Artificial pore blocker acts specifically on voltage-gated potassium channel isoform Kv1.6

Among voltage-gated potassium channel (Kv) isoforms, Kv1.6 is one of the most widespread in the nervous system. However, there are little data concerning its physiological significance, in part due to the scarcity of specific ligands. The known high-affinity ligands of Kv1.6 lack selectivity, and conversely, its selective ligands show low affinity. Here, we present a designer peptide with both high affinity and selectivity to Kv1.6. Previously, we have demonstrated that Kv isoform-selective peptides can be constructed based on the simplistic α-hairpin scaffold, and we obtained a number of artificial Tk-hefu peptides showing selective blockage of Kv1.3 in the submicromolar range. We have now proposed amino acid substitutions to enhance their activity. As a result, we have been able to produce Tk-hefu-11 that shows an EC50 of ≈70 nM against Kv1.3. Quite surprisingly, Tk-hefu-11 turns out to block Kv1.6 with even higher potency, presenting an EC50 of ≈10 nM. Furthermore, we have solved the peptide structure and used molecular dynamics to investigate the determinants of selective interactions between artificial α-hairpins and Kv channels to explain the dramatic increase in Kv1.6 affinity. Since Kv1.3 is not highly expressed in the nervous system, we hope that Tk-hefu-11 will be useful in studies of Kv1.6 and its functions.

Ion channels play a crucial role in physiology, underlying signal transduction in excitable cells, muscle contraction, regulation of cell volume, release of hormones and neurotransmitters, etc. Potassium (K⁺) channels are the most abundant in humans with ~80 genes encoding the pore-forming α-subunits. Voltage-gated K⁺ channels (Kᵥ) have six transmembrane segments (S1–S6) in their α-subunits, and functional channels contain four identical or different α-subunits. The first four transmembrane segments (S1–S4) of each α-subunit form a voltage-sensing domain, whereas the fifth and sixth transmembrane segments (S5 and S6) from all four subunits come together to form the centrally located pore domain. These transmembrane segments are joined by a so-called re-entrant P-loop, which contains a short pore (P) helix and the selectivity filter region. The major function of Kv channels is to provide the repolarization stage of the action potential (1).

Kv1.6 is a member of the Shaker-related subfamily of voltage-gated K⁺ channels (Kᵥ-1) encoded by the KCNA6 gene in humans or Kcnal6 in mice or rats. This isoform is widely expressed in the nervous system representing one of the major K⁺ channels found in the brain and peripheral neurons (2). Kv1.6 is also expressed in muscles and nonelectroexcitable cells and tissues including the ophthalmic artery (3) and nephrons (4). Additionally, there is evidence that this isoform contributes to forming heteromeric K⁺ channels with other Kv1 subunits (5, 6). Changes in KCNA6 expression are linked to some pathologies, for instance, amyotrophic lateral sclerosis (7). However, Kv1.6 is not well studied compared to some other members of the Kv1 subfamily such as Kv1.1–1.3. Ligands with high affinity and selectivity are needed to reveal the blind spots of Kv1.6 physiological functions and its role in diseases. Unfortunately, among the available repertoire, ligands with high (nanomolar) affinity show little selectivity, and conversely, ligands with higher selectivity are active at high concentrations (hundreds of nanomoles or micromoles) (8–13).

Previously, we have developed a computational approach to the design of selective peptides that block KvV channels (14–16). In particular, we noticed that plant α-hairpins are structurally similar to some α-helical cone snail and scorpion toxins. The similarity of Tk-APM-X2 from the wheat Triticum kiharae to k-hefutoxin-1 from the scorpion Heterometrus fulvipes allowed us to design and obtain Tk-hefu, an artificial blocker of Kv1.3 (17). We then built computer models of Tk-hefu with Kv1.3 and other channels, applied protein surface topology to assess the binding interfaces and molecular dynamics (MD) to analyze the interactions, and proposed amino acid replacements to enhance the affinity (18). As a result, we produced Tk-hefu-10, which is selective to and active against Kv1.3 in the submicromolar range (16).

Based on our previous experiments, here we analyzed the relations between amino acid substitutions and the affinity of
Tk-hefu derivatives to KV1.3. We chose to retract some substitutions that we had introduced in Tk-hefu-7 and 9 to further increase the activity of the new peptide Tk-hefu-11 to KV1.3. As expected, we obtained a 2-fold increase in the affinity to that isoform. Surprisingly, we also observed a dramatic increase in affinity toward KV1.6. With an EC50 of ≈ 10 nM on KV1.6, Tk-hefu-11 is > 500-fold more active on this isoform compared to any other Tk-hefu peptide. This new KV1.6 ligand combines both high activity and high selectivity, outperforming all other known ligands to date, and is therefore a valuable tool for neurobiology.

