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A BIFUNCTIONAL SEA ANEMONE PEPTIDE WITH KUNITZ TYPE PROTEASE AND POTASSIUM CHANNEL INHIBITING PROPERTIES.

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Abstract Sea anemone venom is a known source of interesting bioactive compounds, including peptide toxins which are invaluable tools for studying structure and function of voltage-gated potassium channels. APEKTx1 is a novel peptide isolated from the sea anemone *Anthopleura elegantissima*, containing 63 amino acids cross-linked by 3 disulfide bridges. Sequence alignment reveals that APEKTx1 is a new member of the type 2 sea anemone peptides targeting voltage-gated potassium channels (K_V's), which also include the kalicludines from *Anemonia sulcata*. Similar to the kalicludines, APEKTx1 shares structural homology with both the basic pancreatic trypsin inhibitor (BPTI), a very potent Kunitz-type protease inhibitor, and dendrotoxins which are powerful blockers of voltage-gated potassium channels. In this study, APEKTx1 has been subjected to a screening on a wide range of 23 ion channels expressed in *Xenopus laevis* oocytes: 13 cloned voltage-gated potassium channels (K_V1.1-K_V1.6, K_V1.1 triple mutant, K_V2.1, K_V3.1, K_V4.2, K_V4.3, hERG, the insect channel *Shaker* IR), 2 cloned hyperpolarization-activated cyclic nucleotide-sensitive cation non-selective channels (HCN1 and HCN2) and 8 cloned voltage-gated sodium channels (Na_V1.2-Na_V1.8 and the insect channel DmNa_V1). Our data shows that APEKTx1 selectively blocks K_V1.1 channels in a very potent manner with an IC₅₀ value of 0.9 nM. Furthermore, we compared the trypsin inhibitory activity of this toxin with BPTI. APEKTx1 inhibits trypsin with a dissociation constant of 124 nM. In conclusion, this study demonstrates that APEKTx1 has the unique feature to combine the dual functionality of a potent and selective blocker of K_V1.1 channels with that of a competitive inhibitor of trypsin.

Keywords *Anthopleura elegantissima* · K_V channel inhibitor · sea anemone toxin · protease inhibitor ·

1. Introduction

Among ion channels, potassium channels are the most diverse class of ion channels and they are key determinants of neuronal excitability. They regulate a variety of cellular processes and functions such as heart rate, neurotransmitter release and nerve conduction, insulin secretion, blood pressure and muscle contraction [1,2]. In the mammalian genome more than 100 genes are encoding the pore forming α subunits and auxiliary β subunits of K⁺ channels. Together with these numerous genes, the presence of spliced variants, association with chaperone and scaffolding molecules and the formation of heteromultimeric channels contribute greatly to the diversity of the K⁺ channel family [2]. This family has been divided into 15 subfamilies of which the voltage gated potassium channels (K_V) represent one of these subfamilies [3]. They determine neuronal intrinsic electrical excitability by repolarization of the membrane after initiation of the action potential [4]. Upon depolarization of the membrane, K_V channels will open within 1 ms, allowing the flux of K⁺ ions driven by their electrochemical gradient [1,2]. Functional K_V channels are formed by a complex of four pore forming α subunits associated with one or

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4 more auxiliary β subunits. Each α subunit consists of six transmembrane spanning segments (S1-S6). The
5 first four transmembrane segments form the voltage sensing domain with S4 serving as the voltage
6 sensor. This S4 segment has been modeled using the crystal structure of the bacterial K_v channel K_vAP
7 and contains 4 conserved Arg residues. These positively charged basic residues display a net movement
8 outwards upon depolarization allowing conformational changes resulting in the opening of the pore [5,6].
9 The pore region of the channels is formed by the S5 and S6 segments which are connected by a re-entrant
10 P-loop. The P-loop contains an extremely well conserved domain among the K_v channels, the so-called
11 signature sequence (-TXGYGD-) and comprises the selectivity filter, the pore helix and the turret region
12 [7]. The auxiliary β subunits of K_v channels ($K_v\beta$) are cytoplasmic proteins, capable of modifying K_v
13 channel biophysical properties.
14

