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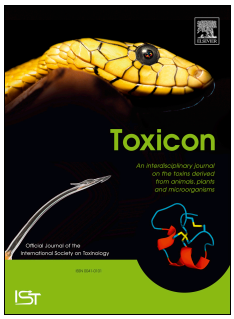

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T. Olamendi-Portugal, A. Csoti, J.M. Jimenez-Vargas, F. Gomez-Lagunas, G. Panyi, L.D. Possani

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Pi5 and Pi6, two undescribed peptides from the venom of the scorpion *Pandinus imperator* and their effects on K^+-channels

Olamendi-Portugal, T.[a,+], Csoti, A.[b,+], Jimenez-Vargas, J.M.[a], Gomez-Lagunas, F.[c], Panyi, G.[b], Possani, L.D.[a,*]

[a]Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autonoma de Mexico, Avenida Universidad, 2001 Cuernavaca, Morelos 62210 Mexico

[b]Departamento de Fisiología. Facultad de Medicina, Universidad Nacional Autonoma de Mexico, Ciudad de Mexico 04510, Mexico

[c]Department of Biophysics and Cell Biology, Faculty of Medicine, Research Center for Molecular Medicine, University of Debrecen, 1 Egyetem ter, Debrecen, 4032, Hungary.

[+]Contributed equally to the work

*Corresponding author

Lourival D. Possani, E-mail: possani@ibt-unam.mx

ABSTRACT

This work reports the isolation, chemical and functional characterization of two previously unknown peptides purified from the venom of the scorpion *Pandinus imperator*, denominated Pi5 and Pi6. Pi5 is a classical K^+-channel blocking peptide containing 33 amino acid residues

with 4 disulfide bonds. It is the first member of a new subfamily, here defined by the systematic number α-KTx 24.1. Pi6 is a peptide of unknown real function, containing only two disulfide bonds and 28 amino acid residues, but showing sequence similarities to the κ-family of K-channel toxins. The systematic number assigned is κ-KTx2.9. The function of both peptides was assayed on Drosophila *Shab* and *Shaker* K^+-channels, as well as four different subtypes of voltage-dependent K^+-channels: hKv1.1, hKv1.2, hKv1.3 and hKv1.4. The electrophysiological assays showed that Pi5 inhibited *Shaker B*, hKv1.1, hKv1.2 and hKv1.3 channels with Kd=540 nM, Kd= 92 nM and Kd= 77 nM, respectively, other studied channels were not affected. Of the channels tested only hKv1.2 and hKv1.3 were inhibited at 100 nM concentration of Pi6, the remaining current fractions were 68% and 77%, respectively. Thus, Pi5 and Pi6 are high nanomolar affinity non-selective blockers of hKv1.2 and hKv1.3 channels.

KEYWORDS

Amino acid sequence; ion-channel; K^+-channel blocker; *Pandinus imperator*; scorpion toxin

1. INTRODUCTION

Most scorpion venoms from the family Buthidae, lethal to humans, contain mainly two types of toxic peptides: the long-chain Na^+-channel specific toxins with 60-76 amino acid residues, and the short-chain peptides with 23-41 amino acid residues, specific for K^+-channels (Possani et al.,1999; Garcia et al., 1998). Toxins from scorpion venom specific for K^+-channel have been widely described (Tytgat et al., 1999; Rodriguez de la Vega and Possani, 2004; Bartok et al, 2015; Kuzmenkov et al, 2016). The venom from the African scorpion *Pandinus imperator*, from

the family Scorpionidae, is different because it is not lethal to humans and instead of having mammalian specific Na^+-channel toxins it contains many proteins and peptides with interesting biological activities, such as imperatoxin activatory (IpTxa) and imperatoxin inhibitory (IpTxi), both specific for the Ryanodine sensitive Ca^{2+}-channels (Zamudio et et al., 1997a,b), phospholipin, a heterodimeric phospholipase (Conde et al., 1999) and scorpine, an antibacterial and antiparasitic peptide (Conde et al., 2000). Also interesting, more than 10% of its venom is composed by various peptides that recognize K^+-channels (Olamendi-Portugal et al. 1996; Goméz-Lagunas et al., 1996). The first one described was Pi1, which contains four disulfide bonds instead of three usually found on K^+-channel specific toxins, at the time when it was described (Olamendi-Portugal et al., 1996). Then Pi2 and Pi3 were found, which differ only by one amino acid residue in their primary structure, but have a 17 fold difference of activity, assayed in *Shaker* B K^+ channels (Gomez-Lagunas et al., 1996). Additionally, Pi4 and Pi7 were described, the first with a Kd of 8.2 nM, whereas the second had no activity against the *Shaker* K^+-channel (Olamendi-Portugal el al., 1998). The three-dimensional structure (3D) of Pi1 and Pi4 were determined (Delepierre et al., 1997; Delepierre et al, 1998; Guijarro et al., 2003) Additionally the 3D structure of Pi7, that lacks a K^+ channel blocking potency, was also solved (Delepierre et al., 1999) in order to reveal possible differences on the folding pattern of the peptides. The general 3D folding of Pi7 turned out to be identical to the other known K^+-channel specific toxins, however an Arg in position 27 (equivalent to lysine-27 of Charybdotoxin) was taken as one of the possible differences among these toxins, that would render Pi7 ineffective against the tested channels. Approximately at the same time, two additional peptides were identified in this venom, and were called Pi5 and Pi6. The trivial name comes from the abbreviations of scorpion species (*Pandinus imperator*) and the number of purified peptides, at

that time. Systematic nomenclature is now being assigned for both peptides. Pi5 is a new member of the family α-KTx. The number of amino acids, disulfide bonds and sequence similarities indicate that it is the first member of the sub-family 24 (systematic number α-KTx 24.1). Pi6 belongs to the κ-family, with the systematic number assigned κ-KTx 2.9. Their structures were not published, because the functional activity of the peptides was not clearly determined. In this communication we fill this gap and describe both peptides; their primary structure and their inhibitory potential of several ion channel subtype of the of the Shaker family including *Shaker B*, Kv1.1-Kv1.4 and on the *Shab* channel.

2. Material and Methods

2.1 Source of venom

Living scorpions (approximately 100 animals) of the species *Pandinus imperator*, from Gabon (Africa), were bought from a pet-shop (Pet Supplies Plus, Ann Arbor, Michigan, USA) and kept alive in the laboratory for several years. This occurred before this species became included on the CITES list for danger of extinction. The scorpions were very well adapted; grow well and even reproduced in captivity. The venom from adult animals, at that time, was obtained by electric stimulation under anesthesia with CO_2, dissolved in double distilled water and centrifuged 10,000 g for 15 min. The supernatant was freeze-dried and stored at -20 °C until use.

2.2 Purification procedure and amino acid sequence determination

The soluble venom was initially fractionated by gel filtration on Sephadex G-50 column. Sub-fractions were further separated by high performance liquid chromatography (HPLC), using a C18 reverse-phase column (Vydac, Hysperia, CA) of a Waters 600E HPLC system, equipped

with a variable wavelength detector, and a WIPS 712 automatic sample injector, as described (Olamendi-Portugal et al., 1996).

The homogeneity of the purified peptides was confirmed by a step-gradient HPLC (only one symmetric peak), molecular mass determination on LCQ^{DUO} Finnigan mass spectrometer (San Jose, CA), and by direct Edman degradation using an automatic ProSequencer (Millipore model 6400/6600); Olamendi-Portugal et al., 1996).

The primary structure of both peptides Pi5 and Pi6 was determined using native samples, samples reduced and alkylated with iodoacetic acid, and after digesting alkylated peptides with enzymes, such as chymotripsin and *Staphyloccocus aureus* endopeptidase V8, from which pure peptides were obtained by HPLC fractionation, essentially as earlier described (Olamendi-Portugal et al., 1996; 2016), and used for completion of the entire amino acid sequence.

The protein sequence data reported in this paper will appear in the UniProt Knowledgebase under the accession number C0HKB2 for Pi5 and C0HKB3 for Pi6.

2.3 Functional characterization of pure Pi5 and Pi6 on Drosophila Shab and Shaker B K^+-channels

Physiological assays were initially conducted using the expression of *Shab* and *Shaker* B K^+-channels in Sf9 cells. The insect Sf9 cells were kept in culture at 27 ºC in Grace's media (Gibco BRL). The cells were transfected by infecting with a recombinant baculovirus (*Autographa californica* nuclear polyhidrosis virus) containing the cDNA of either *Shab* or *Shaker B* K^+-channels, as previously reported (Klaiber et al., 1990; Islas and Sgiworth, 1999). The cells were used for the experiments two days after the infection.

