The potassium channel Kv1.3 is an attractive pharmacological target for immunomodulation of T cell-mediated autoimmune diseases. Potent and selective blockers of Kv1.3 are potential therapeutics for treating these diseases. Here we describe the design of a new peptide inhibitor which is potent and selective for Kv1.3. Three residues (Gly11, Ile28 and Asp33) of a scorpion toxin BmKTX were substituted by Arg11, Thr28 and His33, resulting in a new peptide, named ADWX-1. The ADWX-1 peptide blocked Kv1.3 with picomolar affinity (IC₅₀=1.89 pM), showing a 100-fold increase in activity compared to the native BmKTX toxin. The ADWX-1 also displayed good selectivity on Kv1.3 over related Kv1.1 and Kv1.2 channels.

Furthermore, alanine-scanning mutagenesis was carried out to map the functional residues of ADWX-1 in blocking Kv1.3. Moreover, computational simulation was used to build a structural model of the ADWX-1-Kv1.3 complex. This model suggests that all mutated residues are favorable for both the high potency and selectivity of ADWX-1 toward Kv1.3. While Arg11 of ADWX-1 interacts with Asp386 in Kv1.3, Thr28 and His33 of ADWX-1 locate right above the selectivity filter-S6 linker of Kv1.3. Together, our data indicate that the specific ADWX-1 peptide would be a viable lead in the therapy of T cell-mediated autoimmune diseases, and the successful design of ADWX-1 suggests that rational design based on the
structural model of the peptide-channel complex should accelerate the development of diagnostic and therapeutic agents for human channelopathies.

INTRODUCTION

In human T cells, Kv1.3 potassium channel plays an essential role in regulating the resting membrane potential and Ca\(^{2+}\) signaling (1-3). When the autoimmune disease-related CCR7\(^{-}\) effector memory T-cells are activated in the inflammation sites, expression of Kv1.3 channel in these cells significantly increases from approximately 250 channels to about 1500–2000 channels per cell. Such increased expression of Kv1.3 channel proteins does not occur in the naïve or central memory T cells homed in lymphoid organs (4,5). These findings suggest that selective suppression of effector memory T cells with specific Kv1.3 inhibitors could efficiently suppress immune responses to alleviate the diseases. Moreover, the therapeutic efficacy of Kv1.3 channel blocking was validated by \textit{in vitro} assays and by \textit{in vivo} animal models of T-cell mediated autoimmune diseases such as multiple sclerosis, type-I diabetes, rheumatoid arthritis and psoriasis (1,4,6-8). Progress in this area has promoted the extensive development of highly potent and selective Kv1.3 channel inhibitors, which may lead to a new class of drugs for autoimmune diseases.

Toxin peptides from natural venomous animals comprise the largest families of ion channel blockers, and they are becoming increasingly valuable sources of new drugs for channelopathies (9,10). With respect to the Kv1.3 channel, many structurally diverse peptide toxins, such as ChTX (11,12), ShK (13,14) and OSK1 (15), have been identified. Although these inhibitors can block potassium currents at the nM or pM concentrations, they lack sufficient specificity to distinguish between Kv1.3 and other related Kv1.x channels. Recently, chemical modifications and the deletion mutagenesis method have been used to improve the selectivity of these toxin peptides (16,17). For example, ShK-L5, derived from the sea anemone toxin ShK, exhibited a 100-fold selectivity for Kv1.3 \((IC_{50} = 69 \text{ pM})\) over Kv1.1 and more than 250-fold selectivity over all other tested channels (6). A series of peptide analogs derived from the scorpion toxin OSK1 blocked Kv1.3 with improved selectivity through progressive deletions of N- and C-terminal sequences (15,17). Although the potency and selectivity of these peptide inhibitors are much better than those of small chemical molecules (18,19), the rational design of potent and selective blockers still remains a huge challenge.