15 Since the beginning of last century sea anemones have been studied with an increasing interest. Although
16 a number of sea anemone toxins have been isolated and characterized, these animals remain poorly
17 studied in comparison with other venomous animals such as scorpions, spiders, cone snails or snakes. Sea
18 anemones are a known pharmacological treasure of biological active compounds acting upon a diverse
19 panel of ion channels such as TRPV1, voltage-gated sodium and potassium channels [8-11]. Of these
20 different toxins those that target sodium channels are the best studied group with more than 100 known
21 toxins [12]. In contrast, no more than 12 potassium channel toxins have been characterized to date. Based
22 on structural differences and activity profile, these potassium channel toxins can be divided into 4
23 structural classes [11,13-15]. Up to date 6 toxins from *A. elegantissima* have been isolated and
24 characterized: APE1-1, APE1-2, APE2-2 and ApC which are type 1 sodium channel toxins; APETx1 a
25 selective modifier of the human ether a go-go related gene K^+ channel (hERG) and APETx2 which
26 specifically inhibits the Acid Sensing Ion Channel (ASIC3) [15-18]. In this work we present the
27 purification, biochemical analysis and electrophysiological characterization of a very potent and selective
28 $K_v1.1$ blocker which represents the newest member of the sea anemone type 2 potassium channel toxins.
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31 32 **2. Material and methods**

33 34 2.1. Toxin purification.

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36 The toxin APEKTx1 was purified as described previously [16,17,19]. The fraction containing APEKTx1
37 was further purified by RP-HPLC with a semi preparative Vydac C18 column (4.6X250 mm). Solvent
38 A was 0.1% TFA in water, solvent B was 0.085% TFA in acetonitrile. A linear gradient from 0 to 80%
39 solution B was developed for 80 min at a flow rate of 1ml/min. A second purification was performed in
40 the same conditions as described above. All final fractions were dried by speed-vac evaporation and
41 stored at -20°C.
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44 45 2.2. Biochemical characterization of toxins.

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47 The mass of the intact toxin was measured by MALDI-TOF (4800 Analyzer, Applied Biosystems, USA)
48 as well as by ESI-QUAD analysis (4000QTRAP, Applied Biosystems, USA).

49 The toxin peptide was subjected to Edman degradation on an Applied Biosystems Procise instrument. The
50 toxin was digested with legumain (R&D Systems, USA). A MALDI TOF/TOF instrument (4800
51 Analyzer, Applied Biosystems, USA) was used for *de novo* sequencing analysis of the obtained
52 fragments. These fragments were subjected to MS/MS analysis and the spectra were manually interpreted.
53

54 55 2.3. Expression of voltage-gated ion channels in *Xenopus laevis* oocytes.

56
57 For the expression of the VGPCs (r $K_v1.1$ (Accession number: NM173095), r $K_v1.2$ (NM012970), h $K_v1.3$
58 (L23499), r $K_v1.4$ (NM012971), r $K_v1.5$ (NM012972), r $K_v1.6$ (NM575671), Shaker IR (CG12348),
59 r $K_v2.1$ (NM013186), h $K_v3.1$ (NM004976), r $K_v4.2$ (NM031730), r $K_v4.3$ (NM031739), hERG
60 (NM000238), mHCN 1 (NM010408), mHCN 2 (NM008226) and the VGSCs (r $Na_v1.2$ (NM012647),
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4 rNav_v1.3 (NM012647), rNav_v1.4 (M26643), hNav_v1.5 (NM198056), mNav_v1.6 (L39018), rNav_v1.7
5 (AF000368), rNav_v1.8 (NM017247), rβ1 (NM017288), hβ1 (NM001037) and the insect channel DmNav_v1
6 (NC004354) in *Xenopus* oocytes, the linearized plasmids were transcribed using the T7 or SP6
7 mMESSAGE-mMACHINE transcription kit (Ambion, USA). The harvesting of stage V-VI oocytes from
8 anaesthetized female *Xenopus laevis* frog was previously described [20]. Oocytes were injected with 50 nl
9 of cRNA at a concentration of 1 ng/nl using a micro-injector (Drummond Scientific, USA). The oocytes
10 were incubated in a solution containing (in mM): NaCl, 96; KCl, 2; CaCl₂, 1.8; MgCl₂, 2 and HEPES, 5
11 (pH 7.4), supplemented with 50 mg/l gentamycin sulfate.

