and valuable therapeutic potential encourages the scientists to explore the different scorpion venoms from various geographic regions for novel peptides.

2.6 Fluorescent toxins as probe for K_V1.3 channel

Upregulation of K_V1.3 in immune and CNS cells has emerged as an appealing target for autoimmune and neurological diseases and its role is beginning to be understood in non-immune cells during pathological states. Therefore, a selective molecular visualization tool is much needed to investigate the contribution of K_V1.3 to pathogenesis, to study the expression of K_V1.3 in intact cells from various tissues, for all stages of therapeutic development and as a diagnostic tool.

The venom-derived toxins bind selectively and with extremely high affinities to channel thus, these toxins have been exploited to create fluorescently labeled probes for K_V1.3. However, unlike K_V1.3 specific antibodies, which bind to the epitopes located on the outer vestibule of single subunit, peptide toxins bind to the tetrameric functional channel and can easily recognize folded from unfolded channel or distinguish homotetrameric from heterotetrameric assembly of channel. Previously, K_V1.3-targeted probes were developed by chemically conjugating a fluorophore with toxins or recombinantly linking to the fluorescent proteins. For example, fluorescein-6-carboxyl (F6CA) was conjugated to N-terminus of ShK

toxin which showed picomolar affinity for $K_V1.3$ with IC_{50}= 48 pM. MgTx, AgTx and OSK1 were conjugated with green fluorescent protein and resulting chimera exhibit nanomolar affinity for $K_V1.3$. So, there is still space to expand the available toolkit for visualization of $K_V1.3$. A fluorescently labeled analogue of highly potent and selective toxin for $K_V1.3$ can be designed as a tool for *in vivo* and *ex vivo* imaging, to track the biodistribution of drug peptides and for diagnostic of chronic inflammatory diseases.

2.7 Recombinant production of peptide toxins

The major challenge in characterizing the pharmacological and structural properties of peptide toxins and their therapeutic development and application is the constraint of the native toxin material that can only be extracted from animal venom in extremely limited amounts. Due to the complex structural features and presence of multiple disulfide-bridges the preparation of peptide toxins faces several technical challenges. The chemical synthesis offers an approach to artificially produce disulfide rich peptides and their analogs. However, it is expensive and yields a small quantity of active conformers of peptide toxins. On the other hand, heterologous protein expression system is a cost-effective and most widely used technique to produce large quantities of recombinant proteins. However, recombinant expression of proteins of eukaryotic origin in bacteria needs post translational modifications and *in vitro* refolding for proper activity of peptides. Although some engineered *E. coli* strains are capable of disulfide bond formation and refolding the peptide, their yield is still very low. For example, margatoxin was previously produced in *E. coli* yielding 3−4 mg/L of functional peptide. The yeast expression system can over overcome all these limitations.

2.7.1 Yeast expression system

Pichia pastoris, a methylotrophic yeast, was developed as a heterologous expression system and has become widely popular because of its numerous advantages over bacterial system. It offers an economical and better approach for higher yields of correctly folded recombinant peptides. Foreign genes are expressed under the alcohol oxidase 1 (*AOX1*) promoter, tightly induced by methanol (MeOH) which regulates alcohol oxidase expression, an enzyme involved in MeOH metabolism. High biomass production in simple medium, ease of genetic manipulation and capability of performing posttranslational modifications are other advantages of this system. Additionally, the recombinant proteins are secreted directly into the medium with very few endogenous proteins which simplifies the downstream processing. For example, Anangi *et al*. expressed and purified the His-tagged margatoxin and agitoxin in *Pichia* expression system with a yield of 12−15 mg/L and 14−18 mg/L, respectively. Our group has also reported a high yield of AnTx and Ts6 peptide toxin in the *P. pastoris* system recently.

Pichia pastoris has the tendency to incorporate multiple copies of foreign gene cassettes at a single locus through homologous recombination, facilitating high level heterologous expressions. Moreover, the overexpression of foreign proteins can be enhanced considerably by codon-optimization, screening for multiple copy integrant and choice of efficient promoter. Similarly, several studies have proved that optimization of the cell fermentation conditions such as biomass production, pH of the medium, induction duration and percentage of MeOH induction for individual protein resulted in an improved expression of heterologous proteins in *Pichia pastoris* system.

3. AIMS OF THE STUDY

3.1 Pharmacological and functional characterization of a novel $K_V1.3$ inhibitor toxin from *C. margaritatus* scorpion

Scorpion venom is a rich source of K_V channel affecting peptide toxins. In quest for novel $K_V1.3$ blockers a unique new peptide toxin, named Cm28, was identified from the Colombian scorpion *Centruroides margaritatus* by our collaborators. Our aims were:

1. To characterize the pharmacological properties of Cm28 against a battery of K^+ and Na^+ channels through single-cell electrophysiology
2. To investigate the mechanism of block of $K_V1.2$ and $K_V1.3$ channels by Cm28
3. To study the biological effect of Cm28 on human T lymphocyte activation

3.2 Characterization of a fluorescent peptide toxin as a probe for $K_V1.3$

A fluorescent analogue of a potent scorpion toxin HsTX1[R14A] as a tool to visualize the $K_V1.3$ expression was developed by conjugating the cyanine dye Cy5 to the chemically synthesized toxin by our collaborators. Our aims were:

1. To analyze the binding affinity of Cy5-HsTX1[R14A] with various K^+ channels including the $K_V1.3/K_V1.5$ hetrotetrameric channel using single-cell electrophysiology
2. To demonstrate the ability of Cy5-HsTX1[R14A] to detect $K_V1.3$ expression level in flow cytometry

3.3 Optimization of recombinant production of margatoxin and the functional characterization of the tagged and untagged versions of the peptide

Due to structural complexities, preparation of larger amounts of toxins faces a number of technical challenges. Meanwhile the produced peptides should maintain their biological functions. Our aims were:

1. To improve the yield of recombinant MgTx in the *Pichia pastoris* (yeast) expression system through optimizing multiple factors at genetic level and the fermentation conditions
2. To test the effect of conjugation of MgTx with the His-tag on the binding to $K_V1.2$ and $K_V1.3$ channels
3. To validate the biological activity of rMgTx variants on human T lymphocyte activation

4. MATERIALS AND METHODS

4.1 Toxins

The native Cm28 peptide toxin was isolated from the venom of the Colombian scorpion *Centruroides margaritatus* in Prof. Lourival Possani's laboratory (our collaborator from UNAM, Mexico) following three-step purification. (i) size exclusion chromatography (ii) ion-exchange chromatography (IEC) and RP-HPLC using analytical C_{18} column. The amino acid sequencing of Cm28 peptide was performed by automatic Edman degradation using Biotech PPSQ-31A Protein Sequencer equipment.

HsTX1[R14A] is a modified scorpion toxin originally isolated from the venom of *Heterometrus spinifer*. A fluorescent analogue of this toxin was developed by Prof. Raymond Norton's laboratory (our collaborators from University of Monash, Australia). HsTX1[R14A] was prepared using solid phase chemical synthesis and the cyanine dye Cy5 was conjugated at its N-terminus, and spectral properties of Cy5-HsTX1[R14A] were characterized using fluorescence spectroscopy.