The currents were recorded under whole-cell patch-clamp with an Axopatch 1D (Axon Instruments INC). Borosilicate glass (KIMAX 51) electrodes were pulled to a 1.5 MOhm resistance, and used without further treatment. Eigthy percent of the series resistance was electronically compensated. The currents were filtered in line at 5 KHz, and sampled at 100 µs/point with DIGIDATA 1322A (Axon Instruments, INC). The solutions used were: external side (in mM): 145 NaCl, 10 $CaCl_2$, 10 HEPES-Na buffer, pH 7.1; internal solution (in mM): 95 KF, 30 KCl, 10 EGTA, 10 HEPES-K buffer, pH 7.1.

2.4 Functional characterization of pure Pi5 and Pi6 on four voltage-dependent K^+-channels

Human peripheral lymphocytes were drawn from healthy volunteers. Mononuclear cells were isolated using Ficoll-Hypaque density gradient separation technique and were grown in 24-well culture plates in a 5% CO_2 incubator at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum (Sigma-Aldrich), 100 µg/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (density, 5 x 10^5 cells per ml) for 2 to 5 days. 5, 7.5 or 10 µg/ml phytohemagglutinin A (Sigma-Aldrich) was added to the medium to increase K^+ channel expression. CHO cells were grown under standard condition as described previously (Bagdany et al., 2005; Corzo et al., 2008; Grissmer et al., 1994).

Vectors encoding the human Kv1.1, Kv1.2 and Kv1.4 channels were expressed in CHO cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. hKv1.1 and hKv1.2 genes are coded in pCMV6-GFP plasmid (OriGene Technologies, Rockville, MD), the vector for hKKv1.4 lacking the N-terminal inactivation domain was a gift from David Fedida (University of British Coulmbia, Vancuver, Canada). This latter gene was transiently co-transfected with a plasmid encoding the green fluorescence protein

(GFP). Currents were recorded 24 h after transfection. GFP positive transfectants were identified in a Nikon TE2000U fluorescence microscope and used for current recordings (>70% success rate for co-transfection). For the measurements of hKv1.3 currents activated lymphocytes were used (Bartok et al., 2014).

Measurements were carried out using whole-cell patch-clamp recordings using Multiclamp 700B amplifier and Axon Digidata 1440 digitizer (Molecular Devices, Sunnyvale, CA). Micropipettes were pulled from GC 150 F-15 borosilicate capillaries (Harvard Apparatus Kent, UK) resulting in 3- to 5-MΩ resistance in the bath solution. The extracellular solution consisted of 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5.5 mM glucose, 10 mM HEPES, pH 7.35. Bath solutions were supplemented with 0.1 mg/ml BSA when toxins were dissolved. The osmolarity of the extracellular solutions was between 302 and 308 mOsM/L. The pipette filling solution contained 140 mM KF, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES and 11 mM EGTA, pH 7.22. The osmolarity of the intracellular solutions was 295 mOsM/L.

Whole cell currents were elicited using by voltage steps to +50 mV for variable durations, ranging between15 ms to 500 ms depending on the channel type, from a holding potential of -100 mV every 15 s. The pClamp10 software package was used to acquire and analyze the data. Current traces were lowpass-filtered by the analog four-pole Bessel filters of the amplifiers. The sampling frequency was 2-50 kHz, at least twice the filter cut-off frequency. The effect of the toxins in a given concentration was determined as remaining current fraction ($RF = I/I_0$, 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 Kd was determined from the double reciprocal plot of the blocked fraction of the current (B=1-RF) where 1/B was plotted against the reciprocal of the

toxin concentration (1/C). Fitting a straight line to the data points gave Kd as the slope of the line (1/B=(Kd*1/C)+1). The fit assumed 1:1 stoichiometry for the toxin-channel interaction.

3. Results and Discussion

3.1 Purification and amino acid sequencing determination

After separation of the whole soluble venom by gel filtration into Sephadex G-50 column, as described earlier (Olamendi-Portugal et al. 1996), fraction III containing the peptides of interest was separated by HPLC. Several batches of fraction III (0.5 mg of protein content) was separated independently, given always the same profile. Figure 1A shows the results obtained. Eight sub-fractions were selected for further purification and characterization. Fractions 1 to 4 of Fig.1A were characterized as Pi1 to Pi4, and fraction 7 as Pi7 (Olamendi-Portugal et al., 1998; Delepierre et al., 1999). Components labeled 5 and 6 (marked with asterisks) were used for sequence determination, and named Pi5 and Pi6. The amino acid sequence of Pi5 is shown in Fig.1B. Native peptide and the same peptide in its iodoacetic alkylated format permitted the identification of the first 32 amino acids; the last residue was obtained after sequencing the peptide labeled “Ch”, which overlaps with residues of the C-terminal region of the peptide, completing the sequence. This peptide was obtained after digesting 50 µg of the reduced and alkylated peptide by HPLC (Fig.1A, left side labeled Pi5). The peptide marked with asterisk gave the sequence from 26-33. The experimental determined molecular weight (average molecular mass) of the native Pi5 peptide was 3334.00 Da and the theoretically expected was 3334.81 Da, confirming the full sequence, within the experimental error of the mass spectrometer used for this analysis. The peptide contains 8 cysteines that are forming 4 disulfide bonds. Using the same procedure the amino acid sequence of peptide Pi6 was determined as

183 shown in Fig.1C. The first 24 amino acid residues were identified by direct Edman degradation
184 of native and native-alkylated samples. Alkylation of cysteines was essential for the correct
185 identification of the thiol containing amino acids. The complete sequence was obtained after
186 digesting 50 µg of peptide Pi6 with endopeptidase V8, whose separation profile on HPLC is
187 shown on the right inset panel of Fig.1A, labeled Pi6 and marked with asterisk. This peptide
188 gave the sequence from amino acid Ala21 to Pro28. The molecular weight experimentally
189 determined (average molecular mass) for this peptide was 3126.5 Da and the theoretical
190 molecular weight was 3127.2, within the experimental error of the spectrometer used for this
191 analysis. This peptide has 4 cysteines forming only two disulfide bonds.