14 2.4. Electrophysiological recordings.

16 Two-electrode voltage-clamp recordings were performed at room temperature (18-22°C) using a
17 Geneclamp 500 amplifier (Molecular Devices, USA) controlled by a pClamp data acquisition system
18 (Axon Instruments, USA). Whole cell currents from oocytes were recorded 1-4 days after injection. Bath
19 solution composition was ND96 (in mM): NaCl, 96; KCl, 2; CaCl₂, 1.8; MgCl₂, 2 and HEPES, 5 (pH 7.4)
20 or HK (in mM): NaCl, 2; KCl, 96; CaCl₂, 1.8; MgCl₂, 2 and HEPES, 5 (pH 7.4). Voltage and current
21 electrodes were filled with 3M KCl. Resistances of both electrodes were kept between 0.5-1.5 MΩ. The
22 elicited currents were filtered at 1 kHz and sampled at 0.5 kHz (for potassium currents) or at 20 kHz (for
23 sodium currents) using a four-pole low-pass Bessel filter. Leak subtraction was performed using a -P/4
24 protocol. K_v1.1-K_v1.6 and *Shaker* currents were evoked by 500 ms depolarizations to 0 mV followed by
25 a 500 ms pulse to -50 mV, from a holding potential of -90 mV. Current traces of hERG channels were
26 elicited by applying a +40 mV prepulse for 2 s followed by a step to -120 mV for 2 s. K_v2.1, K_v3.1 and
27 K_v4.2, K_v4.3 currents were elicited by 500 ms pulses to +20mV from a holding potential of -90 mV.
28 Sodium current traces were, from a holding potential of -90 mV, evoked by 100 ms depolarizations to V_{max}
29 (the voltage corresponding to maximal sodium current in control conditions). In order to investigate the
30 current-voltage relationship, current traces were evoked by 10 mV depolarization steps from a holding
31 potential of -90 mV. g_v curves were calculated from IV relationships as follows: $g_K = I_K / (E_m - E_K)$ with
32 $E_K = (RT/zF) \ln([K^+]_o/[K^+]_i)$. In these equations g_K represents the conductance, I_K the potassium current,
33 E_m the membrane potential, E_K the reversal potential, R the gas constant (8.31 J/K.mol), T the
34 temperature, z the charge of the ion (for K⁺ ions: z = 1), F the Faraday's constant (96.500 C/mol), [K⁺]_o
35 and [K⁺]_i respectively are the extracellular and intracellular K⁺ ion concentrations. The values of I_K or g_K
36 were plotted as function of voltage and fitted using the Boltzmann equation: $g_K/g_{max} = [1 + (\exp(Vg - V)/k)]^{-1}$,
37 where g_{max} represents maximal g_K, Vg is the voltage corresponding to half-maximal conductance,
38 and k is the slope factor. To assess the concentration dependency of the APEKTx1 induced inhibitory
39 effects, a concentration-response curve was constructed, in which the percentage of current inhibition was
40 plotted as a function of toxin concentration. Data were fitted with the Hill equation: $y = 100/[1 + (IC_{50}/[toxin])^h]$,
41 where y is the amplitude of the toxin-induced effect, IC₅₀ is the toxin concentration at half-maximal efficacy,
42 [toxin] is the toxin concentration, and h is the Hill coefficient. To investigate the bimolecular kinetics of
43 toxin inhibition, oocytes expressing wild type K_v1.1 channels were depolarized to 0 mV for 0.5 s from a
44 holding potential of -90 mV every 5 s, both in the absence and presence of different concentrations of toxin.
45 The obtained current values were plotted as a function of time. Data were fitted with the following
46 exponential equation: $y = Ae^{-t/\tau}$, where t represents the time, A is the amplitude of the current and τ is
47 the time constant for toxin binding (τ_{on}) or toxin unbinding (τ_{off}). The first-order association rate constant
48 (k_{on}) was calculated using following equation: $\tau_{on} = (k_{on} + k_{off})^{-1}$. The first-order dissociation constant
49 (k_{off}) was calculated using the equation: $\tau_{off} = k_{off}^{-1}$. Comparison of two sample means was made using a
50 paired Student's t test (p < 0.05). All data represent at least 3 independent experiments (n ≥ 3) and are
51 presented as mean ± standard error.