Here we have rationally designed a highly specific Kv1.3 inhibitor peptide, named ADWX-1 (Autoimmune Drug from WenXin group) based on the scorpion toxin BmKTX (20), using a structure-based strategy of manipulating three important residues. The new ADWX-1 peptide blocked Kv1.3 currents with picomolar affinity \((IC_{50}=1.89 \text{ pM})\), showing a 100-fold increase in inhibitory activity compared to native BmKTX peptide. Furthermore, ADWX-1 peptide displayed specificity on Kv1.3 over Kv1.1 and Kv1.2, for \(\sim 340\)-fold and \(>10^5\)-fold, respectively. Moreover, the structure-function relationship of ADWX-1 peptide was characterized through
alanine-scanning mutagenesis and computational modeling. Our work will facilitate the future structure-based design of specific peptide immunomodulators for the therapeutic target of Kv1.3 channels in T-cell mediated autoimmune diseases.

MATERIALS and METHODS

Site-directed Mutagenesis
ADWX-1 was generated by one overlapping PCR from BmKTX (21). A second PCR reaction used the products of the overlapping PCR as templates. The primers used were: Sense primer 1, 5’-GTGAATTCGATGACGATGACAAGGTGGGTATTAATGTGAA-3’; Sense primer 2, 5’-GGTATTCAATGTGAAATGTAAGCATAGCCGCAATGCTGAAACCATGCAAGGATGCTG-3’; Antisense primer 1, 5’-GGTACAATGGCATTGGCATTGCAATTACAATGGGATGC-3'; Antisense primer 2, 5’-TAGCTCGAGTCATCTGGGTTACAATGGGATTTC-3’. The restriction enzyme sites are in bold, and an enterokinase cleavage site is underlined.

All ADWX-1 mutants were generated by overlapping PCR from ADWX-1, the PCR strategy used was the same as constructing ADWX-1.

Purification and Characterization of Toxin Peptides
The ADWX-1 and its mutant plasmids were digested with EcoRI and XhoI, and the cDNAs were subcloned into pGEX-6P-1. After transformed into E. coli Rosetta (DE3) cells, cells were cultured at 37°C in LB medium with ampicillin (100 μg/ml). When cell density reached OD=0.6, 1.0 mM isopropyl thio-b-D-galactoside (IPTG) was added to induce the expression at 28°C. Cells were harvested after 4 hours and resuspended in 50 mM Tris-HCl (pH 8.0)/10 mM Na2EDTA. Supernatant from the bacterial cell lysate was loaded to a GST-bind column. The purified fusion protein was then desalted by using centrifugal filter (Millipore, USA), and cleaved by enterokinase (Biowisdom, China) at 25°C for 16 h. Protein samples were then separated by HPLC on a C18 column (10×250 mm, 5 μm) (Elite-HPLC, China) using a linear gradient from 10% to 80% acetonitrile with 0.1% TFA in 60 min, with detection at 230 nm. Peptides were eluted as major peaks at 21-24% acetonitrile. The molecular masses of the purified peptides were obtained by MALDI-TOF-MS (Voyager-DESTR, Applied Biosystems). The secondary structures of purified ADWX-1 and its mutants were measured by Circular Dichroism (CD) spectroscopy. Measurement was carried out in the UV range of 250-190 nm at 25°C in water on a Jasco-810 spectropolarimeter, with a 0.2-0.4 mg/ml concentration. For each peptide, spectra were collected from 3 separate recordings and averaged after subtracting the blank spectrum of pure water.

Electrophysiology
The cDNAs encoding mKv1.1 in pBSTA, hKv1.2 in pcDNA3/Hygro(+) and mKv1.3 in pSP64 (from Prof. Stephan Grissmer, University of Ulm, Ulm, Germany) were subcloned into the XhoI/BamH I sites of pIRES2-EGFP (Clontech). The constructs were verified by DNA sequencing (Sunbiotech, Wuhan, China).

HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal calf serum (Invitrogen) supplemented with ampicillin 100 units/ml and streptomycin 100 μg/ml. Plasmids containing mKv1.1, hKv1.2 or
mKv1.3 were respectively transfected into HEK293 cells using SofastTM Transfection Reagent (Sunma). Currents were recorded 1 to 3 days later in green fluorescent protein-positive cells.

Electrophysiological experiments were carried out at 22–25°C using the patch-clamp whole-cell recording mode. Cells were bathed with mammalian Ringer’s solution: 5 mM KCl, 140 mM NaCl, 10 mM HEPES, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM D-Glucose, pH 7.4 with NaOH. When ADWX-1 and the mutant toxin peptides were applied, 0.01% BSA was added to the Ringer’s solution. A multichannel micro perfusion system MPS-2 (INBIO Inc, Wuhan, China) was used to exchange the external recording bath solution. The pipette solution contained: 140 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM Na₂ATP, 5 mM HEPES (pH 7.4 with NaOH). All channel currents were elicited by depolarizing voltage steps of 200 ms from the holding potential -80 mV to +50 mV. Membrane currents were measured with an EPC 10 patch clamp amplifier (HEKA Elekt-ronik, Lambrecht, Germany) interfaced to a computer running acquisition and analysis software (Pulse).

Data analyses were performed with IgorPro (WaveMetrics, Lake Oswego, OR), and IC₅₀ values were deduced by fitting a modified Hill equation to the data: \[ \frac{I_{\text{toxin}}}{I_{\text{control}}} = 1 + \left( \frac{[\text{toxin peptide}]}{IC_{50}} \right) \], where \( I \) is the peak current to the normalized data points obtained with at least four different toxin peptide concentrations. Results are mostly shown as mean ± S.E, \( n \) being the number of experiments.

Molecular Modeling and Docking

The structures of mKv1.3 and ADWX-1 were modeled using KcsA (PDB code: 1BL8) and BmKTX (PDB code: 1BKT) as templates through the SWISSMODEL server (22). Using the modeled ADWX-1 and Kv1.3 structures, ZDOCK(23) program was used to generate the models for the ADWX-1-Kv1.3 complex. Several conformations of ADWX-1 modeled from BmKTX were used to improve the rigid performance of ZDOCK. Each docking group gives 2,000 predicted complex structures. Through clustering analysis with the mutagenesis results, some possible hits were screened out, followed by a 500-steps energy minimization and a 500 ps unrestrained molecular dynamics performed on each possible toxin peptide-channel complex using the SANDER module in the AMBER 8 suit programs (24).

**Molecular Dynamic Simulations and Calculation of Binding Free Energies by MM-PBSA**

All MD simulations were performed by using the Amber 8 program on a 32-CPU Dawning TC4000L cluster (Beijing, China). The final ADWX-1-Kv1.3 complex structure was sufficiently equilibrated by 7 ns unrestrained molecular dynamic simulations to introduce enough flexibility for both the channel and the toxin peptide. Temperature was set at 300 K with the cutoff distance of 12 Å used for unbounded interaction. The ff99 force field (Parm99)(25) was applied throughout the energy minimization and MD simulations. Before the unrestrained MD simulation was performed, we also employed enough equilibration steps for 400 ps from a larger force constant 5.0 (kcal/mol)/Å² for restraining all heavy atoms and then gradually reduced it to 0.02 (kcal/mol)/Å² for only heavy atoms in the backbone. Furthermore, for more sufficient simulation, the generalized Born solvation model in
macromolecular simulations (26,27) was used instead of explicit water.

The MM-PBSA module of AMBER 8 was used to calculate the binding free energies for ADWX-1 binding to Kv1.3 (24,28). This MM-PBSA module also contains a computational alanine scanning approach for evaluating the importance of residues functioning in protein-protein interactions. This program was employed for identifying the most reasonable candidate complexes. The detailed method of calculating binding free energies between inhibitory peptides and potassium channels was previously described (29,30).