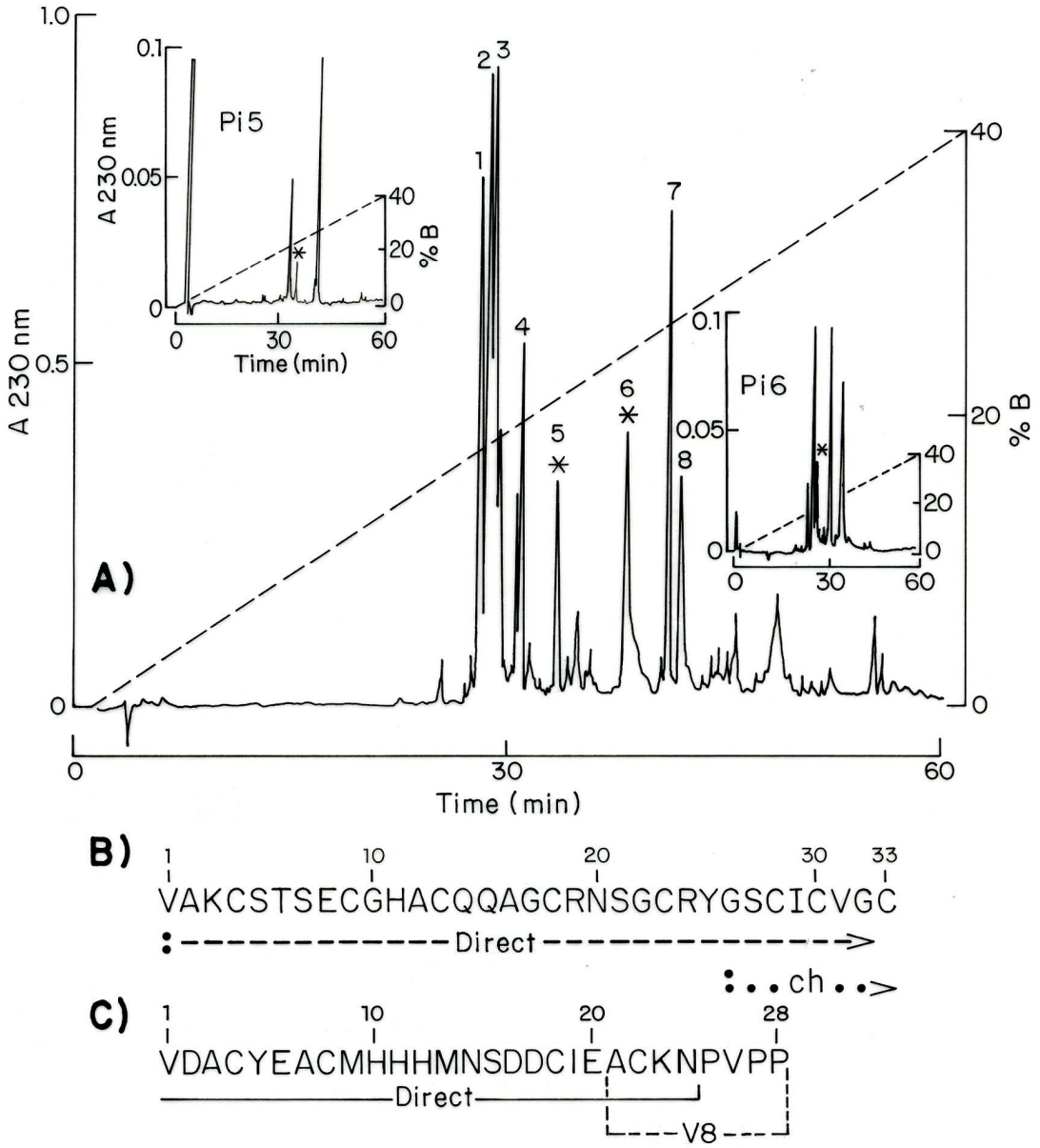


Figure 1: **Purification and amino acid sequence of Pi5 and Pi6**

A. Fraction III from Sephadex G-50 column (0.5 mg) was separated by HPLC using an analytical C18 reverse-phase column (4.6 x 250 mm), with a linear gradient from solution A (0.12% trifluoacetic acid –TFA- in water) to 40% solution B (0.10% TFA in acetonitrile). Sub-fraction 5 (labeled with asterisk) was homogeneous Pi5, which was digested with chymotripsin and separated again in the same conditions, as indicated in the inset figure labeled Pi5. The

asterisk indicates the peptide whose sequence allowed completion of the amino acid sequence shown in letter B, from position 26 to 33 (underlabeled with .Ch.). Similarly, the sub-fraction 6 (labeled with asterisk) was the homogeneous peptide Pi6, which was digested with Protease V8 from *Staphylococcus aureus*, and separated in the same conditions, as indicated in the insert labeled Pi6. The labeled peptide with asterisk was sequenced and permitted completion of the sequence from amino acids in position 21 to 28 (underlined .V8.). Letters B and C show the full amino acid sequence for both peptides, where "Direct" means amino acid sequence obtained with a reduced and alkylated sample of each peptide.

3.2 Effects of Pi5 and Pi6 on Shab and Shaker K^+-channels

Fig. 2A demonstrates that Pi5 is a toxin that reversibly blocks, *Drosophila Shaker B* K^+ channels with low affinity. The left panel presents a family of control K^+ currents (see Figure legend), note that upon addition of 1.5 µM Pi5, to the extracellular solution, the size of I_K was drastically reduced (~80%, middle panel). Inhibition was fully reversed by washing the cell with the control solution (right panel). Experiments repeated at lower Pi5 concentrations show that Pi5 simply scales down the currents without changing its kinetics. The I-V relationship of the traces shown in Fig. 2B demonstrates that, as commonly found with Kv channel blocker toxins, the fraction of the channels blocked by Pi5 is independent of the applied voltage. The fit to the dose-response relationship resulted in a Kd of 540 nM (Fig. 2C) with Hill coefficient close to 1 indicating that the Pi5 blocks *Shaker* channels with a 1:1 stoichiometry. The double reciprocal plot (Fig. 2 C inset) could be well-fit using linear regression (r=0.995), the Kd values obtained by the two methods were comparable. In contrast to the above observations, addition of 1.5 µM Pi5 to the extracellular solution did not inhibit significantly *Drosophila Shab* K^+ channels (Figures 2D and 2E). Pi6 on the other hand did not inhibit the either *Shaker B* or *Shab* (See Supplementary Figure 1).

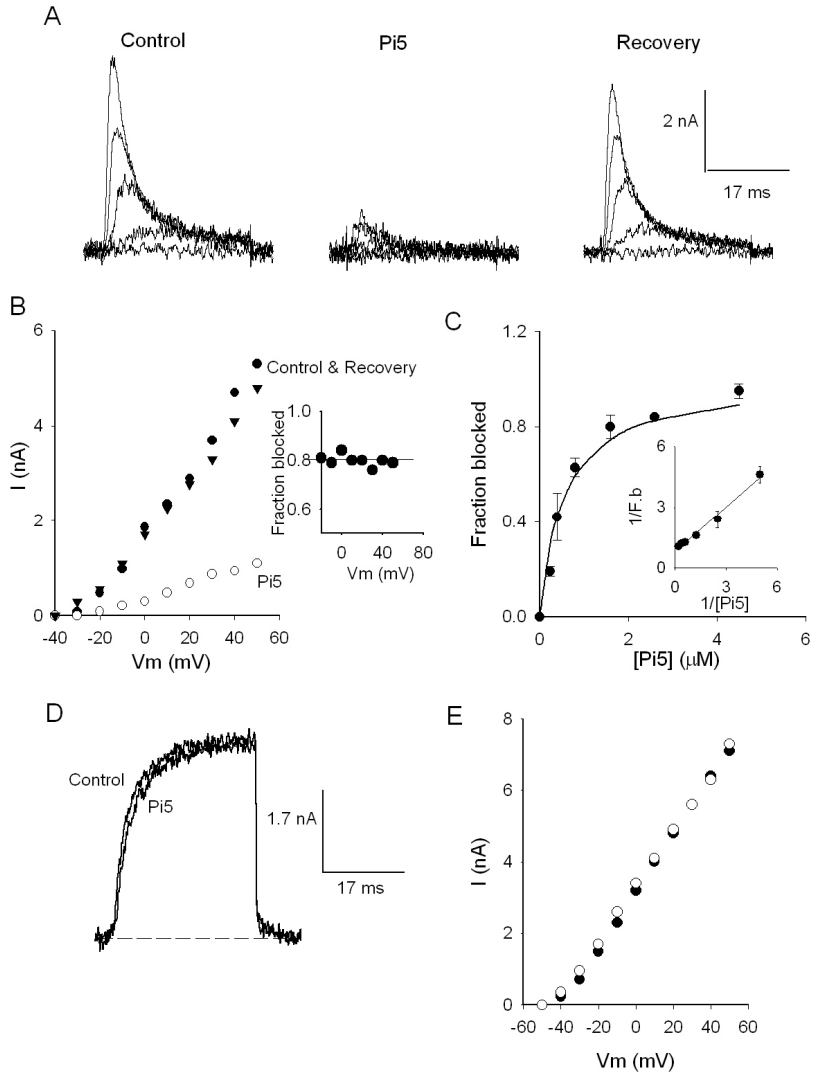


Figure 2: Effect of Pi5 on *Drosophila* K^+ channels

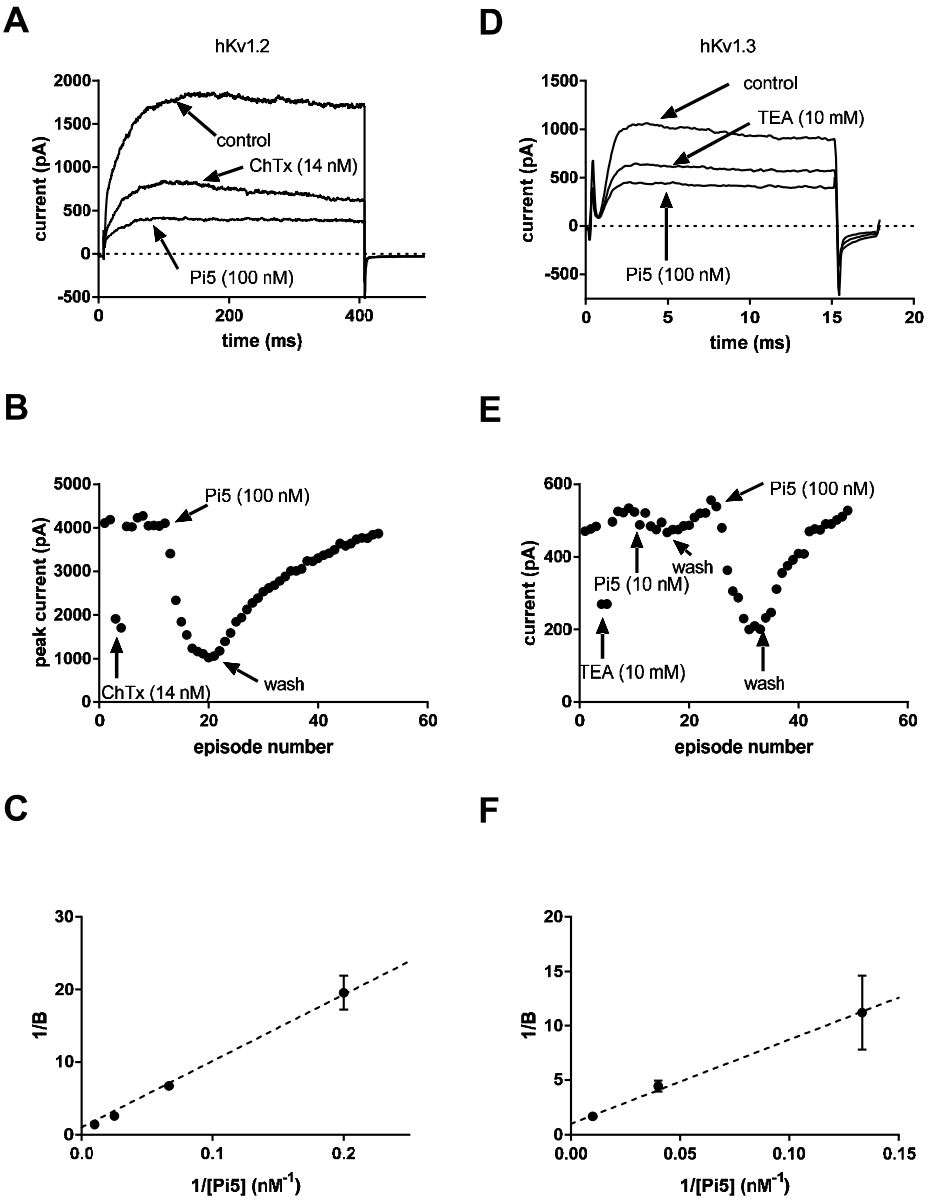
(A) K^+ currents through *dShaker B* channels. K^+-currents were elicited every 15-sec by 30-ms pulses from -20 to +60 mV applied in 10 mV increments from a holding potential of -80 mV, before (left panel, labeled Control), during (middle panel, labeled Pi5) and after (labeled Recovery) the addition of 1.5 µM Pi5 to the external solution. (B) Current-Voltage relationship of the traces in A: closed circles: control I_K, open symbols: I_K with 1.5 µM Pi5 in the external solution, closed triangles: I_K after washing the cell with the control solution. The inset shows that the block (1.5 µM Pi5) is not voltage dependent. (C) Fractional block vs. [Pi5], the fractional block (F.b) was calculated as $F.b=1-(I/I_0)$, where I_0 and I are the peak current in the control and

