Structural Basis of a Potent Peptide Inhibitor Designed for Kv1.3 Channel, a Therapeutic Target of Autoimmune Disease*

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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 that 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 with 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.

In human T cells, the Kv1.3 potassium channel plays an essential role in regulating the resting membrane potential and Ca2+ signaling(1–3). When the autoimmune disease-related CCR7– effector memory T cells are activated in the inflammation sites, expression of the Kv1.3 channel in these cells significantly increases from ~250 channels to about 1,500 ~ 2,000 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 diseases. Moreover, the therapeutic efficacy of Kv1.3 channel blocking was validated by in vitro assays and by in vivo animal models of T cell-mediated autoimmune diseases such as multiple sclerosis, type-1 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 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 (IC50 69 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 (IC50 1.89 pm), showing a 100-fold increase in inhibitory activity compared with the native BmKTX peptide. Furthermore, the ADWX-1 peptide displayed specificity

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4 The abbreviations used are: ADWX, autoimmune drug from Wenxin group; BSA, bovine serum albumin; PDB, Protein Data Bank; GST, glutathione S-transferase; MD, molecular dynamics; wt, wild type; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight-mass spectrometry.
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**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'-GTGAATTCGATGACGATGACGAAAGGGTTGTTAATATGTGAA-3'; Sense primer 2, 5'-GTGATTAACTGGAATGGATTTGCCATTGGTGCATTTACCAAATCTCAT-3'; Antisense primer 1, 5'-GGTACGATGACGATGACGCCATTGGTGCATTTACCAAATCTCAT-3'; Antisense primer 2, 5'-TAGCTCGAGTCCAGCATCTTG-3'; Antisense primer 3, 5'-GGTACGATGACGATGACGCCATTGGTGCATTTACCAAATCTCAT-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. ADWX-1 and its mutant plasmids were digested with EcoRI and XhoI, and the cDNAs were subcloned into pGEX-6P-1. 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.

**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/BamHI sites of pIRE62-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 1–3 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. After being transformed into *Escherichia coli* Rosetta (DE3) cells, cells were cultured at 37 °C in LB medium with ampicillin (100 μg/ml). When the cell density reached an OD of 0.6, 1.0 mM isopropyl thio-β-D-galactoside was added to induce the expression at 25 °C. Cells were harvested after 4 h 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-
Data analyses were performed with IgorPro (WaveMetrics, Lake Oswego, OR), and IC$_{50}$ values were deduced by fitting a modified Hill equation to the data as shown in Equation 1,

$$\frac{I_{\text{toxin}}}{I_{\text{control}}} = \frac{1}{1 + \left(\frac{[\text{toxin peptide}]}{IC_{50}}\right)^{H1}}$$

(Eq. 1)

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., $n$ is 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 SWISS-MODEL 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-step energy minimization and a 500-ps unrestrained molecular dynamics (MD) 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 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 simulation to introduce enough flexibility for both the channel and the toxin peptide. The 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)/Å$^2$ for restraining all heavy atoms and then gradually reduced it to 0.02 (kcal/mol)/Å$^2$ 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, it was selected as a template for designing a new analog with higher potency in this work. 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 $\sim$5 Å between the two Ca atoms from Asp$^{33}$ and Lys$^{26}$ residues (Fig. 1B). Such a close distance impedes the side chain of the conserved Lys$^{26}$ to block channel pores because of 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 for a conserved histidine residue among $\alpha$-KTx3 scorpion toxins (31) (Fig. 1, A–C). Second, we strengthened the polar interaction...
between polar residues from the peptide and Kv1.3. The side chain of Lys26 is expected to insert into the channel pore. We replaced the adjacent hydrophobic Ile28 of BmKTX with the polar threonine residue for a more favorable interaction (Fig. 1, A–C).

Third, we introduced a positively charged residue in the beginning of the H9251-helix domain. There are four negatively charged residues in the turret of Kv1.3. Arg11 was introduced for a potential salt-bridge interaction between K1.3 and the toxin peptide (Fig. 1, C). With these three changes to improve the potency of BmKTX, a new ADWX-1 peptide was rationally designed (Fig. 1, A).

**Pharmacological Activities of the 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 the ADWX-1 peptide was collected and subsequently identified by mass spectrometry analysis (Fig. 2C). The molecular weight of ADWX-1 was 4,072.8, which is close to the calculated molecular mass of 4071.1 Da.