RESULTS

Design strategy of a potent Kv1.3 channel inhibitor

Native BmKTX toxin peptide is a potent Kv1.3 blocker with a K_d value of 0.2 nM (20,21), therefore, was selected as a template for designing a new analog with higher potency in this work. In order to obtain more potent BmKTX peptide analogs, we adopted the following strategies. First, we adjusted the distribution of negatively charged residues in BmKTX peptide. In the BmKTX structure (PDB code: 1BKT), there is ~5 Å between the two Ca atoms from Asp^{33} and Lys^{26} residues (Fig. 1B). Such close distance impedes the side chain of the conserved Lys^{26} to block channel pore due to the strong electrostatic repulsion between Asp^{33} and the conserved aspartic acid in the S6-filter linker (Fig. 1B). In this work, Asp^{33} was substituted by a conserved histidine residue among \( \alpha \)-KTx3 scorpion toxins (31) (Fig. 1A-C); Second, we strengthened the polar interaction between polar residues from the peptide and Kv1.3. The side chain of Lys^{26} is expected to insert into the channel pore. We replaced the adjacent hydrophobic Ile^{28} of BmKTX with the polar threonine residue for a more favorable interaction (Fig. 1A-C). Third, we introduced a positively charged residue in the beginning of the \( \alpha \) helix domain. There are four negatively charged residues in the turret of Kv1.3, Arg^{11} was introduced for a potential salt-bridge interaction between K1.3 and the toxin peptide (Fig. 1C). With these three changes to improve the potency of BmKTX, a new ADWX-1 peptide was rationally designed (Fig. 1A).

Pharmacological activities of ADWX-1 peptide on Kv channels

To experimentally test the ADWX-1 peptide, we have characterized the pharmacological activity of ADWX-1 on Kv1.3 channels. Recombinant ADWX-1 peptide was purified from E. coli. Fig. 2A showed a peptide of 4 kDa cleaved by enterokinase from the 30 kDa fusion protein. The mixture of ADWX-1 peptide and GST protein was completely separated by reversed-phase HPLC (Fig. 2B). The peak at 12-13 min corresponding to ADWX-1 peptide was collected and subsequently identified by mass spectrometry analysis (Fig. 2C). The molecular weight of ADWX-1 was 4072.8 Da, which is close to the calculated molecular mass of 4071.1 Da.

Next we investigated the effect of recombinant ADWX-1 peptide on Kv1.3 channel together with Kv1.1 and Kv1.2 channels. All channels were expressed in the transiently transfected HEK293 cells. Fig. 3A and 3B show the effects of ADWX-1 peptide on Kv1.3 and Kv1.1 currents elicited by 200-ms depolarizing pulses from a holding potential of -80 to 50 mV. As shown in Fig. 3A, ADWX-1 had a very high potency towards Kv1.3 channels with IC_{50} of 1.89 \pm 0.53 pM, showing a ~100-fold increase in binding affinity compared with
that of BmKTX (20,21). In addition, ADWX-1 also exhibited over 340-fold selectivity for Kv1.3 over Kv1.1 (IC$_{50}$=0.65 ± 0.25 nM) (Fig. 3B and C). Furthermore, 100 nM ADWX-1 peptide only blocked about 10% of the peak current of Kv1.2 channels (Fig. 3C and data not shown). These data showed that the designed ADWX-1 peptide was the most selective and potent peptide inhibitor among identified Kv1.3-specific inhibitors, such as AgTX2 (32), ShK-L5 (6), AOSK1 and [△36-38]-AOSK1 (17).

Functional sites of ADWX-1 identified by alanine-scanning mutagenesis

To identify the functional sites of ADWX-1 involved in the interaction with Kv1.3 channels, eight residues were individually mutated to alanine, based on the model of the ADWX-1 peptide-Kv1.3 channel complex. Compared with the ADWX-1 peptide, the CD spectra of eight mutants showed no significant changes in the secondary structure (Fig. 4), indicating that ADWX-1 and its mutant peptides all adopted the same overall structural topology. Furthermore, we also expressed and purified recombinant BmKTX, whose CD spectrum was measured and compared with that of ADWX-1 (Fig. 4A). Although three residues of BmKTX were changed, the structure of the resulted ADWX-1 peptide was almost the same as that of BmKTX peptide.