in the presence of Pi5, respectively. The line is the least squares fit of the points with the Michaelis-Menten equation, with Kd= 540 nM. The inset shows the expected linear double-reciprocal plot of the points (r=0.995). (D) K^+ currents through dShab channels. The figure shows two superimposed currents evoked by a 30mV/30ms pulse applied from -80 mV, before and during addition of 1.5 µM Pi5, as indicated. (E) Current-voltage relationship before and in the presence of 1.5 µM Pi5 from traces as in D. There was no blockage.

3.3 Effects of Pi5 and Pi6 on hKv1.1, hKv1.2, hKv1.3 and hKv1.4 channels.

Based on the similarity of Pi5 and Pi6 with other known α-KTx toxins (see also below) and the literature available for the selectivity of similar peptides ((Camargos et al., 2011; Gomez Lagunas et al., 1997; Mouhat et al., 2004; Peter et al., 2000)), the following *Shaker*-related human ion channels were selected for the study: Kv1.1, Kv1.2, Kv1.3 and Kv1.4. These channels were either expressed heterologously in CHO cells (Kv1.1, Kv1.2 and Kv1.4) or were recorded in human peripheral blood lymphocytes where the dominant voltage-gated K^+ channel is Kv1.3. Our recording condition (no Ca^{2+} in the pipette to evoke Ca^{2+}-activated K^+ channels) and the stimulation of the K^+ channel expression (activation of the cells by PHA, see methods) ensured that the voltage-gated current recorded in these cells is a K^+ current through Kv1.3 (Peter et al., 2001). The toxins were applied using a custom built microperfusion system with a very small perfusion rate (200 µL/min). At this perfusion rate the speed and the completeness of the solution exchange had to be tested repeatedly. The positive controls for perfusion were either quick and fully reversible blockers of the channels (e.g. TEA for Kv1.3 and Kv1.1 and ChTx for Kv1.2 (Bartok et al., 2014)) or the recording chamber was perfused with a modified bath solution containing 150 mM K^+ and reduction of the peak currents was used as an indicator of the solution exchange.

The effect of Pi5 on the whole cell current carried by hKv1.2 channels is shown in Fig. 3A. The figure shows that Pi5 in 100 nM concentration inhibits ~80% of the whole-cell current upon reaching block equilibrium (perfusion of the recording chamber with ChTx was used as a positive control of the solution exchange). The comparison of the currents recorded in the presence and absence of the blocker indicates that the kinetics of the whole-cell currents are unaffected by the application of Pi5. The block of the hKv1.2 channels was fully but slowly reversible by perfusing the recording solution with toxin-free extracellular solution (Fig. 3B, indicated by the arrow labeled wash). The interepisode time during the recording was 15s and the wash-out is complete in ~50 episodes which correspond to ~12-13 min. The kinetics of the recovery from block followed a single exponential time-course (fit not shown) with a time constant of 245+/- 26 s (n=3). The limited amount of the purified peptides allowed the dose-response relationship to be determined over a limited range of Pi5 concentrations, between 5 and 100 nM (Fig. 3C). The best fit linear regression to the double reciprocal plot shown in Fig. 3C resulted in a Kd of 92 nM (R^2=0.99). Pi5 inhibited the hKv1.3 current expressed in human T cells with similar potency, the remaining current fractions of hKv1.2 and hKv1.3 currents were 0.29+/-0.03 (n=4) and 0.39+/-0.02 (n=5), respectively, in the presence of 100 nM Pi5. Fig. 3D shows the raw current traces in the presence and absence of 100 nM Pi5, 10 mM TEA was used as a positive control for solution exchange. The kinetics of the blocked hKv1.3 current did not



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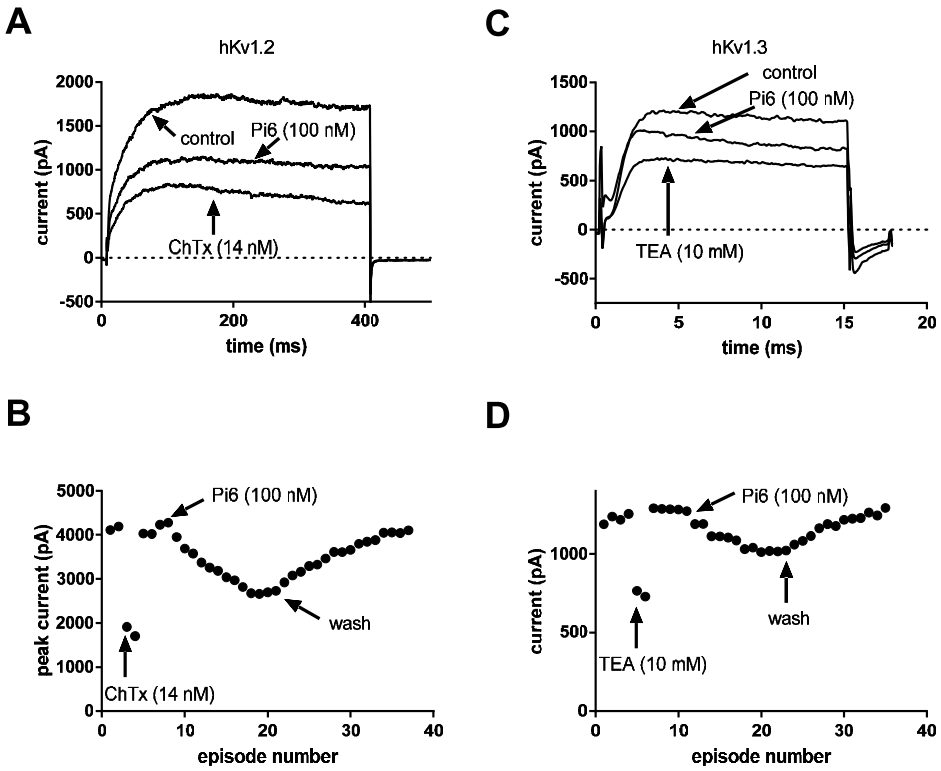
280 **Figure 3: Block of Kv1.2 and Kv1.3 channels by Pi5**

281 A) Whole-cell potassium currents through hKv1.2 channels were evoked from a transiently
282 transfected CHO cells in response to depolarizing pulses to +50 mV from a holding potential of -
283 100 mV every 15s. The current traces were recorded in the absence of the toxins (control,
284 indicated by arrow), after equilibration of the block in the presence of Pi5 (indicated by arrow) in