Results

Design of new Tk-hefu derivative

Our previous works (16–18) aimed at crafting a high-affinity KV1.3 pore blocker and culminated in Tk-hefu-10 production. On the first stage, we created Tk-hefu-1 based on 3D structure similarity between α-hairpins and κ-hefutoxin-1 (19) (Table 1) (17). Next, we introduced one amino acid replacement and produced Tk-hefu-2 with ten times greater potency (16). Although Tk-hefu-10 features a dramatically improved affinity to KV1.3 (IC50 = 150 nM), close inspection of the activity of all intermediate Tk-hefu derivatives (Table 1) indicates that some amino acid substitutions actually had a neutral or even negative effect. In particular, when moving from Tk-hefu-6 to 7, we observed a decrease in activity. And when we went on from Tk-hefu-8 to 9, the activity was not affected. Apparently, the D2Q, Y6K, and R7Q mutations were either neutral or reduced the affinity. Our idea was then to reverse those substitutions and produce a new Tk-hefu derivative with enhanced activity. This new derivative was named Tk-hefu-11.

Production of Tk-hefu-11

To obtain Tk-hefu-11 (Table 1), we used the Escherichia coli SHuffle T7 Express strain as an expression system. A synthetic gene encoding the derivative was cloned into the pET-32b expression vector, and thioredoxin (Trx) was used as the fusion partner to ensure a high yield of the disulfide-containing peptide. The target peptide was produced as a result of fusion protein cleavage by enterokinase followed by separation using reversed-phase HPLC and identification by MALDI mass spectrometry (MS) (Fig. 1). The final yield of Tk-hefu-11 was ~ 6 mg per 1 l of bacterial culture.

Tk-hefu-11 activity

Tk-hefu-11 was tested against four KV isoforms. The calculated EC50 values on hKV1.3 and rKV1.6 were 70.4 ± 2.9 nM and 10.0 ± 0.5 nM, respectively, while 1 μM Tk-hefu-11 inhibited only 41.2 ± 2.9% of hKV1.1 and 50.1 ± 0.8% of hKV1.2-mediated currents. At concentrations higher than 2 μM, lysis of oocytes was detected. These data indicate a stronger inhibition of KV1.6 compared to other KV isoforms tested, making Tk-hefu-11 a unique peptide with an ability to inhibit this channel in the nanomolar range and a selectivity factor of > 7 (Fig. 2, A–C and Table 1). To determine if Tk-hefu-11 inhibits Kv1 channels as a pore blocker or gating modifier, we used two approaches. In one, Tk-hefu-11 was tested on mutants of KV1.3 previously described by us (20) (Fig. 2, D–G). The constructs named mut1 (423DPTSG427 → 423ERDSQP427), mut2 (451HPV453 → 451VPT453), and mut3 (425TSG427 → 425DSQ427) represent hKV1.3 channels that harbor residues from hKV1.2 in the S5-P and P-S6 segments (residue numbering is according to UniProt accession number P22001). Significant differences in the blocking potency of Tk-hefu-11 were observed for all constructs (Fig. 2H), suggesting that the peptide interacts with the channel pore. Of note, mut3 comprises a small fragment of mut1 (underlined in its sequence) with only two replacements. A slightly higher level of current inhibition by Tk-hefu-11 for mut3 compared to wild type KV1.3 evidences that these replacements play a minor but beneficial role in the binding. Conversely, the dramatically reduced inhibitory effect observed for mut1 suggests the importance of D424, P424, or S429 for the interaction of the peptide with KV1.3.

Table 1

Amino acid sequences of Tk-hefu derivatives and their activity against KV channels

| Name       | Sequence* | Potassium channels |
|------------|-----------|--------------------|
|            |           | KV1.1        | KV1.2        | KV1.3        | KV1.6        |
| x-KVhefxin-1| ·GHAACYRCW-EGNDEETCKECK-· | 2 a | 150,000 b | 40,000 c | 4 d |
| Tk-AMP-X2   | ADDRCRAMCQYRDREKKOMGGCRYG | -   | 40,000/8 e | 36,000 ± 2800 f | 40,000/7 |
| Tk-hefu-1   | ADDRCRAMCQYRDREKKQQQKCGYG | -   | 40,000/12 | 200 ± 400   | 40,000/17 |
| Tk-hefu-2   | ADDRCRAMCQYRDREKKQQQKCGYG | -   | 5000/75   | 5000/69    | 700 ± 100   | 5000/58 |
| Tk-hefu-3   | ADDRCRAMCQYRDREKKQQQKCGYG | 5000/62 | 5000/64 | 700/39 | 5000/68 |
| Tk-hefu-4   | ADDRCRAMCQYRDREKKQQQKCGYG | 5000/72 | 5000/60 | 700/59 | 5000/50 |
| Tk-hefu-5   | ADDRCRAMCQYRDREKKQQQKCGYG | 5000/79 | 5000/64 | 700/62 | 5000/51 |
| Tk-hefu-6   | ADDRCRAMCQYRDREKKQQQKCGYG | 5000/54 | 5000 ± 200 | 152.7 ± 34.2 | 5000/30 |
| Tk-hefu-7   | ADDRCRAMCQYRDREKKQQQKCGYG | 1000/41  | 1000/50  | 70.4 ± 2.9 g | 10.0 ± 0.5 h |

a Gray shading highlights cysteine residues that form disulfide bonds; amino acid substitutions introduced in Tk-AMP-X2 to attain Tk-hefu peptides are in bold.