4.2 Recombinant margatoxin production

The amino acid sequence of margatoxin (MgTx) was retrieved from an online protein (Uniprot P40755) database. The MgTx gene cassette was designed by placing the 6x His-tag at N-terminus to facilitate the purification, and factor Xa protease site was introduced in-between them to obtain native N-terminus MgTx. The codon-optimized MgTx cassette was cloned into yeast expression vector pPICZαA using *EcoRI* and *XbaI* restriction sites following the standard cloning procedures and transformed into *P. pastoris* X-33 competent cells using Pichia EasyComp Transformation Kit following the protocol specified by the manufacturer. Transformants were further screened against progressively increasing Zeocin 0.5, 1 and 2 mg/ml for the selection of clone showing hyper-resistance against Zeocin.

Selected clone from the YPD plate containing 2 mg/ml of Zeocin was grown overnight in 5 ml of YPD medium and next day, it was diluted to an OD_{600} = 0.2 in 5 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin and 2% glycerol) for biomass production at 30 ℃ with constant shaking (230 rpm) until the OD_{600} reached between 15 and 20 (after 24-36 h). Cells were collected by centrifugation, re-suspended in 5 ml of BMMY induction medium (same as BMGY with 0.5% MeOH instead of glycerol) and grown for five days at 28 ℃ with constant shaking (230 rpm). 0.5% (v/v) MeOH induction was maintained by adding absolute MeOH every 24 h to maintain the induction except when MeOH concentration dependence of induction was studied. To find the suitable concentration for MeOH induction, cells were induced with 0.5, 1 and 1.5% (v/v) MeOH and for pH optimization, cells were grown in media of different pH values of pH 5, pH 6, and pH 7 and in unbuffered media. 15 µl of supernatant samples were taken from indicated time points and analyzed on 16% tricine SDS-PAGE. The amount of TrMgTx in the gel image was determined by comparing the band intensities with the standards (TrMgTx with known concentration) using Image Lab tool (Bio-Rad). All the experiments were run in triplicates.

4.2.1 Large-scale Fed-batch fermentation and purification of TrMgTx

Large-scale flask-level production was executed following the optimized conditions as described above. Two-step purification was employed to efficiently isolate secreted TrMgTx from the culture. First, the filtered supernatant was subjected to affinity chromatography using His-trap column packed with Ni^{2+} Sepharose™ High Performance and liquid chromatography system. Then, fractions eluted from affinity column using different concentration of imidazole were directly applied on reversed phase (RP) C_{18} semi-prep column (10 mm x 250 mm, 5 µM bead size, 300 Å pore size, Vydac® 218TP, HiChrom, UK) using Prominence HPLC System. A linear gradient of 10-30% of solvent B (0.1 % TFA in 95% acetonitrile) in solvent A (0.1% TFA in deionized distilled water) was run over 30 min. Peak fractions were collected manually and tested on 16% tricine-SDS PAGE. Purity level was judged by reloading the fraction on analytical C_{18} column and calculated using equation [(area under the peak of interest)/ (cumulated area under all peaks) x 100]. Purified peptide was stored at –20 ℃ for further assays.

Hexahistidine residues fused at N-terminus of TrMgTx were cleaved using factor Xa protease to generate UrMgTx. 16% tricine/6M urea-SDS PAGE was performed to confirm the successfully removal of His-tag and digested sample were subjected to RP-HPLC using C_{18} analytical column to purify UrMgTx.

4.2.2 Tricine SDS-PAGE and Western blot

16% tricine SDS-PAGE was performed as described hitherto (Schägger, 2006). Protein sample was mixed with tricine sample buffer in 1:1, incubated at 95℃ for 5 min and subsequently centrifuged at 10,000 rpm for 30 seconds before loading. Electrophoresis was carried out at constant 120 V for 90 min. For protein visualization, the gel was stained with Coomassie Brilliant Blue (CBB) R-250 for 45 min and de-stained using 40% MeOH and 10% acetic acid mixture for 2-3 h.

For Western blotting, the resolved proteins were electro-transferred in wet conditions onto charged Immobilon-P PVDF membrane. Non-specific binding of antibody in the subsequent steps was prevented by membrane blocking with 5% (w/v) skim milk in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for overnight at 4 ℃. The washed membrane was probed with mouse anti-histidine monoclonal antibodies conjugated with horseradish peroxidase in TBST (1:2500) and incubated for 1 h at room temperature. The bands were visualized using Pierce™ enhanced chemiluminescent (ECL) substrate.

4.3 Cell culture

Chinese hamster ovary (CHO) cells and Human embryonic kidney (HEK) 293 cells were cultured following standard procedures. Human venous blood from anonymized healthy donors was obtained from blood bank. Peripheral blood mononuclear cells (PBMCs) were isolated through Histopaque1077 density gradient centrifugation. Cells obtained were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal calf serum (Sigma-Aldrich), 100 µg/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine, seeded in 24-well culture plate at density of $5\ x\ 10^5$ cells per ml and grown in a 5% CO_2 incubator at 37 ℃ for 3 to 6 days. Phytohemagglutinin A (PHA, Sigma-Aldrich) was added

in 5, 7 and 10 µg/ml concentration to the medium to active the PBMCs and boost up the $K_V1.3$ channel expression.

4.4 Single-cell electrophysiology

4.4.1 Plasmid Construction for Tandem Dimers

To construct the $K_V1.3$-$K_V1.3$ tandem dimer, the KCNA3 gene (GenBank accession no. NM_002232.5) excluding the stop codon was amplified by PCR and cloned into the pEGFP-C1 vector using *BglII* and *EcoRI* restriction sites. A second copy of the KCNA3 gene (including the stop codon) was amplified and cloned downstream of the first copy of KCNA3 in the pEGFP-C1-KV1.3 plasmid using *EcoRI* and *HindIII* restriction sites. The $K_V1.3$-$K_V1.5$ tandem dimer was made by replacing the second domain copy of KCNA3 gene in pEGFP-C1-$K_V1.3$-$K_V1.3$ plasmid with KCNA5 gene (GenBank accession no. NM_002234.4) using *EcoRI* and *HindIII* endonucleases. In-frame ligation and gene sequences were confirmed by DNA sequencing.

4.4.2 Heterologous expression of ion channels

CHO cells were transiently transfected using Lipofectamine 2000 kit according to manufacturer's protocol with the vectors coding for respective ion channels. Cells were co-transfected with a GFP expressing plasmid when the target ion channel gene lacked the fluorescent tag. GFP or YFP-positive transfectants were identified with a Nikon TS-100 fluorescence microscope, and currents were recorded 20−30 h post-transfection.

Human embryonic kidney (HEK) 293 cells stably expressing h$K_V11.1$ (*hERG1*, *hKCNH2* gene, a kind gift from H. Wulff, University of California, Davis, CA), m$K_{Ca}1.1$(BK_{Ca}, *mKcnma1*, a kind gift from C. Beeton, Baylor College of Medicine, Houston, TX) and h$Na_V1.4$ (*hSCN4A* gene, a kind gift from P. Lukács, Eötvös Loránd University, Budapest, Hungary) were used.