57 2.5. Kunitz-type inhibition activity.

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4 The possible trypsin inhibition activity of APEKTx1 was measured spectrophotometrically as described
5 previously [21]. Briefly, aliquots containing a final concentration of 3 μ M trypsin (from bovine pancreas,
6 TPCK treated, Sigma-Aldrich, USA) were incubated with various concentrations of peptides in 5 mM
7 CaCl_2 and 50 nM Tris-HCl, pH 7.8. After incubation for 3 h at 25°C the remaining trypsin activity was
8 determined by addition of 5 μ l of a 5 mM $\text{N}\alpha$ -benzoyl-DL-arginine p-nitroanilide (BAPNA, Sigma-
9 Aldrich,USA). Paranitroaniline release was measured at 405 nm.

10 11 12 2.6. Molecular modeling.

13
14 APEKTx1 was modeled using the publicly available program MODELLER9v8
15 (<http://www.salilab.org/modeller>) using textilin-1, a Kunitz-type serine protease inhibitor from the
16 venom of the Australian common brown snake *Pseudonaja textilis* (PDB code 3BYB;
17 <http://www.pdb.org/pdb>) as template.

18 19 20 3. Results

21 22 3.1. Purification of APEKTx1.

23
24 The screening of fractions obtained from the sea anemone *A. elegantissima*, after anion and cation
25 exchange and gel filtration, yielded one fraction which was able to fully block $\text{K}_v1.1$ channels. This
26 fraction was further purified in 2 steps using reversed-phase HPLC. A linear gradient from 0 to 80%
27 solution B was developed for 80 min at a flow rate of 1ml/min. A second purification was performed in
28 the same conditions as described above (fig.1).

29 30 31 3.2. Biochemical properties.

32
33 With MALDI analysis in the linear mode, a broad mass peak with an apex mass of 7468.8 Da was
34 measured. This experimental mass of 7475 Da corresponds well with the theoretical mass of 7468.5 Da.
35 During ESI analysis, a mass of 7484.8 Da was observed, suggesting an oxidation of Met32. The intact
36 toxin was subjected to Edman degradation. Since only the 20 first N-terminal amino acids could be
37 reliably sequenced, we subjected the toxin to a proteolytic treatment in order to obtain fragments
38 amenable to *de novo* sequencing analysis by MALDI TOF/TOF instrument. Digestion of the toxin by
39 trypsin, gluC, thermolysin turned out to be unsuccessful. Finally, an asparaginylendopeptidase was found
40 to cut the toxin peptide into smaller fragments. The digestion was executed according to the protocol of
41 the manufacturer. After digestion, the resulting peptides were separated and desalted by HPLC. The
42 HPLC fractions were dried by speed-vac evaporation and resolubilized in alfa-cyano 4-hydrocinnamic
43 acid matrix solution before analysis by MALDI TOF/TOF. The protein sequence data reported in this
44 paper will appear in the UniProt Knowledgebase under the accession number P86862.

45 46 47 48 3.3. Electrophysiological experiments.