100 nM concentration. Charybdotoxin (ChTx, arrow, 14 nM) was used as a positive control. B) Development of and recovery from block of Kv1.2 by Pi5. Peak currents were determined during repeated depolarizations to +50 mV, arrows indicate the start of the bath perfusion with 100 nM Pi5 or with the toxin-free bath solution (labeled "wash"). C) Double-reciprocal plot of the dose-response of Pi5 on hkV1.2. See methods for details. The linear regression (1/B=(Kd*1/C)+1) resulted in a Kd of 92 nM (R^2=0.99). D) Whole-cell potassium currents through hKv1.3 channels were evoked from in activated human peripheral lymphocytes in response to depolarizing pulses to +50 mV from a holding potential of -100 mV every 15s. The current traces were recorded in the absence of the toxins (control, indicated by arrow), after equilibration of the block in the presence of Pi5 in 100 nM concentration (arrow). Tetraethylammonium (TEA, arrow, 10 mM) was used as a positive control. D) Development of and recovery from block of Kv1.3 by Pi5. Peak currents were determined during repeated depolarizations to +50 mV, arrows indicate the start of the bath perfusion with 10 nM Pi5 or 100 nM Pi5 or the toxin-free bath solution (arrow labeled wash). E) Double-reciprocal plot of the dose-response of Pi5 on hKv1.3. See methods for details. The linear regression (1/B=(Kd*1/C)+1) resulted in a Kd of 77 nM (R^2=0.99).

differ from the control one, similar to the results obtained for the inhibition of the hKv1.2 currents. The block of the Kv1.3 current by Pi5 was fully reversible, as indicated by the full recovery of the peak currents following the application of Pi5 in 10 nM and 100 nM concentrations (Fig. 3 E, indicated by arrows). The wash-out of the peptide was complete in ~15 episodes corresponding to ~225 s. The kinetics of the recovery from block followed a single exponential time-course (fit not shown) with a time constant of 80+/-6 s (n=4). The best fit linear regression to the double reciprocal plot shown in Fig. 3F resulted in a Kd of 77 nM (R^2=0.99). The same set of experiments was repeated using Pi6 and the effect of this peptide at 100 nM concentration was studied on the hKv1.2 and hKv1.3 currents. Panels A and C of Fig. 4 show the raw current traces whereas panels B and D show the change in the peak currents upon application and wash-out of Pi6 when cells expressing hKv1.2 (A, B) or hKv1.3 (C,D) channels



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314 **Figure 4: Block of Kv1.2 and Kv1.3 channels by Pi6**

315 A) Whole-cell potassium currents through hKv1.2 channels were evoked from a transiently
316 transfected CHO cells in response to depolarizing pulses to +50 mV from a holding potential of -
317 100 mV every 15s. The current traces were recorded in the absence of the toxins (control,
318 indicated by arrow), after equilibration of the block in the presence of Pi6 (indicated by arrow) in
319 100 nM concentration. Charybdotoxin (ChTx, arrow, 14 nM) was used as a positive control. B)
320 Development of and recovery from block of Kv1.2 by Pi6. Peak currents were determined during
321 repeated depolarizations to +50 mV, arrows indicate the start of the bath perfusion with 100 nM
322 Pi6 or with the toxin-free bath solution (labeled "wash"). C) Whole-cell potassium currents
323 through hKv1.3 channels were evoked from in activated human peripheral lymphocytes in
324 response to depolarizing pulses to +50 mV from a holding potential of -100 mV every 15s. The
325 current traces were recorded in the absence of the toxins (control, indicated by arrow), after
326 equilibration of the block in the presence of Pi6 in 100 nM concentration (arrow).

Tetraethylammonium (TEA, arrow, 10 mM) was used as a positive control. D) Development of and recovery from block of Kv1.3 by Pi6. Peak currents were determined during repeated depolarizations to +50 mV, arrows indicate the start of the bath perfusion with 100 nM Pi6 or the toxin-free bath solution (arrow labeled wash).

in whole-cell patch-clamp. The raw traces recorded at +50 mV test potential show that Pi6 is a low affinity blocker of both hKv1.2 and hKv1.3. The statistical analysis of the remaining current fractions upon reaching block equilibrium resulted in RF= 0.68+/-0.02 (n=5) and RF=0.77+/-0.01 (n=4) for hKv1.2 and hKv1.3 currents, respectively. The recovery from the block of both hKv1.2 (B) and hKv1.3 (D) was complete in ~300 s. The amount of the purified peptides was not sufficient to obtain the dose-response relationship of inhibitions for either hKv1.3 or hKv1.3. Supplementary Figure 2 shows on the other hand, that neither Pi5 nor Pi6 inhibits hKv1.1 or hKv1.4 channels in 100 nM concentration. To obtain a more precise determination of the peak currents in hKv1.4 expressing cells we transfected the N-terminal inactivation particle deleted hKv1.4 construct (Kv1.4-ΔN). Supplementary Figure 2A and Supplementary Figure 2C show hKv1.1 and hKv1.4 currents, respectively, recorded before application of the peptides and after the 10^{th} pulse in the toxin-containing solution. The overlapping current traces indicate the lack of block, similarly to the constant peak currents regardless of the presence or absence of the peptides shown in Supplementary Figure 2B and Supplementary Figure 2D.

The effect of Pi5 and Pi6 in 100 nM concentration on the ion channels included in this study is summarized in Fig. 5. The data show that these peptides inhibit significantly, the human Kv1.2 and Kv1.3 channels and that Pi5 is slightly more potent blocker of these channels than Pi6. Furthermore, Pi5 also inhibits Shaker B channels with low affinity.

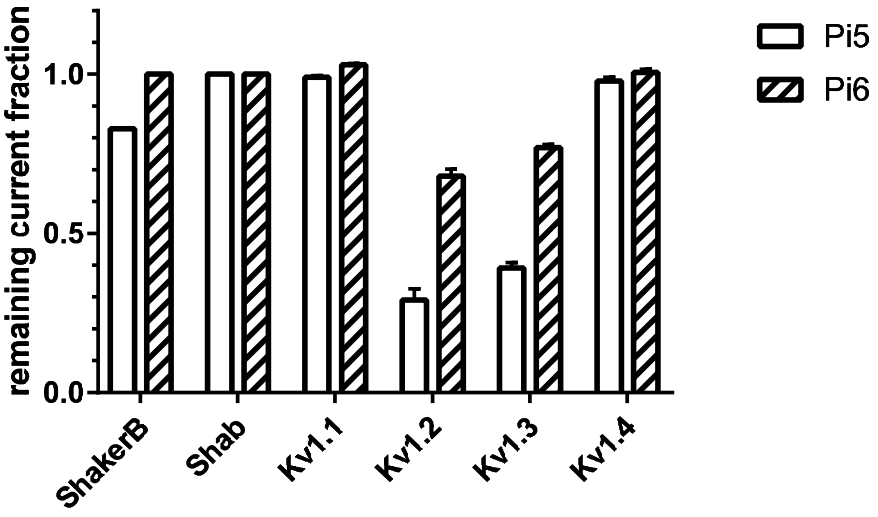


Figure 5: Selectivity profile of Pi5 and Pi6

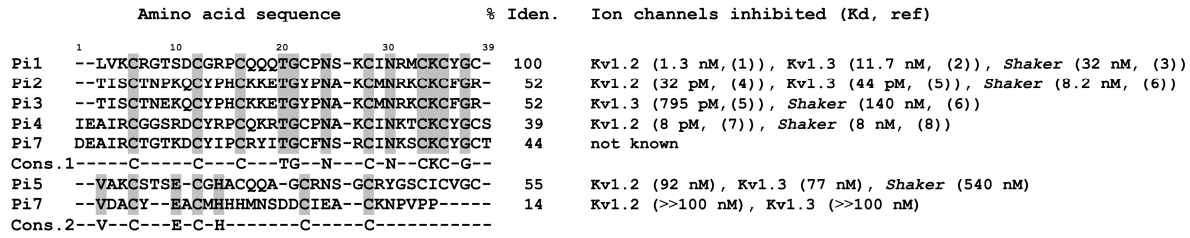
A) Bars indicate the remaining current fractions at equilibrium block of the indicated channels by 100 nM Pi5 (empty bars) or 100 nM Pi6 (hatched bars). For the expression systems, solutions and voltage protocols see Materials and Methods and Figs 2-4 and supplementary Fig. 2. Error bars indicate SEM (n=3-6).

3.4 Comparative analysis of amino acid sequence of the Pi toxins

Fig.6 shows the amino acid sequence of K^+-channel blocking peptides isolated from *Pandinus imperator* venom. The similarities of these sequences are relatively low (39 to 55% identity taking Pi1 as the reference), and it is practically nonexistent for Pi6 (14%). The peptides Pi1, Pi4, Pi5 and Pi7 have four disulfide bonds; Pi2 and Pi3 have 3 and Pi6 only 2. The longest ones are Pi4 and Pi7, containing 37 amino acid residues, whereas Pi6 has only 28. The conserved residues are shaded shown, mainly cysteines. Pi2 and Pi3 differ only by one amino acid in position 7, which is a Proline for Pi2 and Glutamic acid for Pi3.