The blocking activities of these ADWX-1 mutant peptides were then tested on Kv1.3 channels. Fig. 5 A-H showed the representative current traces before and after treatments with mutant peptides. Table 1 listed the average IC$_{50}$ values for these mutants. As illustrated in Table 1 and Fig. 5, all mutants showed decreased activities compared with the ADWX-1 peptide. The substitution of alanine for the three designed residues (Arg$^{11}$, Thr$^{28}$ and His$^{33}$) altered the binding affinity by 178-, 30- and 40-fold, respectively (Table 1, Fig. 5 I). These data indicated that these mutated residues were not only the important channel-interacting residues, but also could be responsible for the enhanced potency of ADWX-1 on Kv1.3 channels. Furthermore, the Arg$^{23}$ and Phe$^{24}$ mutants exhibited the most significant drop of ADWX-1 affinity on Kv1.3 channels for over 3800- and 2100-fold, from 1.89 ± 0.53 pM to 7.34 ± 3.95 nM and 4.01 ± 0.85 nM, respectively (Table 1, Fig. 5 J), suggesting Arg$^{23}$ and Phe$^{24}$ were the major functional residues in the high-affinity binding of ADWX-1 with Kv1.3. Taking into account that the similarity of ADWX-1 and other α-KTX toxin peptides (31,33), an alanine substitution of the conserved pore-blocking residue Lys$^{26}$ was also produced (31). This mutation resulted in a dramatic loss of potency, with IC$_{50}$ value (IC$_{50}$=0.96 ± 0.72 nM) decreasing for over 510-fold compared to that of ADWX-1 (Table 1, Fig. 5 J). Another important channel-interacting site was Asn$^{29}$, and the ADWX-1-N29A mutant was 240-fold less active than ADWX-1 in blocking Kv1.3 channels, with an IC$_{50}$ of 454.4 ± 40.7 pM (Table 1, Fig. 5 J). In addition, the modification of Thr$^{35}$ caused a negligible decrease in the mutant’s affinity for Kv1.3 channels (IC$_{50}$= 28.0 ± 19.2 pM) (Table 1, Fig. 5 J). All these results showed that just like the classical Kv channel toxin peptides, the positively charged residues of ADWX-1 played essential role in mediating the recognition process towards Kv1.3 channels.

Molecular mechanism of ADWX-1 recognizing Kv1.3 channel

Given the characterized functional residues of ADWX-1, a reasonable model of
the ADWX-1-Kv1.3 complex was obtained to reveal the recognition mechanism of ADWX-1 peptide toward Kv1.3 channels through combined computational approaches (29,30). First, the structures of the ADWX-1 peptide and the Kv1.3 channel were respectively modeled based on the structural similarity with their templates. Second, molecular docking was performed on the modeled ADWX-1 and Kv1.3 proteins using the ZDOCK program (23). The docking results were then filtered by scoring combined with detailed mutagenesis information and interaction energy analysis. Third, to introduce flexibility for the rigid docking results, an energy minimization followed by 500 ps molecular dynamics (MD) simulation was performed on each selected complex to better discriminate among them. Finally, an ADWX-1-Kv1.3 complex structural model was screened out using the computational alanine-scanning method in MM-PBSA (24,28), which was compared with the experimental alanine-scanning mutagenesis results. An additional 7 ns unrestrained MD simulation was performed to sufficiently equilibrate this model. Fig. 6A indicated that both the peptide and the channel were pre-aligned and induced to fit in their nanosecond-scale diffusional encounter, and then led to a specifically stable complex. An overall high degree of correlation was found between the calculation and the experiments on mutational effects (Fig. 6B and C). In Fig. 6B, the calculated results are normalized values of $\Delta \triangle G_{\text{binding}}$ from MM-PBSA analysis as we previously described (24,28). Experimental results are obtained as $k_bT \ln[IC_{50(\text{mutant})}/IC_{50(\text{wt})}]$. Fig. 6C showed that functional residues of ADWX-1 located in the peptide-Kv1.3 channel interface. The correlation between the experimental and computational data indicates the structural model of the complex is reasonable.