49
50 Sequence alignment (fig. 2A) indicated that APEKTx1 is a new member of the type 2 sea anemone
51 peptides directing against voltage-gated potassium channels, which also include the kalicludines from *A.*
52 *sulcata* and SHTX II from *Stichodactyla haddoni* [21,22]. From figure 2B it can be seen that APEKTx1
53 also shares homology with protease inhibitors from other sea anemones and with the potent Kunitz-type
54 protease inhibitor Bovine Pancreatic Trypsin Inhibitor (BPTI). Previous electrophysiological experiments
55 in *Xenopus laevis* oocytes have shown that the kalicludines block $\text{K}_v1.2$ channels with IC_{50} values around
56 1 μ M. SHTX II was reported to inhibit the binding of ^{125}I - α -DTX to synaptosomal membranes with an Kd
57 value of 650 nM [21,22]. As shown in figure 3A, the pure peptide APEKTx1 was subject of a wide
58 screening on 23 ion channels: 12 cloned voltage-gated potassium channels ($\text{K}_v1.1$ - $\text{K}_v1.6$, $\text{K}_v1.1$ mutant,
59 $\text{K}_v2.1$, $\text{K}_v3.1$, $\text{K}_v4.2$, $\text{K}_v4.3$, hERG, the insect channel Shaker IR); 2 cloned hyperpolarization-activated
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4 cyclic nucleotide-sensitive cation non-selective channels (HCN1 and HCN2) and 8 cloned voltage-gated
5 sodium channels ($\text{Na}_v1.2$ - $\text{Na}_v1.8$ and the insect channel DmNa_v1). Surprisingly, at concentrations up to 1
6 μM , no significant effect could be observed on the other ion channel isoforms tested. Figure 3B shows
7 original traces for wild type $\text{K}_v1.1$ channels with application of 0.3, 1 and 3 nM APEKTx1 and for mutant
8 $\text{K}_v1.1$ channels with application of 100 μM APEKTx1. A concentration response curve was constructed
9 in order to determine the concentration at which half of the channels were blocked by APEKTx1. The
10 IC_{50} value yielded 0.9 ± 0.1 nM (fig. 4A). Thus APEKTx1 inhibits $\text{K}_v1.1$ channels with the same potency
11 as DTX I and α -DTX and is approximately 700 to 3000 times more potent than other known 2 sea
12 anemone peptides directed against K_v channels. Moreover, this also means that at concentrations which
13 are a 1000-fold the IC_{50} value of APETx1 on $\text{K}_v1.1$, this toxin still discriminates between subtype
14 isoforms.

15
16 The inhibition of $\text{K}_v1.1$ channels by APEKTx1 was not voltage-dependent as in a range of test potentials
17 from -30 mV to +40 mV no difference in the degree of APEKTx1 induced block could be observed (n=8)
18 (fig. 4B).

19
20 In order to investigate if the observed current inhibition is due to pore blockage or rather to altered
21 channel gating upon APEKTx1 binding, the IV and gV curves in ND96 and HK solution were constructed
22 (fig. 4C and D). Application of 10 nM APEKTx1 caused $80.45 \pm 2.23\%$ and $84.21 \pm 3.74\%$ inhibition of
23 the potassium current in ND96 and HK, respectively (n=10). In ND96, the g_{max} curve in control and in the
24 presence of 10 nM toxin was characterized by a $V_{1/2}$ value of -15.7 ± 1.23 mV and -12.71 ± 0.5 mV (n=8)
25 respectively. In HK, the $V_{1/2}$ was respectively, -25.84 ± 2.53 mV and -19.89 ± 3.2 mV (n=8). It can be
26 concluded that in both solutions no significant difference in $V_{1/2}$ values occurred ($P>0.05$). Furthermore,
27 the IV relationship in HK solution shows that the reversal potential, E_K is not significantly influenced by
28 APEKTx1 ($P>0.05$; n=8). E_K values yielded -7.56 ± 2.45 mV in control and -7.96 ± 1.92 mV after
29 application of toxin. All together, these experiments imply that current inhibition upon APEKTx1 binding
30 does not result from changes in the voltage-dependence of channel gating. Presumably, APEKTx1 acts by
31 blocking the pore in the open state of the K_v channel.

32
33 Blockage of $\text{K}_v1.1$ channels occurred rapidly and its binding was reversible since the current recovered
34 quickly and completely upon washout (n=10) (fig. 4E). Both characteristics suggest an extracellular site
35 of action. The reversibility of current inhibition is in contrast with DTX K binding. Toxin K binding is
36 reversible at low concentrations but becomes irreversibly at concentrations higher than its IC_{50} value.
37 However, alanine substitution of the Lys3 or Lys26 rendered DTX K binding reversible, possibly
38 explaining the reversible binding of APEKTx1 since this toxin also possess neutral residues at these
39 positions. Another observed difference between both toxins is that DTX K cannot fully inhibit the
40 potassium current, even at very high concentrations. APEKTx1 however, is able to completely inhibit the
41 current.