The general pattern of ion channel inhibition by Pi5 matches to those of the *Pandinus imperator* toxins, these peptides inhibit Kv1.2 and Kv1.3 channels with nM-pM affinities without

significant selectivity for either of the channels (Fig. 6). Pi6, on the other hands, is different from the other members since it does not inhibit *Shaker*, and has very low affinity to Kv1.2 and Kv1.3. These properties of Pi6 match to the systemic nomenclature (see below).



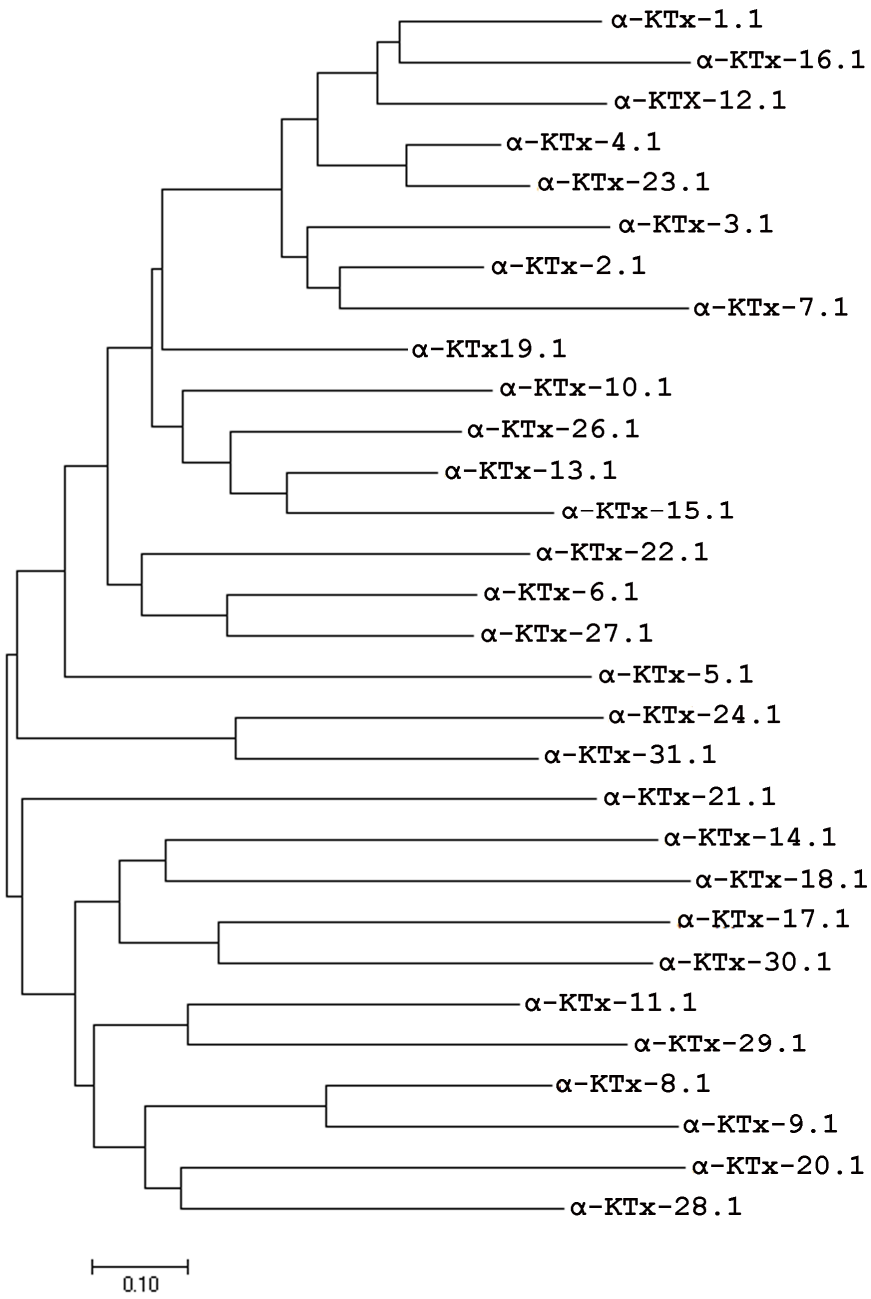
	Amino acid sequence	% Iden.	Ion channels inhibited (Kd, ref)
	1 10 20 30 39		
Pi1	--LVKCRGTSDCGRPCQQQTGCPNS-KCINRMCKCYGC-	100	Kv1.2 (1.3 nM,(1)), Kv1.3 (11.7 nM, (2)), *Shaker* (32 nM, (3))
Pi2	--TISCTNPKQCYPHCKKETGYPNA-KCMNRKCKCFGR-	52	Kv1.2 (32 pM, (4)), Kv1.3 (44 pM, (5)), *Shaker* (8.2 nM, (6))
Pi3	--TISCTNEKQCYPHCKKETGYPNA-KCMNRKCKCFGR-	52	Kv1.3 (795 pM,(5)), *Shaker* (140 nM, (6))
Pi4	IEAIRCGGSRDCYRPCQKRTGCPNA-KCINKTCKCYGCS	39	Kv1.2 (8 pM, (7)), *Shaker* (8 nM, (8))
Pi7	DEAIRCTGTKDCYIPCRYITGCFNS-RCINKSCKCYGCT	44	not known
Cons.1	-----C-----C---C---TG--N---C-N--CKC-G--		
Pi5	--VAKCSTSE-CGHACQQA-GCRNS-GCRYGSCICVGC-	55	Kv1.2 (92 nM), Kv1.3 (77 nM), *Shaker* (540 nM)
Pi7	--VDACY--EACMHHHMNSDDCIEA--CKNPVPP-----	14	Kv1.2 (>>100 nM), Kv1.3 (>>100 nM)
Cons.2	--V--C---E-C-H-------C-----C----------		

Figure 6: Comparative sequences of *Pandinus imperator* toxins

Amino acid sequences of the K^+-channel peptides isolated from *Pandinus imperator* and percentage of identity (Iden.) compared to Pi1. Cons.1 and Cons.2 stand for consensus on the relative positions of the cysteine residues in Pi1-Pi4, Pi7 and Pi5, Pi6, respectively. Amino acids in identical positions are highlighted in gray. References (ref) in this figure correspond to: (1) (Mouhat et al., 2004), (2) (Peter et al., 2000), (3) (Gomez Lagunas et al., 1997), (4) (Rogowski et al., 1996), (5) (Peter et al., 2001), (6) (Gomez-Lagunas et al., 1996), (7) (M'Barek et al., 2003) and (8) (Olamendi-Portugal et al., 1998).

The databank KALIUM (see reference Kuzmenkov et al..2016), by November 2016 listed 174 distinct amino acid sequence belonging to α, β, δ, κ and λ families, from which Pi5 and Pi6 belong to α and κ families, respectively. When this manuscript was been prepared another new sub-family (α-KTx 31.1) was described (ElFessi-Magouri et al., 2016). Pi5 is the first example of a new subfamily, and the systematic number assigned was α-KTx 24.1 (See Supplementary Fig.3). We found that the subfamily number 25 was never used. New coming sequences, that according to the initial proposed nomenclature (Tytgat et al, 1999) justifies the assignation of

389 new member for systematic classification, should use the number 25. The physiological effects
390 of Pi5 described here, certainly justifies including this sequence as a *bona fide* α-KTx peptide.
391 This classification was supported in addition by the phylogenetic analysis conducted, using the
392 number one sequence of each subfamily described until now.



393

Figure 7: Unrooted phylogenetic tree of α-KTx

A multiple sequence alignment of 30 sequences were retrieved from public databases, literature or unpublished results from our laboratory. The evolutionary history was inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA7.

```
κ-KTx2.1 OmTx1      -DPCYEVCLQQHGNVKECEEACKHPVE
κ-KTx2.2 OmTx2      -DPCYEVCLQQHGNVKECEEACKHPVEY
κ-KTx2.3 OmTx3      NDPCEEVCLQHTGNVKACEEACQ-----
κ-KTx2.4 OmTx4      -DPCYEVCLQQHGNVKECEEACKHP---
κ-KTx2.5 OcyC8      YDACVNACLEHHPNVRECEEACKNPVPP
κ-KTx2.6 OcyC9      FPPCVEVCVQHTGNVKECEAACGE----
κ-KTx2.7 HSP053C.1 -NACIEVCLQHTGNPAECDKACDK-----
κ-KTx2.8 HSP053C.2 GNACIEVCLQHTGNPAECDKPCDK-----
κ-KTx2.9 Pi6        VDACYEACMHHHMNSDDCIEACKNPVPP
```

Figure 8: Amino acid sequence of the family κ-KTx 2

Pi6 is the number 9 of this subfamily.