From the structural model of the ADWX-1-Kv1.3 complex, the changes of peptide pharmacological activities of the eight ADWX-1 mutants could be well explained. The most functionally essential Arg$^{23}$ residue formed a considerably strong electrostatic interaction with the Asp$^{386}$ residue in Kv1.3 channel (Fig. 6D and Fig.7A), and gave the biggest $\Delta \triangle G_{\text{binding}}$ value of 5.11 kcal/mol among all mutants. The conserved pore-blocking Lys$^{26}$ residue was found to plug its side chain into channel selectivity filter during the whole simulation as predicted (Fig. 6D and Fig.7A), and the $\Delta \triangle G_{\text{binding}}$ value of 3.58 kcal/mol correlated well with the mutation effect. Furthermore, the Phe$^{24}$ residue was surrounded by a “pocket” formed by many nonpolar and polar residues (Ser$^{379}$, Gly$^{380}$, Phe$^{381}$, Asp$^{402}$, Met$^{403}$ and His$^{404}$) of Kv1.3 channels within a contact distance of 4 Å (Fig.7B), which could well explain the significant loss of potency for ADWX-1 peptides by replacing Phe$^{24}$ with alanine residue. Thr$^{28}$, Asn$^{29}$ and His$^{33}$ residues located right above the filter-S6 linker (Fig. 7C and 7D), thus were important for the peptide-channel interaction. Arg$^{11}$ also interacted with the negatively charged Asp$^{386}$ residue in Kv1.3 channels (Fig. 7D). The binding affinity of ADWX-1 to Kv1.3 was affected little by the Thr$^{35}$ mutation, which was in accord with the observation that this residue located away from the interface. Hence, the model of the complex well elucidated the molecular basis of the major determinants for the high affinity of ADWX-1 binding to Kv1.3 channels.

DISCUSSION

In the current study, we have designed the ADWX-1 peptide, an analog of the
scorpion toxin BmKTX. ADWX-1 exhibited high potency and selectivity towards Kv1.3 channels. This design was based on many factors affecting the toxin peptide-potassium channel interactions, such as the distribution of peptide functional residues, residue polarity, and especially the negatively charged residues in toxin peptides. There is a strong electrostatic repulsion between the negatively charged Asp33 in the toxin peptide and the conserved aspartic acid in the S6-filter linker of Kv channels (Fig. 1B). The side chain of the conserved Lys26 residue is likely unable to insert into the channel selectivity filter. Thus, the pharmacological activities and profile of toxin peptides could be altered through changing the distribution of negatively charged residues together with modifying the possible contact residues with the structure-guided approach.

We addressed the above issues by producing a new ADWX-1 peptide, through mutating Asp33, Gly11 and Ile28 of the parent BmKTX into His33, Arg11 and Thr28 (see Fig. 1). The resulting ADWX-1 peptide could potently block Kv1.3 with an IC50 of 1.89 pM, which is ~100-fold lower than that of BmKTX (20). ADWX-1 also displayed selectivity toward Kv1.3 over Kv1.1 (by 340-fold) and over Kv1.2 (by >105-fold). The importance of the three mutated Arg11, Thr28 and His33 residues was confirmed by mutagenesis studies and structural analysis. The substituted His33 was surrounded by Gly401, Asp402 and His404 in the D chain of Kv1.3 channels within a contact distance of 4 Å, and a hydrogen bond formed between His33 of the peptide and His404 of the Kv1.3 channel (Fig. 7D). In the model of the ADWX-1-Kv1.3 complex, Thr28 faced a polar interface formed by channel residues (Gly401, Asp402, Met403, and His404) together with a hydrogen bond between Thr28 and Asp402 in the D chain of Kv1.3 channels. Such local environment is more favorable for the polar Thr28 residue than the hydrophobic Ile28 residue (Fig. 7C). Arg11 of ADWX-1 could closely contact the negatively charged Asp386 in Kv1.3 through a salt-bridge interaction (Fig. 7D). All these favorable residue-residue contacts make ADWX-1 a highly potent inhibitor for Kv1.3 channels. In addition, His404 in Kv1.3 affects the selectivity of toxin peptides among different Kv channels (34). The interaction of Thr28 and His33 in ADWX-1 with His404 in Kv1.3 likely contributed to the specificity of ADWX-1 among Kv1.1 (with a Tyr residue at the position 404), Kv1.2 (with a Val) and Kv1.3 channels.