4.4.3 Patch-clamp electrophysiology recording conditions

Whole-cell currents were measured using patch-clamp technique in voltage-clamp mode following standard protocols (Hamill *et al.*, 1981). All recordings were performed using Multiclamp 700B amplifier connected to a personnel computer with Axon Digidata1440 digitizer and for data acquisition, Clampex 10.7 software was used (Molecular Devices, Sunnyvale, CA). In general, current traces were lowpass-filtered by using the built-in analog four-pole Bessel filters of the amplifier and sampled (4-50 kHz) at least twice the filter cutoff frequency. Micropipettes were pulled from GC150F-7.5 borosilicate capillaries (Harvard Apparatus, Kent, UK) with tip resistance typically ranging 3-6 MΩ in the bath solution. Only those records were used for data analysis when the leak current at holding potential was <10% of peak current at the test potential. Recordings were carried out at room temperature (20-25°C). Control and test solutions were perfused to the cell through gravity flow perfusion system. Excess bath solution was removed constantly with vacuum suction.

For the measurement of $K_V1.x$, m$K_{Ca}1.1$ and $Na_V1.x$ currents the normal bath solution consisted of (in mM) 145 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 5.5 glucose, and 10 HEPES, pH 7.35. To record the tail current of $K_V1.2$ the bath solution (HK-20) contained 130 mM NaCl

and 20 mM KCl, other components remained unchanged. In the HK-150 bath all Na^+ was substituted by K^+ to yield 150 mM K^+ concentration. In the Na^+-free extracellular solution all Na^+ was substituted by Choline-Cl, other components remained unchanged. Equimolar substitution of Na^+ for tetraethylammonium-Cl was used in the various TEA^+-containing solutions. The bath solution for $K_V11.1$ consists of (in mM) 140 Choline-Cl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 20 glucose, 0.1 $CdCl_2$, pH 7.35; for $K_{Ca}3.1$ (in mM)145 L-Aspartic Na^+ salt, 5 KCl, 10 HEPES, 5.5 glucose, 2.5 $CaCl_2$, 1 $MgCl_2$, pH 7.4 and for hH_V1 (in mM) 60 L-Aspartic acid Na^+ salt, 80 MES, 5.5 glucose, 6 $MgCl_2$, pH 7.4. The osmolarity of the extracellular solutions was between 302 and 308 mOsm/L. All the bath solutions were supplemented with 0.1 mg/ml bovine serum albumin (BSA) to prevent toxin adsorption to the plastic surfaces of the perfusion system. The composition of internal solution was (in mM) 140 KF, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES and 11 EGTA, pH 7.22 for recording $K_V1.x$ and $mK_{Ca}1.1$ currents. For $Na_V1.4$- $Na_V1.5$ currents the internal solution consisted of (in mM) 10 NaCl, 105 CsF, 10 HEPES, 10 EGTA pH 7.2 and for $K_V11.1$ (in mM) 140 KCl, 2 $MgCl_2$, 10 HEPES, 10 EGTA, pH 7.3. The internal solution for $K_{Ca}3.1$ recording contained (in mM) 150 K-Asp, 5 HEPES, 8.5 $CaCl_2$, 1.0 $MgCl_2$ pH 7.22 and for hH_V1 (in mM) 90 L-Aspartic acid with Na, 80 MES, 6 $MgCl_2$, 3.3 glucose pH 6.17. The measured osmolarity of internal solutions was ~ 295 mOsm/L.

In general, for all the measurements the holding potential (V_h) was kept at −120 mV and the pulses were delivered every 15 s except when indicated. For recording the currents of $K_V1.x$ and tandemly linked $K_V1.3$-$K_V1.5$ channels, 15-2000 ms long voltage pulses to +50 mV were applied. To record the $K_V1.3$ currents for conductance-voltage (G-V) relationship, activated T cells were depolarized to voltages ranging from -70 mV to +50 mV in steps of 10 mV every 15 s. For instantaneous current-voltage (I-V) relationships of $K_V1.2$ and $K_V1.3$, currents were evoked with 200-ms-long voltage ramps to +50 mV. For recording $K_V11.1$ current, the voltage step to +20 mV for 1.25 s from a V_h of −80 mV was followed by a step to −40 mV for 2 s, applied every 30 s, and the peak (tail) currents were recorded during the latter step. $mK_{Ca}1.1$ currents were evoked by depolarizing the cells to +100 mV for 600 ms from a V_h of −100 mV. For $K_{Ca}3.1$ currents, 150-ms-long voltage ramp to +50 mV from −120 mV was applied every 10 s. Current through human proton channel (hH_V1) was elicited by applying 1.0-s-long voltage ramp to +100 mV from a V_h of −60 mV every 15 s. For the recording of Na^+ currents through $Na_V1.4$ and $Na_V1.5$, 15-ms-long voltage steps to 0 mV were applied every 10 s.

4.4.4 Patch-clamp data analyses

For patch-clamp data analyses, pClamp 10.7 software package (Molecular Devices) was used. Current traces were digitally filtered with a 3-poins boxcar filter and were corrected for ohmic leakage when needed. The inhibitory effect of a peptide toxin at a given concentration was calculated as remaining current fraction (RCF = I/I_0, where I_0 is the peak current in the absence of the toxin, and I is the peak current at equilibrium block at a given toxin concentration). The data points (average of 3-5 individual records) in the dose-response curve were fitted with the Hill equation.

$$\mathrm{RCF} = \frac{K_d^H}{K_d^H + [TX]^H}$$

where [TX] is the concentration of the toxin, K_d is the dissociation constant, and H is the Hill coefficient. To construct the voltage-dependance of steady-state activation of $K_V1.3$, peak conductance (G) at each test potential was calculated from peak current (I) at a test potential (E_m) and the K^+ reversal potential (E_K) using chord-conductance equation $G = I/(E_m - E_K)$. Normalized conductance (G_{norm}) values were calculated by dividing the G values obtained at different test potentials by the maximum value of G and G_{norm} values were plotted as a function of the test potential. The Boltzmann sigmoidal equation was fitted to the data points:

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

where V_{50} is the midpoint voltage, E_m is the test potential, and k represents slope factor of the function. For determination of voltage dependence of steady-state activation of $K_V1.2$, the membrane was depolarized to different test potentials (ranging from −70 to +80 in 10 mV steps) for 300 ms and the tail currents were recorded at −120 mV. Peak tail currents recorded following various test potentials were normalized to the maximum tail current value and plotted against the corresponding test potential. Data points were fitted with a Boltzmann sigmoidal equation as stated above.

To examine the binding kinetics, normalized peak currents ($I_{norm} = I_t/I_0$, where I_t is peak current in the presence of the toxin at time t and I_0 is peak current in the absence of toxin) were plotted as function of time. Association and dissociation time constants (τ_{on}, τ_{off}) were determined by fitting the data points during the wash-in and wash-out procedures, respectively, with the single exponential decay function and single exponential rise function.

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

These time constants were utilized to calculate the association rate constant (k_{on}), dissociation rate constant (k_{off}) and dissociation coefficient (K_d), considering a simple bimolecular interaction between the channel and the toxin and using following equations:

$$k_{on} = \frac{1 - (\tau_{on} \times k_{off})}{\tau_{on} \times [toxin]}, \qquad k_{off} = \frac{1}{\tau_{off}}, \qquad K_d = \frac{k_{off}}{k_{on}}$$

4.5 Biological assays

4.5.1 Isolation of $CD4^+$ Effector memory T lymphocyte

PBMCs were isolated from healthy donor's blood and cultured as explained above. Dead Cell Removal Microbead Kit (Miltenyi Biotec B.V & CO. KG, Bergisch Gladbach, Germany) was used to eliminate the dead cells and $CD4^+$ T_{EM} lymphocytes were isolated through magnetic cell sorting (negative selection) with the $CD4^+$ Effector Memory T Cell Isolation Kit (Miltenyi Biotec B.V & CO. KG, Bergisch Gladbach, Germany) following manufacturer's instructions.