42
43 Because APEKTx1 did not alter channel gating and binding was reversible we investigated whether
44 APEKTx1 blockage followed the kinetic behavior of a bimolecular reaction [23]. Figure 4F shows the
45 effects of increasing concentrations APEKTx1 on a $\text{K}_v1.1$ channel. The apparent first order association
46 rate constant k_{on} (open circles) increased linearly with toxin concentration, while the second order
47 dissociation constant k_{off} (closed circles) remained constant. Both values are in concordance with the
48 scheme of a bimolecular reaction. The influence on toxin-channel binding by through-space electrostatic
49 forces between charged residues of the $\text{K}_v1.1$ channel and of APEKTx1 was also examined. This was
50 done by measuring the effectiveness of APEKTx1 binding on $\text{K}_v1.1$ channels in solutions of different
51 ionic strength. As described above, the IC_{50} value of APEKTx1 for $\text{K}_v1.1$ homomeric channels was $0.9 \pm$
52 0.1 nM. However, when using an iso-osmotic solution with a 48 mM NaCl concentration (sucrose
53 substitution) the obtained IC_{50} value was 0.43 ± 0.07 nM. This 2 fold increase in APEKTx1 potency
54 underlines that through-space electrostatic interaction is involved in APEKTx1 binding. These results are
55 in corroboration with previously described similar observations for charybdotoxin binding to Shaker
56 channels and for α -DTX binding to $\text{K}_v1.1$ channels [23-25].

57
58 In order to define the α -dendrotoxin footprint on mammalian potassium channels, 3 mutations (A352P,
59 E353S and Y379H) were introduced in the dendrotoxin binding site, located in the H5 loop between the
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transmembrane domains S5 and S6 of K_v1.1 subunits (fig. 5). By mutating these 3 particular residues, the pore region of the K_v1.1 triple mutant closely resembles this one of K_v1.3 channels [23,24]. We investigated whether the sensitivity of APEKTx1 for K_v1.1 could be affected by mutating these 3 critical residues. Interestingly, even at extreme high concentrations, APEKTx1 was unable to induce full blockage of current through these mutated K_v1.1 channels. Application of 100 μM APEKTx1 caused only 53 % inhibition (n=4) and the obtained IC₅₀ yielded 10.8 ± 0.6 μM (fig. 4 A and Fig. 3B). This dramatic loss of affinity underlines the crucial interaction of these mutated residues with the toxin. Since it was reported that DTX K only needs one K_v1.1 subunit to cause inhibition of the potassium current through wild type heteromultimeric channels [26], we investigated also if DTX K could block the triple mutant. Concentration response curves were constructed for both K_v1.1 wild type and triple mutant channels (fig. 4A). The observed IC₅₀ value for wild type channels was 0.51 ± 0.064 nM which is in good accordance with previous reported values [27]. For mutant channels the IC₅₀ was 5.28 ± 0.23 nM. This means a 10-fold decrease in sensitivity compared to wild type channels.

3.4. Trypsin inhibition activity.

As all members of the type 2 sea anemone toxins APEKTx1 share sequence homology with the well characterized and potent Kunitz-type protease inhibitor BPTI, we also investigated if APEKTx1 could block trypsin activity. In order to do this, trypsin was incubated with different concentrations of APEKTx1 at room temperature for 3 hours to reach equilibrium. In the same conditions, trypsin was also incubated with DTX I, DTX K or BPTI. The protease inhibition activity was determined spectrophotometrically after incubation by measuring the ability of the remaining free trypsin to release paranitroaniline from BAPNA. It has been shown before that the kalicludines have a very similar inhibition profile as BPTI [21]. Figure 6 shows that APEKTx1 is a trypsin inhibitor with a K_d = 124 nM. Presumably, APEKTx1 is a competitive trypsin inhibitor. A 3-fold molecular excess of APEKTx1 over trypsin completely inhibited the paranitroaniline release. However, this is almost 3 times the amount of BPTI or the kalicludines necessary to reach total inhibition of trypsin activity. The same figure also indicates that DTX I and DTX K, could not inhibit the trypsin activity, even at a 6:1 ratio of toxin over trypsin.