Relatively limited information is available for the inhibition of K^+ channels by κ-KTx peptides albeit the family consists of 9 members now. The only peptide studied in this group so far is κ-KTx 2.5 (OcyC8) where a low affinity inhibition of Kv1.1 (Kd=217 μM) and Kv1.4 (Kd=71 μM) were reported earlier (Camargos et al., 2011). These peptides do not conform to the general pattern of the peptides inhibiting K^+ channels with high affinity, Pi6 has less cysteines to make disulfide bridges and the 2 antiparallel beta sheets plus the α helix cannot be assigned to Pi6. Regardless, it is very interesting that Pi6 does inhibit (albeit with low affinity) voltage gated K^+ channels (this communication) which might be related to an unspecific effect of charged amino acid residues. Thus, the real effect of this peptide remains to be clarified, if any.

In summary, we have identified and biochemically characterized two novel peptides from *Pandinus imperator*, and assigned the systemic names of α-KTx 24.1 (Pi5) and κ-KTx 2.9 (Pi6) to them. Due to the relatively low affinity of Kv1.2 or kv1.3 inhibition the applicability of these peptides as research or therapeutic purposes is unlikely. On the other hand, our results contribute to the description of the biodiversity of the scorpion peptides regarding their primary structure and biological function.

Ethical Statement

This manuscript does not include any studies using human subjects. Authors declare that the described work has not been published previously. All authors approve this manuscript.

Conflict of interest

The authors do not have any conflicts of interest to disclose.

Acknowledgements

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542

543

544 **FIGURE LEGENDS**

545

546 Figure 1: **Purification and amino acid sequence of Pi5 and Pi6**

A. Fraction III from Sephadex G-50 column (0.5 mg) was separated by HPLC using an analytical C18 reverse-phase column (4.6 x 250 mm), with a linear gradient from solution A (0.12% trifluoacetic acid –TFA- in water) to 40% solution B (0.10% TFA in acetonitrile). Sub-fraction 5 (labeled with asterisk) was homogeneous Pi5, which was digested with chymotripsin and separated again in the same conditions, as indicated in the inset figure labeled Pi5. The asterisk indicates the peptide whose sequence allowed completion of the amino acid sequence shown in letter B, from position 26 to 33 (under labeled with .Ch.). Similarly, the sub-fraction 6 (labeled with asterisk) was the homogeneous peptide Pi6, which was digested with Protease V8 from *Staphylococcus aureus*, and separated in the same conditions, as indicated in the insert labeled Pi6. The labeled peptide with asterisk was sequenced and permitted completion of the sequence from amino acids in position 21 to 28 (underlined .V8.). Letters B and C show the full amino acid sequence for both peptides, where "Direct" means amino acid sequence obtained with a reduced and alkylated sample of each peptide.

Figure 2: Effect of Pi5 on *Drosophila* K^+ channels

(A) K^+ currents through *dShaker B* channels. K^+-currents were elicited every 15-sec by 30-ms pulses from -20 to +60 mV applied in 10 mV increments from a holding potential of -80 mV, before (left panel, labeled Control), during (middle panel, labeled Pi5) and after (labeled Recovery) the addition of 1.5 µM Pi5 to the external solution. (B) Current-Voltage relationship of the traces in A: closed circles: control I_K, open symbols: I_K with 1.5 µM Pi5 in the external solution, closed triangles: I_K after washing the cell with the control solution. The inset shows that the block (1.5 µM Pi5) is not voltage dependent. (C) Fractional block vs. [Pi5], the fractional block (F.b) was calculated as $F.b=1-(I/I_0)$, where I_0 and I are the peak current in the control and in the presence of Pi5, respectively. The line is the least squares fit of the points with the Michaelis-Menten equation, with Kd= 540 nM. The inset shows the expected linear double-reciprocal plot of the points (r=0.995). (D) K^+ currents through dShab channels. The figure shows two superimposed currents evoked by a 30mV/30ms pulse applied from -80 mV, before and during addition of 1.5 µM Pi5, as indicated. (E) Current-voltage relationship before and in the presence of 1.5 µM Pi5 from traces as in D. There was no blockage.

Figure 3: Block of Kv1.2 and Kv1.3 channels by Pi5

A) Whole-cell potassium currents through hKv1.2 channels were evoked from a transiently transfected CHO cells in response to depolarizing pulses to +50 mV from a holding potential of -100 mV every 15s. The current traces were recorded in the absence of the toxins (control, indicated by arrow), after equilibration of the block in the presence of Pi5 (indicated by arrow) in 100 nM concentration. Charybdotoxin (ChTx, arrow, 14 nM) was used as a positive control. B) Development of and recovery from block of Kv1.2 by Pi5. Peak currents were determined during repeated depolarizations to +50 mV, arrows indicate the start of the bath perfusion with 100 nM Pi5 or with the toxin-free bath solution (labeled "wash"). C) Double-reciprocal plot of the dose-response of Pi5 on hkV1.2. See methods for details. The linear regression (1/B=(Kd*1/C)+1) resulted in a Kd of 92 nM (R^2=0.99). D) Whole-cell potassium currents through hKv1.3 channels were evoked from in activated human peripheral lymphocytes in response to depolarizing pulses to +50 mV from a holding potential of -100 mV every 15s. The current traces were recorded in the absence of the toxins (control, indicated by arrow), after equilibration of the block in the presence of Pi5 in 100 nM concentration (arrow). Tetraethylammonium (TEA, arrow, 10 mM) was used as a positive control. D) Development of and recovery from block of Kv1.3 by Pi5. Peak currents were determined during repeated depolarizations to +50 mV, arrows indicate the start of the bath perfusion with 10 nM Pi5 or 100 nM Pi5 or the toxin-free bath solution (arrow labeled wash). E) Double-reciprocal plot of the dose-response of Pi5 on hkV1.3. See methods for details. The linear regression (1/B=(Kd*1/C)+1) resulted in a Kd of 77 nM (R^2=0.99).

Figure 4: Block of Kv1.2 and Kv1.3 channels by Pi6

A) Whole-cell potassium currents through hKv1.2 channels were evoked from a transiently transfected CHO cells in response to depolarizing pulses to +50 mV from a holding potential of -100 mV every 15s. The current traces were recorded in the absence of the toxins (control, indicated by arrow), after equilibration of the block in the presence of Pi6 (indicated by arrow) in 100 nM concentration. Charybdotoxin (ChTx, arrow, 14 nM) was used as a positive control. B) Development of and recovery from block of Kv1.2 by Pi6. Peak currents were determined during repeated depolarizations to +50 mV, arrows indicate the start of the bath perfusion with 100 nM Pi6 or with the toxin-free bath solution (labeled "wash"). C) Whole-cell potassium currents

through hKv1.3 channels were evoked from in activated human peripheral lymphocytes in response to depolarizing pulses to +50 mV from a holding potential of -100 mV every 15s. The current traces were recorded in the absence of the toxins (control, indicated by arrow), after equilibration of the block in the presence of Pi6 in 100 nM concentration (arrow). Tetraethylammonium (TEA, arrow, 10 mM) was used as a positive control. D) Development of and recovery from block of Kv1.3 by Pi6. Peak currents were determined during repeated depolarizations to +50 mV, arrows indicate the start of the bath perfusion with 100 nM Pi6 or the toxin-free bath solution (arrow labeled wash).

Figure 5: Selectivity profile of Pi5 and Pi6

A) Bars indicate the remaining current fractions at equilibrium block of the indicated channels by 100 nM Pi5 (empty bars) or 100 nM Pi6 (hatched bars). For the expression systems, solutions and voltage protocols see Materials and Methods and Figs 2-4 and supplementary Fig. 2. Error bars indicate SEM (n=3-6).

Figure 6: Comparative sequences of *Pandinus imperator* toxins

Amino acid sequences of the K^+-channel peptides isolated from *Pandinus imperator* and percentage of identity (Iden.) compared to Pi1. Cons.1 and Cons.2 stand for consensus on the relative positions of the cysteine residues in Pi1-Pi4, Pi7 and Pi5, Pi6, respectively. Amino acids in identical positions are highlighted in gray. References (ref) in this figure correspond to: (1) (Mouhat et al., 2004), (2) (Peter et al., 2000), (3) (Gomez Lagunas et al., 1997), (4) (Rogowski et al., 1996), (5) (Peter et al., 2001), (6) (Gomez-Lagunas et al., 1996), (7) (M'Barek et al., 2003) and (8) (Olamendi-Portugal et al., 1998).

Figure 7: Unrooted phylogenetic tree of α-KTx

A multiple sequence alignment of 30 sequences were retrieved from public databases, literature or unpublished results from our laboratory. The evolutionary history was inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary

636 distances were computed using the Poisson correction method and are in the units of the number
637 of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA7.

638

639 **Figure 8: Amino acid sequence of the family κ-KTx 2**
640 Pi6 is the number 9 of this subfamily.

641

642 **Legend for Supplementary Figures**

643

644 **Supplementary Figure 1: Pi6 effect on Shaker channels**
645 Pi6 does not inhibit *Shaker* channels. *Shaker* I_K evoked by delivering 30-ms activation pulses
646 from -40 to +50 mV, in 10 mV increments, before (left panel), and after addition of 100 nM Pi6
647 to the extracellular solution (right panel). I_K was not significantly inhibited by Pi6. HP= -80 mV.
648 Time between pulses was 20-sec to allow full recovery from inactivation.