b, no activity at 20 μM.

c, KV values as reported in (19).
d, Empty box means that the activity was not tested.
e, “X/Y” means that at concentration of X nM, Y percent of block was observed.
f, X ± Y represent IC50 values in nM.
g, These data represent EC50 values in nM.
K\textsubscript{V}1.3 compared to the corresponding residues in K\textsubscript{V}1.2. In the other approach, we evaluated the conductance-voltage relationship for K\textsubscript{V}1.6 (Fig. 2I). No significant difference in the V\textsubscript{1/2} values was observed: 7.7 ± 0.8 mV and 12.9 ± 0.9 mV for control and in the presence of 10 nM Tk-hefu-11, respectively. For a gating modifier, we would expect a profound shift in the voltage dependence of activation (21, 22). Together, these data indicate that Tk-hefu-11 interacts with the pore of K\textsubscript{V} channels.

Because we observed oocyte lysis at higher Tk-hefu-11 concentrations, we decided to test its antimicrobial activity, which is a hallmark of cytolytic peptides (23). We did not detect any activity at concentrations up to 20 μM against neither Gram-positive nor Gram-negative bacteria. In addition, the peptide did not exhibit any hemolytic or cytolytic activity at the studied concentrations up to 20 μM.

**Spatial structure of Tk-hefu-11**

The 3D structure of the peptide was solved in water (Fig. 3). For this, a \textsuperscript{15}N-labeled analog of the peptide (\textsuperscript{15}N-Tk-hefu-11) was prepared by using a culture medium containing only \textsuperscript{15}N as the source of nitrogen. Hundred structures were calculated using the torsion angle restraints, upper and lower NOE-based distance restraints, as well as hydrogen bond and disulfide bond restraints (data are shown in Table S2). The resulting set of ten NMR structures is characterized by a low RMSD value for backbone atoms (0.39 ± 0.15) and insignificant restraint violations, suggesting that the structure is well defined by the experimental data. Chemical shifts and coordinates are deposited to the Protein Data Bank (PDB) and Biological Magnetic Resonance Data Bank (BMRB) databases under the accession codes 7QXJ and 34703, respectively.

The solution structure of Tk-hefu-11 consists of two anti-parallel α-helices (residues K3–Q10 and R15–G24) joined by a short loop (Figs. 3 and S1), corresponding to the α-hairpin fold that we and others described in many plant peptides (24, 25). The structure is stabilized by two disulfide bridges (C5–C25 and C9–C21) and nine hydrogen bonds formed according to α-helical conformation (Fig. S2). Detailed analysis of the obtained structure reveals that Y6 is likely to form a cation–π contact with K3 and/or K22. We notice that Tk-hefu-11 has a small hydrophobic core formed by the disulfide bonds surrounded by M8 and Y12 on one side and A1, Y6, and Y27 on the other side. Tk-hefu-11 also reveals an anisotropic distribution of electrostatic parameters; a pronounced positively charged face is formed by arginine and lysine residues, and an uncharged and relatively apolar face is formed by the side chains of cystine, methionine, and tyrosine residues.

**Molecular modeling explains the selectivity of Tk-hefu-11**

To uncover the molecular determinants underlying the differences in Tk-hefu-10 and 11 activities on K\textsubscript{V} channels, we performed a computational study of the molecular complexes of these peptides with hK\textsubscript{V}1.3 and rK\textsubscript{V}1.6 (Fig. 4). We took advantage of the solved NMR structure of Tk-hefu-11 and built a homology model of Tk-hefu-10 based on it. As mentioned in Experimental procedures, we aligned K/Y6 and K22 of Tk-hefu-10/11 onto the classical dyad residues Y36 and K27 in ChTx complexed with K\textsubscript{V}1.2 pore (26) to get a starting conformation. MD trajectories were calculated, and computational analysis of contact surfaces and energy contributions of residues to complex formation was carried out.