Natural venoms provide many candidate peptides for designing potential drug leads targeting the Kv1.3 channels. However, present strategies for improving the selectivity and activity of these candidate peptides still remain a significant challenge. Our success with the design of ADWX-1 suggests that rational design based on the model of the peptide-channel complex will accelerate the development of diagnostic and therapeutic agents using venom peptides as scaffolds.

References


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FIGURE LEGENDS

FIGURE 1. Design strategy of ADWX-1 based on BmKTX. A, The sequence alignment for BmKTX and ADWX-1. Shaded residues (in light pink) are different between BmKTX and ADWX-1. The asterisks indicate residues changed in later functional studies. B and C, Models for BmKTX (B) and ADWX-1 (C) towards Kv1.3. The mutated sites and a conserved pore-blocking residue (Lys26) are labeled.

FIGURE 2. Purification and characterization of ADWX-1. A, Tricine/SDS-PAGE analysis of purified ADWX-1 peptide. Lane 1, molecular mass markers. Lane 2, purified GST fusion protein after affinity chromatography and desalting. Lane 3, fusion protein cleaved by enterokinase. Lane 4, purified ADWX-1 peptide by reversed-phase HPLC. B, Purification of ADWX-1 by HPLC on a C18 column. The fractions containing ADWX-1 is indicated by an arrow. C, Mass spectra of ADWX-1 measured by MALDI-TOF-MS. Measured value is 4072.8 Da, and the calculated one is 4071.1 Da.

FIGURE 3. Bioactivity of ADWX-1 on Kv channels. A, Current traces in the absence (control) or presence of 10 pM ADWX-1 on Kv1.3 channels. B, Current traces in the absence (control) or presence of 1 nM ADWX-1 on Kv1.1 channels. C, Normalized current inhibition by various concentrations of ADWX-1 on Kv1.1, Kv1.2 and Kv1.3 channels. Data represent mean ± S.E. of at least three experiments.

FIGURE 4. Circular dichroism spectra of ADWX-1 and its mutants. A, the circular dichroism spectra of recombinant BmKTX (PDB code: 1BKT), ADWX-1, ADWX-1-R11A, ADWX-1-T28 and ADWX-1-H33A peptides. B, the circular dichroism spectra of ADWX-1, ADWX-1-R23A, ADWX-1-F24A, ADWX-1-K26A, ADWX-1-N29A and ADWX-1-T35A peptides. The measurement was carried out in the UV range of 250-190 nm at 25°C in water on a Jasco-810 spectropolarimeter with a concentration of 0.2-0.4 mg/ml.

FIGURE 5. Effects of the ADWX-1 mutants on Kv1.3. A-H, Representative current traces of Kv1.3 showed the block of currents by ADWX-1 mutants: (A) 300 pM ADWX-1-R11A, (B) 100 pM ADWX-1-T28A, (C) 100 pM ADWX-1-H33A, (D) 10 nM ADWX-1-R23A, (E) 10 nM ADWX-1-F24A, (F) 1 nM ADWX-1-K26A, (G) 1 nM ADWX-1-N29A, and (H) 100 pM ADWX-1-T35A. I and J, Concentration-dependent inhibition of Kv1.3 channels by the mutants of ADWX-1: (I) ADWX-1-R11A, ADWX-1-T28A and ADWX-1-H33A; (J) ADWX-1-R23A, ADWX-1-F24A, ADWX-1-K26A, ADWX-1-N29A and ADWX-1-T35A. The IC50 values are listed in Table 1.