4. Discussion

The unique feature of a dual functionality within one molecule is intriguing. However, since the production of toxins is an energy demanding task for an organism, the dual effects contained within one molecule can be seen as an efficient and economic manner to save energy with a maximal pharmacological output. Furthermore, the dual effect has clearly advantages for the sea anemone in both an offensive and defensive point of view: the presence of a trypsin inhibitor will prolong the half-life of the peptides in the venom and therefore optimize the strategy of the venom.

APEKTx1 is the newest member of the type 2 sea anemone toxins directed towards voltage-gated potassium channels. To date, this family exists of only 4 other toxins, 3 kalicludines and SHTX II. Although it is known that these toxins are effective on K_v channels, little is known about their subtype selectivity. Here we report for the first time a screening of a type 2 toxin on a wide range of 23 different ion channels. This broad screening not only reveals a potent activity against K_v1.1 (IC₅₀ = 0.9 ± 0.1 nM) but also points out an impressive selectivity for these channels over the other isoforms tested. Even at concentrations 1000-fold higher than the IC₅₀ value for K_v1.1 channels, APEKTx1 has the ability to selectively inhibit this isoform subtype over others within the K_v1 family. Moreover, A352P, E353S and Y379H substitutions in the K_v1.1 subunit made these channels insensitive for APEKTx1 indicating that these 3 residues are critical for the toxin-channel interaction. Further electrophysiological characterization showed that this toxin does not modulate the voltage dependence of gating of K_v channels. The IV relationship in HK solution showed that ion selectivity was not changed after toxin binding. The observation that there is no difference in the percentage induced block either in ND96 or HK leads to the

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4 conclusion that channel blockage is independent of the direction of the potassium current flux and is not
5 influenced by the extracellular concentration of K^+ ions. Moreover, APEKTx1 did not show voltage
6 dependence in its blockage of channels in a wide voltage range. The site of toxin binding is presumably
7 located at the extracellular side since the inhibition of current through $K_v1.1$ channels occurred rapidly
8 and binding was reversible upon washout. We have shown that APEKTx1 shares not only sequence and
9 structure homology with dendrotoxins (fig. 7) but also a similar activity profile with a comparable
10 potency as these snake toxins. The APEKTx1 blockage followed the kinetic behavior of a bimolecular
11 reaction as previously described for α -DTX. The dendrotoxins have been extensively studied. The X-ray
12 structure of α -DTX and the NMR structure of DTX K have been reported [28,29]. They both possess
13 structures which resemble very well the Kunitz-type serine protease inhibitors such as BPTI. The
14 dendrotoxins have an N-terminal 3_{10} -helix, a β hairpin and a C-terminal α -helix. The residues important
15 for their potent K_v blocking activity have been characterized. For DTX K, site-directed mutagenesis
16 studies have shown that Lys3, Tyr4, Lys6, Pro8, Arg10, Trp25, Lys26 and Lys28 are the main residues
17 involved in the interaction with the $K_v1.1$ channels [30-32]. Among these, the basic residues in the 3_{10} -
18 helix, Lys3 and Lys6, have been pointed out as the key residues for toxin binding since mutation to an
19 alanine led to a dramatically decrease in the ability to inhibit the K^+ current [33]. Mutating the Pro8 and
20 the Arg10 caused a 50- and 140-fold increase in IC_{50} value, respectively. The dramatic loss of affinity
21 observed for DTX K binding to synaptic membranes when Trp25 and Lys26 were mutated to a alanine
22 underlines that these residues are critical in recognition of $K_v1.1$ channels [33,34]. Among these key
23 residues, Pro8, Arg10 and Arg28 are conserved in APEKTx1.