In line with the findings of our recent study of Tk-hefu derivatives (16), the analysis of residual contributions to intermolecular interaction energy yielded expectable results (Fig. 5). We note that the calculated absolute energy values have little physical meaning and should only be considered in comparison with other calculated values. In going from Tk-hefu-10 to 11, the substitution of a neutral residue by a negatively charged one (Q2D), as well as the substitution of a positively charged residue by a neutral one (K6Y) provides a negative contribution to the channel binding (rise in complex energy values). On the contrary, the Q7R substitution results in a positive contribution (drop in energy values). These tendencies are observed in complexes of both K\textsubscript{V}1.3 and 1.6.
Analysis of MD trajectories revealed that in the complexes rKv1.6–Tk-hefu-11, rKv1.6–Tk-hefu-10, and hKv1.3–Tk-hefu-11, the peptides change their positions from the initial state considerably, while the position of Tk-hefu-10 in complex with hKv1.3 changes less significantly (Fig. 6). In the complexes with Kv1.6, the flexibility of the N terminus of the peptides is restrained by hydrogen bonding and salt bridge formation between their N-terminal amino group and the D404 side chain carboxyl group of the channel. The positively charged side chain of R11 tends to approach the rKv1.6 channel-specific negatively charged motif 399EADDVD404 located in the S5-P loop (Fig. 7, A–C). Thus, Tk-hefu-11 rotates slightly around the Kv1.6 pore axis during MD, which results in multiple polar contacts: R11 forms salt bridges with E399 and D404, hydrogen bonds with S405 and Y430, and a cation-π contact with F407. Moreover, the reorientation of Tk-hefu-11 allows R7 to reach D412 and D428 side chains (and form two salt bridges), as well as the side chains of W415 and Y430 of a neighboring channel subunit (and form two cation-π contacts), which stabilizes the complex greatly (Fig. 7B and Table S3). A shorter and uncharged side chain of Q7 in Tk-hefu-10 cannot reach those channel residues, so its position in the complex is less stabilized, and R11 forms only one salt bridge with D404 in Kv1.6 (Fig. 7C).

Notably, in the complex with Kv1.6, residue D2 of Tk-hefu-11 tends to repulse from D404 (Fig. 7B). However, that same residue D2 also repulses the C-terminal carboxyl group from the N terminus of the peptide. In Tk-hefu-11, the C terminus interacts with K23 forming an intramolecular salt bridge that stabilizes the secondary structure.
of the peptide. In Tk-hefu-10 that has Q2 instead of D2, the N- and C-terminal groups interact. This causes peptide secondary structure distortion and brings the C-terminal carboxyl group in close proximity to D404 of Kv1.6, destabilizing the complex (Fig. 7, B and C).

The channel-specific negatively charged motif 420EADDPT425 in the S5-P loop of hKv1.3 has a lower formal charge compared to the aforementioned 399EADDVD404 in rKv1.6 because D404 is substituted by T425 in the former (Fig. 7A). Besides, the side chain of T425 is not long enough to reach and fix the position of the N-terminal peptide residue in a manner described previously. Therefore, the electrostatic attraction from the S5-P loop affects the peptide position to a lesser degree in case of Kv1.3. Tk-hefu-11 in the complex with Kv1.3 moves slightly during MD, and R7 forms a salt bridge with D433, a hydrogen bond with G427, and a cation-π contact with F428 (Fig. 7D). Such a shift does not happen in the complex with Tk-hefu-10 because Q7 is not charged. Instead, it forms a single hydrogen bond with D449 of a neighboring channel subunit. Together with two hydrogen bonds formed by Q2 with D433 and D449, this interaction forces Tk-hefu-10 to disrupt its secondary structure (Fig. 7E).

Analysis of the interactions during MD has shown that Tk-hefu-11 in the complexes with Kv1.3 and 1.6 forms more contacts with the channels than Tk-hefu-10 (Tables 2 and S3), which is in agreement with the results of electrophysiological
recordings. Despite Tk-hefu-10 being involved in a decent number of medium-lived and short-lived contacts (19 and 11, respectively), it forms only 16 long-lived specific interactions with Kv1.6. This indicates that Tk-hefu-10 forms a weak complex with Kv1.6, which is consistent with our experimental data.

Our analysis of the intermolecular contacts during MD shows that the substitutions introduced in Tk-hefu-11 affect the binding to the channels in a complex manner. On the one hand, when moving from Tk-hefu-10 to 11, the Q2D substitution results in a loss of interactions with Kv1.3 due to electrostatic repulsion from the side chains of D433 and D449 (Fig. 7). However, neither Q2 nor D2 in Tk-hefu-10 or 11 forms specific contacts (H-bonds, salt bridges, cation-π, or stacking) with Kv1.6. On the other hand, Tk-hefu-10 residue K6 is involved in a salt bridge, three hydrogen bonds, and two cation-π contacts in the complex with Kv1.3, while Tk-hefu-11 residue Y6 forms just one hydrogen bond and one stacking interaction. In the complex with Kv1.3, that same residue K6 in Tk-hefu-10 is involved in two hydrogen bonds and two cation-π contacts, while Y6 of Tk-hefu-11 forms just one hydrogen bond (Table S2). The substitution Q7R affects contact distribution in the complexes even more prominently. According to our analysis, together with the N terminus and R11, R7 provides the high stability of the Tk-hefu-11 complex with Kv1.6 and 1.3.