4.5.2 Activation of $CD4^+$ T_{EM} lymphocyte

To compare the effect of TrMgTx and UrMgTx on T cell activation, four treatment groups were designated: 1) unstimulated, 2) stimulated, 3) stimulated + TrMgTx (8.5 nM), and 4) stimulated + UrMgTx (5 nM). For Cm28 functional assay, $CD4^+$ T_{EM} lymphocytes were divided into five different treatment groups: 1) unstimulated and non-treated, 2) unstimulated and treated with Cm28 (1.5 µM), 3) stimulated only, 4) stimulated and treated with Cm28 (1.5 µM), 5) stimulated and treated with margatoxin (UrMgTx, 5 nM, used as control here). The high concentrations of peptide toxins were used to ensure the complete blockade of $K_V1.3$ channels throughout the entire treatment duration and to counterbalance peptide adsorption to plastic surfaces and biological degradation. To stimulate lymphocytes through the T cell receptor (TCR), anti-human CD3 monoclonal antibody was bound to the surface of 96-well cell culture plate at 1µg/well or 24-well plate at 5 µg/well in phosphate buffered saline (PBS) at 4 °C overnight. Wells were washed twice with PBS to get rid of unbound antibody. $CD4^+$ T_{EM} cells were suspended at a density of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 100 µg/ml streptomycin and 100 U/ml penicillin-G. To ensure the complete blockade of $K_V1.3$ prior to activation, cells were incubated with the toxins for 5 min (rMgTx versions) or 30 min (Cm28) and the plate was incubated in a humidified incubator at 37 °C in 5% CO_2 for 24 h. Each experiment included two technical duplicates and was performed on three different donors.

4.5.3 Cytotoxicity assay

Cellular cytotoxicity mediated by peptide toxins was determined using Pierce lactate dehydrogenase (LDH) Cytotoxicity Assay Kit following manufacturer's instructions.

4.5.4 Flow cytometry

For assessing the cell viability, Zombie NIR fixable viability Dye was used. Cells were washed with PBS and incubated with Zombie NIR dye (at 1:500 dilution in 100 µl PBS) at room temperature for 20 min in dark. For staining the cells with fluorescent antibodies, cells were washed with PBS supplemented with 1% FBS and stained with PerCP/Cyanine5.5 conjugated anti-human CD25 (IL2R) antibody (clone BC96, BioLegend) and FITC conjugated anti-human CD154 (CD40L) antibody (clone 24−31, BioLegend, San Diego, CA) in 100 µl PBS+1% FBS at 4 °C for 20 min in dark. Cells were then washed with the PBS+1% FBS buffer and finally, resuspended in 150 µl PBS+1% FBS for flow cytometer analysis. For staining the CHO or stably expressing mGFP-$K_V1.3$ CHO cells with Cy5-HsTX1[R14A] cells were washed twice with PBS containing 2% v/v FBS and incubated with 10 nM Cy5-HsTX1[R14A] in the dark at room temperature for 20 min. Cells were then washed with PBS containing 2% v/v FBS and resuspended in same the sbuffer for flow cytometry analysis. For the competition assay, cells were preincubated for 20 min with 100 nM unconjugated HsTX1[R14A] or 1 µM rMgTx before adding the Cy5-HsTX1[R14A].

Samples were measured using a NovoCyte 3000 RYB flow Cytometer (. Zombie NIR dye was excited by red laser (640 nm) and 780/60 nm emission filter was used. FITC and PerCP/Cyanine5.5 were excited by using blue laser (488 nm), and 530/30 nm and 695/40 nm emission filters were used, respectively. GFP and Cy5 were excited with blue (488 nm) and red (640 nm) lasers, and the emission filters 530/30 nm and 660/20 nm were used for GFP and Cy5,

respectively. 10,000–20,000 events were recorded for each sample and data were analyzed using FCS Express 6.0 (De Novo Software, Glendale, CA).

4.6 Statistics

Statistical analyses and graph generation were executed in GraphPad Prism software. For all the experiments, data were presented as mean ± SEM. For pairwise comparison, student's *t* test with Mann-Whitney rank sum test and for multiple comparisons, one-way ANOVA with post hoc Tukey's test was performed. Statistical significance is indicated in terms of P values.

5. RESULTS AND DISCUSSION

5.1 Pharmacological and functional characterization of a novel K_V1.3 inhibitor toxin from *C. margaritatus* scorpion

Our laboratory has a more than 20-year long history of collaboration with Prof. Lourival D. Possani and his colleagues (institute of Biotechnology, UNAM, Mexico). In the past we have described several peptide toxins isolated for scorpion. The collection of scorpions, venom extraction and purification of peptides is performed in Prof. Possani's laboratory, while the pharmacological and biological properties are mainly studied in our facility using single-cell electrophysiology and cellular assays.

Centruroides margaritatus is a scorpion from the *Buthidae* family and its venom has not been explored fully except a well-known peptide MgTx which was isolated and characterized a long time ago. Recently, a detailed proteomic analysis of *C. margaritatus* venom was performed by our collaborators and in addition to a K_V11.1 inhibitor toxin CmERG1 (γ-KTx 10.1) they found a novel peptide with molecular weight (MW) 2820.5 Da. This peptide was named "Cm28" corresponding to the scorpion's name *C. margaritatus* and its MW. Cm28 has a unique and short primary structure with only 27 amino acids residues and three potential disulfide bridges. Sequence comparison with all the known KTx scorpion toxins revealed that it has less than 40% similarity with α-KTxs and lacks the typical functional dyad (Lys-Tyr) required to block K_V channels with high affinity. In this section of my thesis, I characterized the pharmacological and immunological activities of this novel toxin Cm28.

5.1.1 Cm28 inhibits human K_V1.2 and K_V1.3 with low-nanomolar affinity

The primary structural features of Cm28 are unusual and exceptional among other scorpion toxins. Therefore, to assess whether this toxin has any pharmacological activity against K^+ channels, we first aimed to test the effects of Cm28 on two human K^+ channels; K_V1.2 and K_V1.3. The whole-cell K_V1.2 currents were measured in transiently transfected CHO cells. For K_V1.3 current measurements, human peripheral T lymphocytes were activated by Phytohemagglutinin A (PHA) to boost up the expression of K_V1.3 channels and the pipette-filling solution was Ca^{2+} free to avoid K_{Ca}3.1 channel activation (for voltage protocol see Materials and Methods). Cm28 dissolved freshly in the extracellular solution was applied through a custom-built micro perfusion system at rate of 200 µl/min. The complete exchange of solution in the bath chamber *i.e.*, the proper operation of the perfusion apparatus was confirmed frequently using fully reversible inhibitors as positive controls at a concentration equivalent to their K_d values and the approximate 50% reduction in peak current was an indicator of both the ion channel and the proper operation of the perfusion system.