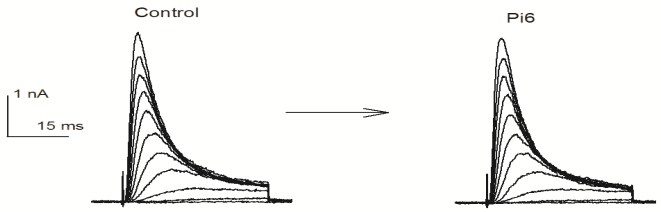
649

650 **Supplementary Figure 2: Lack of inhibition of hKv1.1 and hKv1.4 by Pi5 and Pi6**
651 A, C) Whole-cell potassium currents through hKv1.1 (A) or hKv1.4 channels (C) were evoked
652 from a transiently transfected CHO cells in response to depolarizing pulses to +50 mV from a
653 holding potential of -100 mV every 15s. The current traces were recorded in the absence of the
654 toxins (control, indicated by arrow), after at least 10 episodes in of Pi5 or Pi6 (indicated by
655 arrows) in 100 nM concentration. Tetraethylammonium (TEA, arrow, 0.3 mM) was used as a
656 positive control in A. Positive control for hKv1.4 was obtained by perfusing the recording
657 chamber with a bath solution congaing 150 mM K+ (not shown). B,D) Peak hKv1.1 (B) and
658 hKv1.4 currents (D) in 100 nM Pi5 or Pi6. Peak currents were determined during repeated
659 depolarizations to +50 mV, arrows indicate the start of the bath perfusion with 100 nM Pi5 or
660 100 nM Pi6.

661

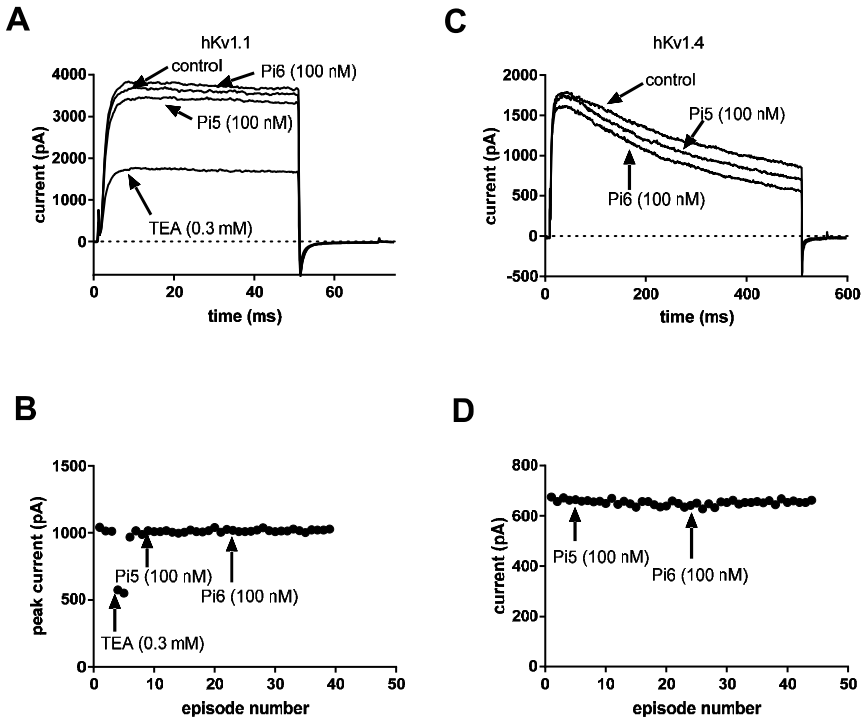
662 **Supplementary Figure 3: Amino acid sequence of α-KTx toxins**
663 Only the number 1 toxin of each subfamily was selected. The number α-KTx 25 is empty.

664



Supplementary Figure 1: **Pi6 effect on Shaker channels**

Pi6 does not inhibit *Shaker* channels. *Shaker* I_K evoked by delivering 30-ms activation pulses from -40 to +50 mV, in 10 mV increments, before (left panel), and after addition of 100 nM Pi6 to the extracellular solution (right panel). I_K was not significantly inhibited by Pi6. HP= -80 mV. Time between pulses was 20-sec to allow full recovery from inactivation.



Supplementary Figure 2: Pi6 does not inhibit Shaker channels. Shaker IK evoked by delivering 30-ms activation pulses from -40 to +50 mV, in 10 mV increments, before (left panel), and after addition of 100 nM Pi6 to the extracellular solution (right panel). IK was not significantly inhibited by Pi6. HP= -80 mV. Time between pulses was 20-sec to allow full recovery from inactivation.

Supplementary Figure 3: Amino acid sequence of α-KTx toxins

•-KTx-1.1	QFTNVSCTTSKECWSVCQRLHNTSRGKCMNKKCRCYS
•-KTx-2.1	TIINVKCTSPKQCSKPCKELYGSSAGAKCMNGKCKCYNN
•-KTx-3.1	GVEINVKCSGSPQCLKPCKDAGMRFGKCMNRKCHCTPK
•-KTx-4.1	VFINAKCRGSPECLPKCKEAIGKAAGKCMNGKCKCYP
•-KTx-5.1	AFCNLRMCQLSCRSLGLLGKCIGDKCECVKH
•-KTx-6.1	LVKCRGTSDCGRPCQQQTGCPNSKCINRMCKCYGC
•-KTx-7.1	TISCTNPKQCYPHCKKETGYPNAKCMNRKCKCFGR
•-KTx-8.1	VSCEDCPEHCSTQKAQAKCDNDKCVCEPI
•-KTx-9.1	VGCEECPMHCKGKNAKPTCDDGVCNCNV

•-KTx-10.1	AVCVYRTCDKDCKRRGYRSGKCINNACKCYPY
•-KTx-11.1	DEEPKESCSDEMCVIYCKGEEYSTGVCDGPQKCKCSD
•-KTx-12.1	WCSTCLDLACGASRECYDPCFKAFGRAHGKCMNNKCRCYT
•-KTx-13.1	ACGSCRKKCKGSGKCINGRCKCY
•-KTx-14.1	TPFAIKCATDADCSRKCPGNPSCRNGFCACT
•-KTx-15.1	QNETNKKCQGGSCASVCRRVIGVAAGKCINGRCVCYP
•-KTx-16.1	DLIDVKCISSQECWIACKKVTGRFEGKCQNRQCRCY
•-KTx-17.1	QTQCQSVRDCQQYCLTPDRCSYGTCYCKTT
•-KTx-18.1	TGPQTTCQAAMCEAGCKGLGKSMESCQGDTCKCKA
•-KTx-19.1	AACYSSDCRVKCVAMGFSSGKCINSKCKCYK
•-KTx-20.1	GCTPEYCSMWCKVKVSQNYCVKNCKCPGR
•-KTx-21.1	GKFGKCKPNICAKTCQTEKGKGMGYCNKTECVCSEW
•-KTx-22.1	EVDGRTATFCTQSICEESCKRQNKNGRCVIEAEGSLIYHLCKCY
•-KTx-23.1	AAAISCVGSPECPPKCRAQGCKNGKCMNRKCKCYYC
•-KTx-24.1	VAKCSTSECGHACQQAGCRNSGCRYGSCICVGC
•-KTx-25.1	Empty
•-KTx-26.1	NFKVEGACSKPCRKYCIDKGARNGKCINGRCHCYY
•-KTx-27.1	QIDINVSCRYGSDCAEPCKRLKCLLPSKCINGKCTCYPSIKIKNCKVQTY
•-KTx-28.1	ACVTHEDCTLLCYDTIGTCVDGKCKCM
•-KTx-29.1	EGDCPISEAIKCVEKCKEKVEVCEPGVCKCSG
•-KTx-30.1	EDKLKCTKTDDCAKYCSQFTDVHPACLGGYCECLRWEGGISS
•-KTx-31.1	AGSMDSCSETGVCMKACSERIRQVENDNKCPAGECICTT

Only the first the number 1 toxin of each subfamily was selected. The number α-KTx 25 is empty

Highligths

1. Two short length peptides: Pi5 and Pi6 were purified and characterized from *Pandinus imperator* scorpion venom.
2. Pi5 has 33 amino acids and constitutes a new subfamily of α-KTx, (systematic number α-KTx 24.1).
3. Pi6 contains 28 amino acids, two disulfide bonds, and is a new member of the κ-KTx (systematic number κ-KTx2.9).
4. Pi5 inhibited *Shaker B*, hKv1, Kv1.2 and hKv1.3 channels at nanomolar concentrations.
5. Pi6 is a non-selective blocker of hKv1.2 and hKv1.3 channels.