Discussion

Quite unexpectedly, Tk-hefu-11, which was designed to target Kv1.3, showed pronounced affinity and selectivity to Kv1.6. Our molecular modeling suggests that the observed Tk-hefu-11 activity is due to (i) direct contacts between the channel and those residues that differ from other Tk-hefu peptides and (ii) an indirect effect of the substitutions.

The 3D structure of Tk-hefu-11 established here (Fig. 3) diverged from the initial structure of Tk-hefu-1 reported previously (PDB ID: 5LM0) (18). The major differences are the angle between the α-helices and the positioning of Y6. While in Tk-hefu-1 the α-helices are more X-crossed with the interhelix angle of ≈160°, Tk-hefu-11 is more planar with this angle being ≈−170°, that is, the α-helices twist by 30°. This twist is explained by a cation-π interaction between Y6 and K3 found in Tk-hefu-11. In Tk-hefu-1, on the other hand, D3 cannot engage in such contact, and Y6 flips to interact with Q10 and K18. These unanticipated changes lead to a different mode of peptide interaction with the channels and should be taken into account in molecular modeling.

Previously, we used a rather straightforward approach to suggest amino acid replacements in Tk-hefu derivatives. We analyzed the interaction energy profiles of the models of the complexes and sought to substitute just those residues that made a negative contribution. Here, in case of Tk-hefu-11, we observe that sometimes such negative contribution to the complex formation may actually lead to a conformational reorganization of the peptide, which in turn affects the binding capacity positively. Thus, we note that in the complex with Kv1.6, although residue D2 of Tk-hefu-11 is repulsed from D404, it also repulses the C-terminal carboxyl group of the peptide. This latter repulsion allows Tk-hefu-11 to assume an optimal position in the pore vestibule of the channel. Conversely, residue Q2 of Tk-hefu-10 is not repulsed from D404, but it does not repulse the C terminus, and this peptide does not fit into the channel vestibule.
optimally. This finding may be utilized in further attempts to improve ligand binding to ion channels.

With an EC$_{50}$ of $\approx 10$ nM, Tk-hefu-11 is one of the most potent KV1.6 ligands. Table 3 compares Tk-hefu-11 with other known peptides showing some selectivity to KV1.6 isoform. As mentioned previously and seen from the table, other ligands have either high affinity or high selectivity toward KV1.6, while our peptide combines both properties. The closest competitor is conopeptide Y-PI1 (11), but it is less potent (IC$_{50}$ = 170 nM) and the activity against KV1.1 is not known.

Previously, using the $\alpha$-hairpinin fold as template, we designed only KV1.3-targeting peptides. Tk-hefu-11 exemplifies the applicability of this fold to obtain selective blockers of other KV channels. Interestingly, Tk-hefu-11 outperforms not only all previously known artificial $\alpha$-hairpins (Table 1) but also natural toxins with the same type of fold (called cysteine-stabilized $\alpha$/$\alpha$ fold or CS$\alpha$/CS$\alpha$) such as $\kappa$-hefutoxin-1 (19) or HelaTx1 (27). In conclusion, we hope that Tk-hefu-11 will be useful as a molecular tool to study the function of KV1.6 isoform.

**Figure 7.** Tk-hefu-10 and 11 interactions with KV1.3 and 1.6 predicted by molecular dynamics. A, amino acid sequence alignment of the extracellular pore region of hKV1.3 and rKV1.6 channels. Residue numbering is above each sequence (UniProt accession numbers P22001 and P17659); identical residues are shaded gray. Functional segments of the channels are marked above the alignment. B–E, orientation of peptides and intermolecular contacts in the complexes rKV1.6–Tk-hefu-11 (B), rKV1.6–Tk-hefu-10 (C), hKV1.3–Tk-hefu-11 (D), and hKV1.3–Tk-hefu-10 (E). Orientations of the peptides in the channel pore vestibule are shown in the up-left corner of each panel; the channel-specific motifs$^{399}$EADDVD$^{404}$ and $^{420}$EADDPT$^{425}$ of the SS-P loop in KV1.6/KV1.3 are shown in red; Tk-hefu-10 or 11 are shown in pink; the initial orientation of the peptides is shown in sand yellow. Interacting amino acid residues of the peptides and channels are shown as sticks; residues not involved in the interactions are shown semitransparently. Intermolecular contacts (hydrogen bonds and salt bridges) are shown as dashed yellow lines.
Experimental procedures

Recombinant peptide production

Tk-hefu-11 was produced by a standard protocol that we used in previous work (17, 18). Shortly, a bacterial expression system was used to produce peptides as fusion proteins with the carrier protein Trx (28), containing a site of cleavage by human enteropeptidase light chain (29), and a His-tag for affinity chromatography purification.