At equilibrium block, Cm28 showed ~70% reduction in current amplitude. The block was almost fully reversible upon washing the perfusion chamber with toxin-free solution. Both the association and dissociation processes of Cm28 were very slow, accordingly, development of equilibrium block and recovery up to ~85% of control current took several minutes. In a similar set of experiments for K_V1.3, 2 nM Cm28 inhibited ~58% of the K_V1.3 current upon reaching the block equilibrium. Like K_V1.2, the block of K_V1.3 was also reversible (90%

recovery took 10 min) upon perfusing the cell with toxin-free solution. The onset of steady-state block and relief from the block took comparatively less time than for $K_V1.2$ indicating that the association and dissociation steps are faster for $K_V1.3$ than $K_V1.2$. The analysis of concentration-dependent block of $K_V1.2$ and $K_V1.3$ channels by Cm28 resulted in dissociation constant (K_d) values of 0.96 nM and 1.3 nM, respectively. Cm28 showed a similar affinity for both channels.

5.1.2 Mechanism of block

Generally, most of the known toxins inhibit the K_V channels either by physically occluding the pore region or by modulating the voltage-sensor of the channel. To determine the mechanism behind the $K_V1.2$ and $K_V1.3$ block by Cm28 we studied the effect of Cm28 on the threshold voltage of activation and voltage dependence of steady-state-activation of both ion channels. Instantaneous current-voltage relationship (I-V) was recorded using CHO cells for $K_V1.2$ and activated T cells for $K_V1.3$ (see Materials and Methods). Cm28 did not shift the threshold voltage of activation. The current traces in control solution and at the equilibrium block with 2 nM Cm28 showed a similar threshold voltage of activation; ~ −23 mV for $K_V1.2$ and ~ −40 mV for $K_V1.3$.

For construction of conductance-voltage (G-V) relationship for $K_V1.2$, isochronal tail current peaks were recorded in CHO cells at −120 mV followed by 300-ms long depolarizations ranging from −70 to +80 in 10 mV steps from V_h −120 mV in HK-bath containing 20 mM K^+ to increase the tail currents. Due to highly variable activation properties, only those records were considered for analysis which had similar gating mode. Normalized tail current peaks currents were fitted Boltzmann sigmoidal function. Cm28 did not introduce any substantial shift in the G-V curve of $K_V1.2$. The midpoint voltage (V_{50}) of the G-V relationship for $K_V1.2$ was 21 ± 3 mV in the control solution (n = 5) and 15 ± 4 mV at equilibrium block with 2 nM Cm28 (n = 5). For the G-V relationship of $K_V1.3$, whole-cell currents in the activated human T lymphocytes were measured and conductance values were calculated for each test potential and normalized for the maximal conductance (see Materials and Methods). The best fit of the Boltzmann sigmoidal function to the averaged data points revealed that there is no change in the voltage dependence of steady state activation of $K_V1.3$ in the presence of Cm28 at 2 nM, like for $K_V1.2$. The V_{50} values for $K_V1.3$ were similar in the control solution (V_{50} = −20 ± 3, n = 4) and at equilibrium block with 2 nM Cm28 (V_{50} = −19 ± 2, n = 4). As the voltage dependence of steady-state-activation and threshold voltage of activation were not affected by the Cm28 for both $K_V1.2$ and $K_V1.3$ ion channels, it suggests that Cm28 is not a gating modifier rather it interacts with the pore region of ion channels.

Furthermore, the binding kinetics of Cm28 toxin were also studied. The time constant for the development of the block (τ_{on}, association or wash-in time constant) was obtained by fitting a single-exponential equation as decay function to the normalized peak currents in the presence of Cm28. The time constant for the recovery from block (τ_{off}, dissociation or wash-out time constant) was determined by fitting a single exponential equation as rising function to the normalized peak currents during the wash-out procedure. With the assumption of a simple bimolecular reaction between the toxin and the channel, the resulting wash-in and wash-out time constants can be expressed as follows:

$$\tau_{on} = \frac{1}{k_{on} \times [Cm28] + k_{off}}, \qquad \tau_{off} = \frac{1}{k_{off}}$$

where k_{on} is second-order association rate constant and k_{off} is the first-order dissociation rate constant and [Cm28] is the toxin concentration. Plotting the $1/\tau_{on}$ and dissociation rate constant ($1/\tau_{off}$ or k_{off}) values as a function of the Cm28 concentrations showed that the $1/\tau_{on}$ increases linearly with toxin concentration, whereas the dissociation rate remains constant with $k_{off} = 0.0028 \pm 2.15 \times 10^{-4}$ s^{-1}, similar to ChTx binding to *Shaker* channel. The slope of superimposed regression line fitted to the $1/\tau_{on}$ data points, using 0.0028 s^{-1} as the y-intercept, corresponds to second-order rate constant of association with $k_{on} = 0.0013 \pm 2.6 \times 10^{-5}$ nM^{-1} s^{-1} ($r^2 = 0.99$).

Similarly, the association (τ_{on}) and dissociation (τ_{off}) time constants of $K_V1.2$ blockade at 2 nM Cm28 were determined and k_{on} and k_{off} rate constant were calculated using the above-mentioned equations and time constants. The dissociation constants ($K_d = k_{off}/k_{on}$) calculated from the block kinetics yielded 1.18 nM for $K_V1.2$ and 2.15 nM for $K_V1.3$, that are in good agreement with the K_d values obtained from equilibrium block.

5.1.3 Selectivity profile of Cm28

Next, to fully characterize the pharmacological properties of Cm28, we investigated its effect on various physiologically crucial ion channels. First, we tested whether two other members of voltage-gated *Shaker* family channels, $hK_V1.1$ and $hK_V1.5$, that are closely related to Cm28, are sensitive to Cm28. Additionally, we also tested the effect of Cm28 on $hK_V11.1$ (hERG1), a voltage-gated cardiac K^+ channel; $hK_{Ca}3.1$ (IKCa1, SK4), the Ca^{2+} activated K^+ channel expressed in T lymphocytes; $mK_{Ca}1.1$ (BK, Slo1, MaxiK), the large conductance voltage- and Ca^{2+}-activated channel; two voltage-gated Na^+ channels, $hNa_V1.4$ and $hNa_V1.5$, expressed in skeletal and cardiac muscles respectively; and hH_V1, a voltage-gated proton channel. We found that, except $K_V1.1$, none of the ion channels tested were inhibited by Cm28 at 150 nM concentration. This concentration is more than150-fold higher than the K_d for $K_V1.2$ and more than 100-fold higher than the K_d for $K_V1.3$. Cm28 caused ~27% reduction in $K_V1.1$ current at 150 nM concentration and the averaged RCF value was 0.73 ± 0.03 (n = 3). The estimated K_d value for $K_V1.1$ from a single concentration, based on a bimolecular interaction, yielded ~0.4 μM.

5.1.4 Cm28 peptide does not compromise cell viability but suppresses the expression of activation markers in $CD4^+$ T_{EM} cells

To investigate whether the pharmacological properties of this unique Cm28 peptide are reflected in biological functional studies, we evaluated the effect of Cm28 on the TCR-mediated activation of $CD4^+$ T_{EM} cells. Before performing the T cell activation assay, we first had to determine whether the Cm28 compromises cell viability. After 24 h treatment with 1.5 μM Cm28 or 50 nM rMgTx, the viability of $CD4^+$ T_{EM} cells were not impaired either in quiescent or TCR-activated cells as analyzed by two different assays. Fixable viability dye Zombie NIR identified in flow cytometric analysis that ≥90% cells were viable in the presence of either toxin. 30% dimethyl sulfoxide (DMSO) was added to the cells for 30 min as positive control for dead cells. In parallel, we found that in lactate dehydrogenase (LDH) activity assay the

cytotoxicity of Cm28 was <1% and that of rMgTx was <2% for either quiescent or TCR-activated T_{EM} cells after 24 h culture period. 50 mM Sodium azide (NaN_3), as positive control, showed 15% cytotoxicity.