Next we investigated the effect of recombinant ADWX-1 peptide on the Kv1.3 channel together with Kv1.1 and Kv1.2 channels. All channels were expressed in the transiently transfected HEK293 cells. Fig. 3 (A and B) shows the effects of the 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 toward Kv1.3 channels with an IC_{50} of 1.89 ± 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. 3, B 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 [Δ^{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 to that of ADWX-1 (Fig. 4A). Although three residues of BmKTX were changed, the struc-
ture of the resulting ADWX-1 peptide was almost the same as that of the 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 lists the average IC50 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 (Arg11, Thr28, and His33) altered the binding affinity by 178-, 30-, and 40-fold, respectively (Table 1 and 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 Arg23 and Phe24 mutants exhibited the most significant drop of ADWX-1 affinity on Kv1.3 channels for over 3,800- and 2,100-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 Arg23 and Phe24 were the major functional residues in the high affinity binding of ADWX-1 with Kv1.3. Taking into account the similarity of ADWX-1 and other α-KTX toxin peptides (31, 33), an alanine substitution of the conserved pore-blocking residue Lys26 was also produced (31). This mutation resulted in a dramatic loss of potency, with the IC50 value (IC50, 0.96 ± 0.72 nM) decreasing over 510-fold compared with that of ADWX-1 (Table 1 and Fig. 5, J). Another important channel-interacting site was Asn29, and the ADWX-1-N29A mutant was 240-fold less active than ADWX-1 in blocking Kv1.3 channels, with an IC50 of 454.4 ± 40.7 pM (Table 1 and Fig. 5, J). In addition, the modification of Thr35 caused a negligible decrease in the mutant affinity for Kv1.3 channels (IC50, 28.0 ± 19.2 pM) (Table 1 and Fig. 5, J). All these results showed that just like the classical Kv channel toxin peptides, the positively charged residues of ADWX-1 played an essential role in mediating the recognition process toward Kv1.3 channels.

### Molecular Mechanism of ADWX-1 Recognizing the 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

**TABLE 1**

| ADWX-1 mutant | IC50 (pM) | n | IC50(mut)/IC50(wt) |
|---------------|----------|---|-------------------|
| Wild type     | 1.89 ± 0.53 | 13 | 1.0 |
| R11A          | 336.3 ± 49.1 | 8 | 177.9 |
| R23A          | 7344.2 ± 3950 | 4 | 3885.8 |
| F24A          | 4013.4 ± 857 | 4 | 2123.4 |
| K26A          | 967.4 ± 726 | 4 | 511.8 |
| T28A          | 58.2 ± 6.5 | 4 | 30.8 |
| N29A          | 454.4 ± 40.7 | 6 | 240.4 |
| H33A          | 77.2 ± 37.4 | 3 | 40.8 |
| T35A          | 28.0 ± 19.2 | 4 | 14.8 |

* Each value represents the mean ± S.E.
* n, number of separate experiments.
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 a 500-ps 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-scan- ning 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. 6, B and C). In Fig. 6B, the calculated results are normalized values of $\Delta \Delta G_{\text{binding}}$ from MM-PBSA analysis as we previously described (24, 28). Experimental results are obtained as $k_bT \ln(\text{IC}_{50(\text{mutant})}/\text{IC}_{50(\text{wt})})$. Fig. 6C showed functional residues of ADWX-1 located in the peptide-Kv1.3 channel interface. The correlation between the experimental and computational data indicates that 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 explained. The most functionally essential Arg23 residue formed a considerably strong electrostatic interaction with the Asp386 residue in Kv1.3 channel (Figs. 6D and 7A), and gave the biggest $\Delta \Delta G_{\text{binding}}$ value of 5.11 kcal/mol among all mutants. The conserved pore-blocking Lys26 residue was found to plug its side chain into channel selectivity filter during the whole simulation as predicted (Figs. 6D and 7A), and the $\Delta \Delta G_{\text{binding}}$ value of 3.58 kcal/mol correlated well with the mutation effect. Furthermore, the Phe24 residue was surrounded by a “pocket” formed by many nonpolar and polar residues (Ser379, Gly380, Phe381, Asp402, Met403, and His404) 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 Phe24 with the alanine residue. Thr28, Asn29, and His33 residues located right above the filter-S6 linker (Fig. 7, C and D), thus were important for the peptide-channel interaction. Arg11 also interacted with the negatively charged Asp386 residue in Kv1.3 channels (Fig. 7D). The binding affinity of ADWX-1 to Kv1.3 was affected little by the Thr35 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...
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![Diagram of Kv1.3 channels and toxin peptides]

high potency and selectivity toward 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 Asp\(^{33}\) 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 Lys\(^{26}\) 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 Asp\(^{33}\), Gly\(^{11}\), and Ile\(^{28}\) of the parent BmKTX into His\(^{33}\), Arg\(^{11}\), and Thr\(^{28}\) (see Fig. 1). The resulting ADWX-1 peptide could potently block Kv1.3 with an IC\(_{50}\) 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 100-fold) and over Kv1.2 (by 5000-fold) (see Fig. 1). The interaction of Thr\(^{28}\) and His\(^{33}\) in ADWX-1 with His\(^{404}\) in Kv1.3 is 100-fold more potent than BmKTX (20). Arg\(^{11}\) of ADWX-1 affects the selectivity of toxin peptides among different Kv channels (34). The interaction of Thr\(^{28}\) and His\(^{33}\) in ADWX-1 with His\(^{404}\) 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.

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