Expression vector construction

DNA sequence encoding Tk-hefu-11 was obtained in two steps by PCR using synthetic oligonucleotides as primers (Table S1). On the first step, all four primers were used for five PCR cycles. Then, the reaction mixture was diluted 1000 times and used as a matrix on the second step with flanking primers (F1 and R1). The resulting PCR fragment was cloned into the expression vector pET-32b (Novagen) using the KpnI and BamHI restriction enzymes to produce pET-32b-Tk-hefu-11.

Fusion protein expression and purification

E. coli SHuffle T7 Express cells (New England Biolabs) were transformed using the expression vector pET-32b-Tk-hefu-11 and cultured at 37 °C in LB medium to the mid-log phase.
Expression was then induced by 0.2 mM IPTG. Cells were cultured at room temperature (RT) (24 °C) overnight (16 h) and harvested by centrifugation. The cell pellet was resuspended in 30 ml of 300 mM NaCl, 50 mM Tris–HCl buffer (pH 8.0), and ultrasonicated. The lysate was applied to a HisPur Cobalt Resin (Thermo Fisher Scientific) and the fusion protein Trx-Tk-hefu-11 was purified according to the manufacturer’s protocol.

To produce 15N-labeled Tk-hefu-11, M9 minimal medium with the addition of ISOGRO and 15NH4Cl (both at 1 g/l; Sigma–Aldrich) was used instead of LB medium. E. coli was first cultured in LB, harvested by centrifugation, and resuspended to A600 = 0.1 in M9 medium with ISOGRO and 15NH4Cl. The bacterial culture was then grown at 37 °C for ~4 h to the mid-log phase (A600 = 0.5) using the New Brunswick BioFlo/CelliGen 115 bioreactor (Eppendorf) with intense aeration and agitation. Transgene expression was induced as aforementioned, and after the induction, the culture was incubated at RT (24 °C) overnight (16 h). 15N-Trx-Tk-hefu-11 was purified as the unlabeled protein aforementioned.

Fusion protein cleavage and purification of recombinant peptides

Fusion proteins were dissolved in 50 mM Tris–HCl (pH 8.0) to a concentration of 1 mg/ml. Protein cleavage with human enteropeptidase light chain (1 U of enzyme per 1 mg of substrate) was performed at 37 °C for 60 min, followed by a quick step to 80%) in the presence of 0.1% TFA. The purity of the target peptides was checked by MALDI MS and analytical chromatography on a Vydac C18 column (4.6 × 250 mm; Separations Group) in a linear gradient of acetonitrile concentration (0%–60% in 60 min, followed by a quick step to 80%) in the presence of 0.1% TFA. The purity of the target peptides was checked by MALDI MS and analytical chromatography on a Vydac C18 column (4.6 × 250 mm; Separations Group) in the same acetonitrile gradient.

MS

MALDI MS was performed on an Ultraflex TOF-TOF (Bruker Daltonik) spectrometer as described previously (30). 2,5-Dihydroxybenzoic acid (Sigma–Aldrich) was used as a matrix. Measurements were performed in the reflectron mode with a mass accuracy error not exceeding 100 ppm. Mass spectra were analyzed with the Data Analysis 4.3 and Data Analysis Viewer 4.3 software (Bruker).

Electrophysiology

Expression of Kv channels in Xenopus laevis oocytes

Voltage-gated potassium channels (hKv1.1, hKv1.2, hKv1.3, and rKv1.6) were expressed in X. laevis oocytes. Frogs were kept in compliance with the regulations of the European Union concerning the welfare of laboratory animals as declared in Directive 2010/63/EU. The use of X. laevis oocytes was approved by the Animal Ethics Committee of KU Leuven with the license number P186/2019. Mature female animals were purchased from Nasco (Fort Atkinson) and housed in the Aquatic Facility at KU Leuven. Stage V–VI oocytes were collected from anaesthetized frogs as described previously (31). Human KCNA1 (GenBank accession number: NM_000217) and KCNA2 (NM_004974) genes were cloned in pcDNA3.1(+) vector, which was linearized using EcoRV. Human KCNA3 (NM_002232) was in pCI-neo, which was linearized by Ndel. Rat KcnA6 (X17621) was in pGEM-HE, which was linearized by Ndel. The linearized plasmids were transcribed using the mMESSAGE mMACHINE T7 transcription kit (Ambion). mRNA was injected into oocytes using a microinjector (Drummond Scientific), with a programmed RNA injection volume of 4 to 50 nl depending on channel subtype. The oocytes were incubated in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 2 mM MgCl2, and 5 mM Hepes, pH 7.4), supplemented with 50 mg/l gentamicin sulfate.

Electrophysiological recordings

Electrophysiological measurements were performed at RT (18–22 °C) using the two-electrode voltage-clamp technique. Data were obtained using a GeneClamp 500 amplifier and Clampex 9 software (Molecular Devices). Micropipettes were produced using borosilicate glass capillaries (1B120-6) and drawn in a manual stretcher (World Precision Instruments). The bath and perfusion solutions were ND96.