TCR-mediated activation of human T lymphocytes leads to the up-regulation of Ca^{2+}-dependent early activation markers such as IL2R and CD40 ligand. These activation markers have been widely used as a readout to assess the $K_V1.3$-dependence of T cell activation. $CD4^+$ T_{EM} cells were pre-incubated for 30 min with either 1.5 µM Cm28 (>1000× concentration of its K_d for $K_V1.3$) or 5 nM rMgTx (100× concentration of its K_d for $K_V1.3$, as positive control) and cells were then activated for 24 h with plate-bound anti-human CD3 antibody in the continuous presence of toxins. The flow cytometric overlayed histograms revealed that Cm28 significantly reduced the fraction of CD40L and IL2R expressing T_{EM} cells, like rMgTx, as compared to the control cells stimulated identically in the absence of toxin. The expression of CD40L and IL2R in Cm28 treated T cells, normalized to that of stimulated but not treated cells, is reduced to ~47% and ~55% respectively. Similarly, positive control rMgTx decreased the CD40L and IL2R expression level to ~51% and ~48%, respectively. There was no change in expression of CD40L or IL2R in unstimulated $CD4^+$ T_{EM} cells regardless of the presence or absence of Cm28.

5.2 Characterization of a fluorescent peptide toxin as a probe for $K_V1.3$

In collaboration with Prof. Raymond S. Norton' laboratory, Monash University, Australia, we created a fluorescent analogue of HsTX1[R14A]. Cyanine dye Cy5 was conjugated to the N-terminus of chemically synthesized HsTX1[R14A] as the N-terminus does not take part in the interaction with the channel and thus its modification is likely to have less effects on $K_V1.3$ binding. The ^1H nuclear magnetic resonance (NMR) studies confirmed the correct folding of the Cy5 labelled peptide toxin. The fluorescence absorption and emission spectra of Cy5-HsTX1[R14A] show an excitation maximum at 650 nm and an emission maximum at 666 nm. These spectral properties were identical to those measured for Cy5 under same conditions indicating that peptide conjugation did not affect the Cy5 excitation/ emission spectra. The chemical synthesis and structural and spectral properties were performed in Norton's laboratory. This section describes the electrophysiological characterization of Cy5-HsTX1[R14A] and demonstrates its ability to detect $K_V1.3$ expression using flow cytometry.

5.2.1 Biophysical characterization of Cy5-HsTX1[R14A]

Although it has been previously known that HsTX1[R14A] holds a great selectivity for $K_V1.3$ over many other physiologically important ion channels, small modification in the peptide core or N- /C- terminus conjugation may change the selectivity profile of the modified peptide. Therefore, patch-clamp electrophysiology was conducted to confirm the retained affinity and selectivity for $K_V1.3$ of Cy5-HsTX1[R14A]. We found that Cy5-HsTX1[R14A] inhibits $K_V1.3$ with K_d = 853 pM which is ~20 times larger than K_d (41 pM) for HsTX1[R14A], however it is still in picomolar range. Further, we experimentally determined the selectivity profile of Cy5-HsTX1[R14A]. We investigated its effect on four other members of K_V1 family, $hK_V1.2$, $hK_V1.4$, $hK_V1.5$ and $hK_V1.6$, which are close relative to $K_V1.3$.We also tested the effect of Cy5-HsTX1[R14A] on $mK_{Ca}1.1$ (BK, MaxiK), the large conductance voltage- and Ca^{2+}-

activated channel, and hK_{Ca}3.1 (IK_{Ca}1, SK4), the Ca^{2+}-activated K^+ channel expresses in T lymphocytes. Cy5-HsTX1[R14A] did not inhibit any of these ion channels tested at 100 nM concentration, which is about 100-fold higher than the K_d for K_V1.3.

When K_V1.3 subunit is co-expressed with other K_V1.x subunits, heterotetramers can be formed with various stoichiometries and intermediate characteristics regarding the biophysical and pharmacological properties. As described earlier, hyperactivated disease-relevant immune cells such as microglia and macrophages express K_V1.3/K_V1.5 heterotetramers. HsTX1[R14A] binds selectively to K_V1.3 homotetramers however, its affinity for heterotetramers has not been investigated previously. The ability of a probe to distinguish between homo- and heterotetrameric assembly of K_V1.3 channels would be highly beneficial.

We directly tested the affinity of Cy5-HsTX1[R14A] for K_V1.3/K_V1.5 homotetramers using tandemly linked dimers of K_V1.3-K_V1.5. To confirm the suitability of the tandem dimers we first generated K_V1.3-K_V1.3 tandem dimers and confirmed that the biophysical hallmarks of K_V1.3 were retained upon channel assembly from the dimers, *i.e.*, inactivation kinetics of the current during long depolarization pulses, and the affinity for the small inorganic blocker TEA^+ and the peptide blocker Cy5-HsTX1[R14A] were similar to those values obtained for channels composed of K_V1.3 monomers. K_V1.3 current measured in a CHO cell expressing tandem dimer K_V1.3-K_V1.3 constructs inactivates following single exponential decay kinetics with the characteristic time constant for this decay of 170 ± 25 ms (n = 5), similar to the reported inactivation kinetics of K_V1.3 composed of four monomers (~150−200 ms). Cy5-HsTX1[R14A] at 1 nM concentration inhibited ~50 % of the current, the RCF value was 0.56 ± 0.02 (n = 4), consistent with the K_d for the monomeric assembly of K_V1.3 (853 pM). Similar results were obtained for the TEA^+ affinity on K_V1.3 assembled from dimers where a ~50% current inhibition was found at 10 mM extracellular TEA^+ concertation, consistent with the data on K_V1.3 assembled from monomers. These results confirm the proper biophysical and pharmacological properties of channels assembled from tandemly linked K_V1.3 dimers.

Upon confirming the suitability of the tandem dimer K_V1.3 constructs for pharmacology, we expressed K_V1.3/K_V1.5 tandem dimers and determined the inactivation kinetics of the current which can be used as an indicator of the heteromeric assembly of the channels. A typical outside-out patch current was recorded in a CHO cell transfected with the K_V1.3/K_V1.5 tandem dimer construct. The inactivation kinetics of the current were well-fit using a single exponential function with a time constant (τ_{in}) of 561 ± 42 ms (n = 6), which are significantly slower than the inactivation kinetics of K_V1.3. This is consistent with the formation of heterotetramers with 1:1 stoichiometry of K_V1.3/K_V1.5 subunits since the very slow inactivating K_V1.5 (inactivation time constant (τ_{in}): 4046 ± 310 ms, n = 8) and relatively quick inactivating K_V1.3 subunits are mixed, and the overall kinetics of inactivation is determined by the cooperative interaction between the subunits. The affinity of the heterotetramers for Cy5-HsTX1[R14A] was significantly reduced, 1 nM of the conjugated peptide blocked a negligible proportion of the current as compared to the inhibition of the K_V1.3/K_V1.3 tandem construct, the peptide concentration had to be increased to 10 nM to see current reduction. A similar effect of reduction in affinity for heterotetramers was observed in

case of TEA^+ The estimated K_d for the inhibition of the $K_V1.3$-$K_V1.5$ heterotetrameric current by Cy5-HsTX1[R14A] was ~18 nM.