FIGURE 6. Computational simulation of the ADWX-1-Kv1.3 complex. A, Root mean square deviations (Å) of the α-carbons of the ADWX-1-Kv1.3 complex fitting with the starting conformation during the 7 ns MD simulations. B, Comparisons of the calculated and experimental effects for the eight ADWX-1 mutants. The calculated data are normalized values of ΔΔGbinding (24,28), whereas the experimental data are obtained as kBTln[IC50(mutant)/IC50(wt)]. C, An overall view of the final ADWX-1-Kv1.3 complex represented as ribbon structures.
The main mutation sites of ADWX-1 are labeled. The four subunits of Kv1.3 are distinguished by color. 

**D.** Molecular surface rendering of the ADWX-1-Kv1.3 complex. Basic residues are colored blue and acidic residues in red. Only two subunits of Kv1.3 are shown for clarity.

**FIGURE 7. Interaction of ADWX-1 with Kv1.3.** **A,** Arg$^{23}$ and Lys$^{26}$ of ADWX-1 are surrounded by residues from Kv1.3 within 4.0 Å. **B,** Phe$^{24}$ of ADWX-1 is “pocketed” by several residues in Kv1.3, which are represented as molecular surfaces. **C** and **D,** Thr$^{28}$, Arg$^{11}$ and His$^{33}$ are surrounded by residues from Kv1.3 pore and turret regions, respectively.
Figure 1

A

BmKTX: VGINVKCKHSQCLPKCDAAGMFGLCINCNGKCDCTPK
ADWX-1: VGINVKCKHSQCLPKCDAAGMFGLCINCNGKCHCTPK

B

EADDPPSSGFNSIPD-386
Turret

G11
D33
I28
K26

C

DMHPVT-407
S6-filter linker

R11
K26
T28
H33
Figure 2

A

B

C
Figure 3

A

Kv1.3

Control

10 pM ADWX-1

2 nA

50 ms

B

Kv1.1

Control

1 nM ADWX-1

2 nA

50 ms

C

Concentrations of toxins (pM)

Normalized currents

ADWX-1 on Kv1.1

ADWX-1 on Kv1.2

ADWX-1 on Kv1.3
Figure 4

A

B

molar ellipticity
10^4 deg cm^2 dmol^-1

Wave Length (nm)

190 200 210 220 230 240 250

BmKTX
ADWX-1
ADWX-1-R11A
ADWX-1-T28A
ADWX-1-H33A

molar ellipticity
10^4 deg cm^2 dmol^-1

Wave Length (nm)

190 200 210 220 230 240 250

ADWX-1
ADWX-1-R23A
ADWX-1-F24A
ADWX-1-K28A
ADWX-1-N29A
ADWX-1-T35A
Figure 5

A, B, C, D: Graphs showing the effects of different concentrations of toxins on normalized currents.

E, F, G, H: Graphs showing the effects of different toxins on normalized currents.

I, J: Graphs showing the relationship between concentrations of toxins and normalized currents.
Figure 6
Figure 7
Table 1. Effect of ADWX-1 and its mutants in blocking Kv1.3 channels

<table>
<thead>
<tr>
<th>ADWX-1 mutant</th>
<th>$IC_{50}$ (pM)</th>
<th>n</th>
<th>$IC_{50}$(mut)/ $IC_{50}$(wt)</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>1.89±0.53</td>
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Each value represents mean ± S.E. n, the number of separate experiments.
Structural basis of a potent peptide inhibitor designed for Kv1.3 channel, a therapeutic target of autoimmune disease

Song Han, Hong Yi, Shi-Jin Yin, Zong-Yun Chen, Hui Liu, Zhi-Jian Cao, Ying-Liang Wu and Wen-Xin Li

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