Whole cell currents were recorded 1 to 2 days after RNA injection. Current and voltage electrodes were filled with 3 M KCl and their resistance was adjusted to 0.7–2.0 MΩ. Currents were sampled at 2 kHz and filtered at 0.5 kHz using a four-pole Bessel low-pass filter. The holding potential was set at −90 mV. Leak subtraction was performed using a P/4 protocol. Kv currents were evoked by a 500 ms depolarization to 0 mV followed by a 500 ms pulse to −50 mV. For conductance–voltage relationship studies, currents were evoked by 5 mV depolarization steps. Potassium conductance was calculated using Ohm’s law:

$$g_K = \frac{I_K}{V_m - V_{rev}},$$

where $I_K$ is the maximal current at the test potential $V_m$, and $V_{rev}$ is the reversal potential. The conductance-voltage data were fitted using the Boltzmann equation:

$$\frac{g_K}{g_{max}} = \left[1 + \frac{e^{(V_m - V_{1/2})/k}}{1}ight]^{-1},$$

where $g_{max}$ represents maximal $g_K$, $V_{1/2}$ is the voltage corresponding to half-maximal conductance, and $k$ is the slope factor.

Different concentrations of the peptides diluted in ND96 were used for the concentration–response assays. These solutions were added to the bath containing the oocyte and
**New artificial Kv1.6-specific pore blocker**

mixed immediately, thereby obtaining the desired final concentrations. Kv currents were then recorded in the presence of the tested peptide, and the data were fitted with the Hill equation:

\[
y = \frac{100}{1 + \left( \frac{[\text{peptide}]}{EC_{50}} \right)^h}
\]

where \(y\) is the amplitude of the compound-induced effect in percent (i.e., the percent of maximal current inhibition for the given compound), \([\text{peptide}]\) is the peptide concentration, \(EC_{50}\) is the half-maximal effective concentration, and \(h\) is the Hill coefficient.

All data were obtained in at least three independent repeats \((n \geq 3)\) using different batches of X. laevis oocytes. Statistical significance was determined using one-way ANOVA with Dunnett's post-test. Experimental values are presented as mean ± SEM. Bar charts present all individual data points and the SD to represent variation.

**Antimicrobial assay**

Determination of antimicrobial activity was performed following a previously described protocol (32). Bacteria (Enterococcus faecalis (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC 29212), E. coli (Migula) Castellani and Chalmers (ATCC 25922), Pseudomonas aeruginosa (Schoeter) Migula (ATCC 27853), and Staphylococcus aureus subsp. aureus Rosenbach (ATCC 29213)) were cultured overnight in LB medium at 37 °C. Determination of the minimal inhibitory concentrations for the peptide was performed using a 2-fold microtiter broth dilution assay in 96-well sterile plates at a final volume of 100 μl. Mid-log phase cultures were diluted to a final concentration of 10^6 colony-forming units/ml. Dried peptide was dissolved in 10 μl of water and added to 90 μl of the bacterium dilution. The peptide, a nontreated control, and a sterility control were tested in triplicate. The microtiter plates were incubated for 24 h at 37 °C; growth inhibition was determined by measuring the absorbance at 620 nm. Minimal inhibitory concentrations are expressed as the lowest concentration of peptide, a nontreated control, and a sterility control were tested in triplicate. The microtiter plates were incubated for 24 h at 37 °C; growth inhibition was determined by measuring the absorbance at 620 nm. Minimal inhibitory concentrations are expressed as the lowest concentration of peptide that caused 100% growth inhibition.

**Cytolytic assay**

Human capillary blood was collected in a tube with heparin (10 units/ml), diluted to \((1.0 \pm 0.1) \times 10^7\) cells/ml with RPMI-1640 medium (PanEco) containing 10% fetal bovine serum (HyClone), and incubated with the peptides (0.6–20 μM, 2-fold dilutions) for 3 h at 37 °C with gentle shaking. Hemoglobin release was measured as described previously (33).

Human lung adenocarcinoma A549 cells were cultured in Dulbecco’s modified Eagle’s medium with addition of 2 mM L-glutamine and 10% fetal bovine serum (complete medium) at 37 °C in humidified atmosphere with 5% CO₂. Cell reseeding was performed twice a week. To study the cytolytic activity of the peptides, cells were seeded in 96-well plates (seeding density of 5 x 10^3 cells per well) 1 day before the experiment. Peptides were added to cells (2.5–20 μM, 2-fold dilutions). The cytotoxicity was estimated after incubation of the cells with peptides for 3 h at 37 °C in humidified atmosphere with 5% CO₂. Cell survival was analyzed by staining cell nuclei with Hoechst 33342 (stains all cells) and propidium iodide (stains dead cells) and examining them with an inverted fluorescence microscope Axio Observer (Zeiss) as described previously (34). At least three independent experiments were carried out \((n \geq 3)\).