5.2.2 Cy5-HsTX1[R14A] can be used to detect $K_V1.3$-expressing cells in flow cytometry

We also determined whether the fluorescent toxin Cy5-HsTX1[R14A] can be used to detect the $K_V1.3$ channel expression in transfected CHO cells using flow cytometry. We stained both control CHO cells and CHO cells expressing GFP-$K_V1.3$. The fluorescence histograms of unstained, non-transfected CHO cells and non-transfected CHO cells stained with 10 nM Cy5-HsTX1[R14A] overlap completely, indicating that staining with Cy5-HsTX1[R14A] at the applied concentration does not bind to the CHO cells nonspecifically in the absence of $K_V1.3$ expression. When CHO cells expressing GFP-$K_V1.3$ were stained with Cy5-HsTX1[R14A], a significant population of the cells showed increased fluorescence in the Cy5 channel. Overall, about ~40% of the cells showed increased Cy5-HsTX1[R14A] signal, which corresponds to ~80% of the cells that display increased GFP signal as well. The specificity of $K_V1.3$ labelling by Cy5-HsTX1[R14A] was also confirmed using a competition assay. In this experiment, GFP-$K_V1.3$ expressing CHO cells were incubated first with either excess unconjugated HsTX1[R14A] or excess recombinant margatoxin (rMgTx) and were subsequently stained with Cy5-HsTX1[R14A]. Pre-incubation of the cells with excess unconjugated pore-blocking toxins resulted in histograms that were superimposable to the control, unstained CHO population. This indicates that excess unconjugated HsTX1[R14A] or rMgTx completely inhibited labelling of the $K_V1.3$ channels with Cy5-HsTX1[R14A].

5.3 Optimization of recombinant production of margatoxin and the functional characterization of the tagged and untagged versions of the peptide

5.3.1 Generation of rMgTx expressing *P. pastoris* X-33 clones

The MgTx expression cassette was synthesized following codon-optimization for *P. pastoris* and cloned under the control of *AOX1* promoter in pPICZαA expression vector. The linearized TrMgTx-pPICZαA plasmid was transformed into *Pichia* X-33 competent cells. After 3 days of incubation at 30 °C, >40 colonies showed prominent growth on YPD agar plate containing 100 μg/ml of Zeocin as antibiotic selection. According to Nordén *et al.* increased resistance against Zeocin directly correlates with higher expression of heterologous protein in *P. pastoris*. The initially chosen colonies were grown under gradually increasing Zeocin concentrations. Most of the clones exhibited strong resistance against antibiotic selection *i.e.*, 0.5 and 1 mg/ml of Zeocin, however, comparatively few *Pichia* clones showed prominent growth at 2 mg/ml of Zeocin and presumably these clones have the high copy number of expression cassettes incorporated in the genome. The clones, survived at highest concentration of Zeocin (2 mg/ml), were subjected to colony PCR which confirmed the integration of TrMgTx-pPICZαA cassette by single crossover at 5′ *AOX1* locus of *P. pastoris* genome.

5.3.2 Optimization of fermentation conditions for improved expression of rMgTx

For expression analysis supernatant samples were collected from the cultures every 24 h post MeOH induction for 5 days and 16% tricine SDS-PAGE was performed. A peptide band

of ~6.5 kDa MW was detectable in the sample collected 24 h post MeOH induction. The intensity of this band increased gradually to maximum after 72 h of induction, however, then it declined on the 4th and 5th day following induction. The molecular mass of the TrMgTx estimated from gel was slightly higher than the predicted mass (5.9 kDa) most likely because of its higher isoelectric point (pI) which is 9.10. The maximum concentration of peptide in supernatant was 78 ± 7 mg/L after 72 h of induction which was significantly higher than any other time point with $P < 0.05$ in pairwise multiple comparison.

To optimize the MeOH concentration for induction, biomass *Pichia* clone was induced with 0.5, 1 and 1.5% MeOH in a medium of pH 6. The gel scanning assay revealed that the highest concentration of TrMgTx was in the supernatant sample induced with 0.5% MeOH for 72 h (71 ± 13 mg/L) and it was substantially higher than other two samples induced with 1 and 1.5% MeOH. Similarly, to find the suitable pH of the medium for improved expression of the peptide, biomass was induced with 0.5% MeOH in media having pH 5, pH 6, pH 7 or "not adjusted" for 72 h. The supernatant sample taken from the culture at pH 6 showed 76 ± 8 mg/L expression of TrMgTx which is greatly higher than other samples from cultures with pH 5 or pH 7 or "not adjusted".

5.3.3 Purification of TrMgTx and UrMgTx

The best *Pichia* X-33 clone in trial expression study was subjected to large-scale shake-flask fermentation under optimized conditions (72h, pH 6 and 0.5% MeOH) and TrMgTx was purified to homogeneity by two-step purification strategy. His-tagged MgTx was efficiently captured using His-trap column packed with Ni^{2+} Sepharose and eluted with imidazole. The tricine SDS-PAGE analysis of fraction from affinity chromatography showed a clear and dense band of TrMgTx around ~6.5 kDa in imidazole eluates. The moderately purified TrMgTx was further purified by RP-HPLC using C_{18} semi-preparative column. Analysis of the HPLC fractions by tricine SDS-PAGE revealed the fraction corresponding to the peak eluted at about 28 min retention time (R_T) contained TrMgTx. To confirm the molecular identity of the product ESI-QTOF-MS analysis was performed in our collaborator's laboratory (Prof. Attila Gáspár, Faculty of Sciences, University of Debrecen). The determined average molecular mass of TrMgTx 5980.86 Da was in full agreement with the theoretical average mass of 5980.96 Da.

Western blot analysis verified the identity of TrMgTx by detecting a single band at the expected MW with the anti-His antibody. The purity of TrMgTx was >98% after the two-step purification as assessed by C_{18} RP-HPLC analytical column. About 9.1 mg of pure TrMgTx was produced with 43% net recovery from 250 ml of *P. pastoris* culture under optimized conditions.

N-terminus His-tag was cleaved by digesting TrMgTx with factor Xa protease. Appearance of a protein band of ~4.1 kDa (theoretical MW of native MgTx) in overnight digested sample in tricine SDS-PAGE confirmed the successful removal of tag from TrMgTx The untagged rMgTx (UrMgTx) was purified using C_{18} RP-HPLC column. The determined average mass of UrMgTx (4178.95 Da), provided by the MS analysis in Prof Gaspar's lab, is similar to the theoretical mass (4179.018 Da) of UrMgTx and is also in full agreement with previously reported MW of native MgTx indicating that there is no additional amino acid at either terminus of UrMgTx.