**NMR spectroscopy**

All NMR experiments were performed using the Avance 700 MHz spectrometer (Bruker Biospin) at 30 °C. 15N-Tk-hefu-11 was dissolved in H2O/D2O (19:1) and pH was adjusted to 5.5. 1H, 15N chemical shift assignments were obtained by the standard procedure based on 2D TOCSY, 2D NOESY (mixing time of 80 ms), 3D NOESY, 15N-heteronuclear single quantum coherence (HSQC), and 13C-HSQC spectra. After recording the set of spectra, the peptide sample was freeze dried and redissolved in 100% D2O (Acros Organics) to measure the proton-deuterium exchange rates and record the 2D NOESY and DQF correlated spectroscopy spectra.

Spatial structure calculation was performed using the simulated annealing/MD protocol as implemented in the CYANA software package version 3.98.13 (L.A. Systems) (35). Upper interproton distance restraints were obtained by 1/r^6 calibration of NOESY crosspeak intensities. Torsion angle restraints and stereospecific assignments were obtained based on the J-couplings and NOE intensities. Hydrogen bonds were added at the final stage of the structure calculation, if they were formed in at least 70% of the obtained conformers. The disulfide linkages were introduced based on the previously published data for α-hairpinins and their derivatives (17, 18) and were additionally tested when calculating the spatial structure. Visual analysis of the calculated structures and figure drawings were performed using PyMOL (Schrodinger, LLC) and MOLMOL (36).

**Molecular modeling**

Structural model of Tk-hefu-10 was generated in PyMOL using the in silico mutagenesis option based on the NMR solution structure of Tk-hefu-11 in a similar way to the procedure described elsewhere (18). rKv1.6 model was generated analogously to the model of hKv1.3 (14, 18, 20) in MODELLER 9.19 (37) using the rKv1.2 structure (PDB ID: 3LUT) (38) as a template. Complexes of Tk-hefu-10 and 11 with Kv channels were modeled based on the crystal structure of the Kv1.2/2.1 paddle chimera in complex with charybdotoxin (ChTx; PDB ID: 4JTA) (26) analogously to the procedures described in (37, 39). Briefly, the model of each channel was structurally superimposed onto Kv1.2/2.1 in the complex with ChTx to get a model of ChTx complex with that channel.

Then, ChTx was replaced in the complex with Tk-hefu-10 or 11 by structural alignment of the appropriate dyads: K/Y6 and...
K22 of the peptide onto the classical Y36 and K27 in ChTx with minor manual adjustment.

**MD simulations**

The resulting complexes of Tk-hefu-10 and 11 with Kv channels were placed inside a lipid bilayer mimicking neuronal membrane in terms of lipid composition. We used a pre-equilibrated fragment of bilayer (7.0 × 7.0 × 13.5 nm³; 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine/1-palmitoyl-2-oleyl-sn-glycero-3-phosphoethanolamine/cholesterol, POPC:POPE:Ch = 100:50:50 molecules, solvated with 14,172 water molecules) that has been described in detail in our previous works (18, 40, 41); some phospholipid and cholesterol molecules were removed to provide room for the protein. The TIP3P model water (42) and the required number of Na⁺ ions were carried out at 37°C. Brieﬂy, H-bonds were assigned using parameters set from the h-bond utility of GROMACS software (https://www.gromacs.org/) (43) (versions 2018/2020) using the AMBER99SB-ILDN parameters set (44) and 1.5 nm cutoff distance for Lennard-Jones or electrostatic interactions were used during the intermolecular short-range nonbonded interaction energy estimation. The latter is the sum of the Lennard-Jones and electrostatic terms. All drawings of 3D structures were prepared with PyMOL. Graphical representation of interaction energy proﬁles was performed using Python built-in libraries and NumPy package.

**Data availability**

All data are contained within the article and supporting information.

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**Author contributions**—Conceptualization: A.A.V.; Data curation: V.M.T., A.A.V.; Formal Analysis: V.M.T., A.V.F., R.G.E., K.S.M., J.T., A.A.V.; Funding acquisition: S.P., J.T., A.A.V.; Investigation: A.M.G., R.G.E., K.S.M., J.T., A.A.V.; Methodology: V.M.T., A.V.F., R.G.E., K.S.M., J.T., A.A.V.; Project administration: J.T., A.A.V.; Resources: A.V.F., R.G.E., K.S.M., J.T., A.A.V.; Software: VMT, AVF, RGE, KSM, JT, AA.V. Visualization: AMG, EL.P.-J., VM.T., AA.I., KSM; Writing – original draft: AMG, EL.P.-J., VMT, AAI, KSM; Writing – review & editing: R.G.E., A.A.V.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: MD, molecular dynamics; MS, mass spectrometry; PDB, Protein Data Bank.

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