5.3.4 Electrophysiological and biological characterization of TrMgTx and UrMgTx

Since the MgTx blocks $K_V1.2$ and $K_V1.3$ channels with high potency, therefore the pharmacological activities of rMgTx analogues were tested against these two channels. The $K_V1.2$ currents were recorded in CHO cells. TrMgTx and UrMgTx at 100 pM inhibited ~60% and 90% of $K_V1.2$ current, respectively. The equilibrium block of $K_V1.2$ developed slower with TrMgTx than UrMgTx at the same concentration (100 pM). The time constants, obtained by fitting the appropriate exponential function to the normalized peak currents during the toxin wash-in procedure, were τ_{on} = 202 ± 2.3 s for TrMgTx and τ_{on} = 191 ± 12 s for UrMgTx. When cells were perfused with toxin-free solution, a slow but full recovery from the block was achieved in case of TrMgTx with τ_{off} = 532 ± 69 s. However, like native MgTx, slow and partial recovery (τ_{off} = 1308 ± 351 s) from the block by UrMgTx was observed. The k_{on} for UrMgTx is slightly higher than that of TrMgTx (unpaired *t* test, n = 3−4, P <0.01), however the k_{off} rate of UrMgTx is considerably lower than that of TrMgTx (unpaired *t* test, n = 3−4, P <0.001). We also determined the dose-dependent inhibition of $K_V1.2$ by both analogues of rMgTx (K_d = 64 pM for TrMgTx and K_d = 14 pM for UrMgTx). TrMgTx exhibited ~5-fold less potency for $K_V1.2$ than the tag-free UrMgTx.

A similar set of experiments were performed for $K_V1.3$ channel. TrMgTx and UrMgTx at 200 pM concentrations inhibited ~70% and ~80% of whole-cell $K_V1.3$ currents in activated human T lymphocytes. Like $K_V1.2$, TrMgTx took more time to reach block equilibrium than UrMgTx upon application of 200 pM either toxin in bath solution (τ_{on} = 168 ± 19 s for TrMgTx and τ_{on} = 116 ± 10 s for UrMgTx were obtained, n = 4). However, unlike $K_V1.2$, the τ_{off} values were similar for both analogues of rMgTx (for TrMgTx τ_{off} = 568 ± 84 s and for UrMgTx τ_{off} = 560 ± 28 s were obtained, n = 4) with almost full recovery of initial peak current. The k_{on} rate of UrMgTx was significantly higher than that of TrMgTx (P <0.001, n = 4), however the k_{off} rates of both peptides were statistically the same (P >0.05, n = 4). In the dose-response relationship the best fit of data points resulted in K_d 86 pM and 50 pM for TrMgTx and UrMgTx, respectively. TrMgTx has slightly lower potency for $K_V1.3$ than the UrMgTx.

In order to demonstrate the biological function of the recombinant peptides we also studied the effect of TrMgTx and UrMgTx on the expression of IL2R and CD40L, early activation markers of $CD4^+$ T_{EM} cells. The presence of either TrMgTx or UrMgTx (at ~100× concentration of their respective K_d values) during the T_{EM} cell stimulation with plate-bound anti-human CD3 antibody for 24 h substantially reduced the upregulation of IL2R and CD40L expression as compared to the control cells stimulated in the same fashion but in the absence of the toxin. The quantitative analysis showed that TrMgTx (8.5 nM) causes ~39% inhibition of IL2R and ~36% of CD40L expression. Similarly, UrMgTx (5nM) hampered expression of both activation markers by ~45%. These results suggest that both analogues of rMgTx are biologically active and the presence of a His-tag at the N-terminus marginally reduces the efficacy of TrMgTx.

6. SUMMARY

The voltage-gated potassium $K_V1.3$ channels express in immune cells and are implicated in a range of autoimmune diseases and neuroinflammatory disorders. Selective blocking of $K_V1.3$ using peptides isolated from scorpion venom holds a great potential in developing immunomodulatory therapies. We discovered and characterized a novel short peptide in the venom of *C. margaritatus* (Cm28). Cm28 obeys a unique primary structure, consists of 27 amino acid residues and has <40% similarity with other known α-KTxs from scorpions. Cm28 reversibly inhibited $K_V1.2$ and $K_V1.3$ channels with K_d values of 0.96 and 1.3 nM, respectively. The biophysical characterization of the block revealed that Cm28 is not a gating modifier, but rather a pore blocker. Cm28 is ~400-fold selective for $K_V1.2$ and $K_V1.3$ over $K_V1.1$ and did not inhibit a variety of other K^+, Na^+ and H^+ channels at 150 nM concentration. Cm28 strongly downregulated the expression of two key early activation markers IL2R and CD40 ligand in stimulated human effector memory T cells. Cm28, due to its unique structure, may serve as a template for the generation of novel peptides targeting $K_V1.3$.

The high affinity and selectivity of peptide toxins for $K_V1.3$ make them suitable for the development of visualization tools to study the expression and the pharmacokinetics of peptide toxins. We developed a fluorescent analogue of HsTX1[R14A] having an N-terminus Cy5 tag. We showed that Cy5-HsTX1[R14A] retained high affinity and selectivity for $K_V1.3$ (K_d ~0.9 nM), even against channels formed by $K_V1.3$-$K_V1.5$ tandem dimers. Furthermore, flow cytometry demonstrated that Cy5-HsTX1[R14A] can identify $K_V1.3$-expressing CHO cells.

To generate ample amounts of $K_V1.3$ inhibitor toxins for pharmacology and therapeutic development processes we optimized *Pichia pastoris* expression system to produce ~36 mg/L of His-tagged margatoxin with >98% purity. This yield, which is 3-fold higher than has been previously reported, was achieved by optimizing the codon, the selection process, and the culturing conditions. Moreover, we showed that the His-tagged MgTx inhibited $K_V1.2$ and $K_V1.3$ channels with similar potency to the untagged MgTx, and significantly inhibited the IL2R and CD40 ligand in activated human effector memory T cells, thus, elimination of the tag removal reduces the cost of the production. These results suggest that *Pichia* expression system is a powerful method to produce the disulfide-rich peptide MgTx, the overexpression of similar peptides could be enhanced noticeably through optimization strategies, making it more cost-effective.

In summary, the data presented in the PhD dissertation resulted in a novel ion channel inhibitor peptide (Cm28), a new visualization tool for $K_V1.3$ (Cy5-HsTX1[R14A]) and an optimized method to produce MgTx in the *Pichia* expression system.

7. PUBLICATIONS

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Registry number: DEENK/40/2023.PL
Subject: PhD Publication List

Candidate: Muhammad Umair Naseem
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. Wai, D. C. C., **Naseem, M. U.**, Mocsár, G., Babu Reddiar, S., Pan, Y., Csóti, Á., Hajdu, P., Nowell, C., Nicolazzo, J. A., Panyi, G., Norton, R. S.: A Fluorescent Peptide Toxin for Selective Visualization of the Voltage-Gated Potassium Channel Kv1.3.
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2. **Naseem, M. U.**, Carcamo-Noriega, E., Beltrán-Vidal, J., Borrego, J., Szántó, G. T., Zamudio, F. Z., Delgado-Prudencio, G., Possani, L. D., Panyi, G.: Cm28, a scorpion toxin having a unique primary structure, inhibits KV1.2 and KV1.3 with high affinity.
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List of other publications

4. **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.
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5. Bosire, R., Fadel, L., Mocsár, G., Nánási, P. P. i., Sen, P., Sharma, A. K., **Naseem, M. U.**, Kovács, A., Kugel, J., Kroemer, G., Vámosi, G., Szabó, G.: Doxorubicin impacts chromatin binding of HMGB1, Histone H1 and retinoic acid receptor.
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IF: 1.65

Total IF of journals (all publications): 33,502
Total IF of journals (publications related to the dissertation): 16,057

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

10 February, 2023

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