24 For α -DTX a mutational approach based on site-directed mutagenesis and chemical synthesis in which 31
25 out of 59 amino acids were mutated, revealed that Arg1, Arg2, Lys3, Leu4, Ile6, Leu7 were crucial for
26 channel blockage activity of this toxin. Substituting Arg1 by alanine resulted in a less than 10-fold
27 decreased affinity, whereas Arg2, Leu4 and Ile6 substitution lowered the affinity 10 to 30 times. The
28 Lys3 and Leu6 seem to be the most critical residues since mutating these amino acids resulted in a
29 thousand times less potent toxins [30,31,35,36]. Of these residues critical for α -DTX binding, the leu4
30 and Ile6 are replaced by a homologous residue in APEKTx1.

31 These Lys3 and Leu6 residues in α -DTX form the so-called dyad. It has been proposed that most toxins
32 that block K_v channels possess a conserved functional core composed of a key basic residue (Lys or Arg)
33 associated with a 6.6 ± 1 Å distant key hydrophobic residue (Leu, Tyr or Phe). Such a functional dyad can
34 be found in a broad range of structurally unrelated peptides from various animals such as scorpions, cone
35 snails, snakes and sea anemones [37,38]. However, it has been reported that besides this dyad, other toxin
36 determinants are required for a high affinity interaction between the toxin and his target [39]. Known
37 examples of toxins lacking a dyad but still capable of blocking the K_v channel (or the other way around
38 toxins with a dyad but incapable of blocking) strongly suggest that the functional dyad on its own cannot
39 represent the minimal pharmacophore or prerequisite for K_v1 binding [40,41]. Docking experiments with
40 the scorpion toxin Pi1 and its homologs have highlighted that a ring of basic residues interacts with acidic
41 residues of the K^+ channel turret located in the most extracellular part of the channel outer vestibule. Pi1
42 analogs, in which the basic residues from the ring were replaced by alanines, show a 50-480-fold
43 reduction in peptide affinity for their target but were still able to block the potassium current [38]. In
44 general, it is assumed that toxins recognize the K_v1 subtypes through the interaction of various toxin
45 residues, among which the basic ring, with certain residues of the K_v1 channel turret. These interactions
46 can be sufficient to inhibit the potassium current. Moreover, these specific molecular contacts determine
47 toxin selectivity towards particular K_v1 channel isoforms. The functional dyad can then be viewed as a
48 secondary anchoring point, providing a higher toxin affinity without altering toxin selectivity. The side
49 chain of the basic key residue enters the ion channel pore and is surrounded by four Asp residues of the P-
50 loop selectivity filter. This Asp residue is highly conserved among all the K_v1 channel isoforms. The
51 carbonyl oxygen atoms of these Asp residues will form a stabilizing interaction with the side chain of the
52 basic residue of the dyad. The key hydrophobic residue of the dyad will interact through both
53 hydrophobic forces and hydrogen bonding with a cluster of aromatic residues in the P-loop. The specific
54 positioning of the 2 residues from the dyad results in a physical barrier, especially because of the
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4 positively charged side chain which is opposed to the K^+ flux [42,43]. Because of the low identity with
5 other K_V channel toxins and because none of the key residues critical for the K_V blockage of DTX K or α -
6 DTX are conserved in APEKTx1, we tried to hypothesize the key residues of APEKTx1 with the help of
7 structural models. As it can be seen from figure 7, the role of a possible dyad in APEKTx1 can be
8 fulfilled by Arg15 and Phe13, a basic and hydrophobic residues which are 6.2 Å separated. Assumably,
9 the side chain of Arg15 enters the $K_V1.1$ channel pore and interacts with the carbonyl oxygen atoms of the
10 Asp377 residues. The Phe13 will most likely interact through hydrophobic forces with the aromatic
11 cluster formed by Phe356, Trp364, Trp365, and Tyr375. Previous work has highlighted Tyr375 in $K_V1.1$
12 as a crucial residue for the selectivity of scorpion and sea anemone toxins [44]. The amino acid at this
13 position is highly variable between K_V1 isoforms and the nature of the side chain of this residue
14 contributes significantly to the subtype selectivity of toxins. The observed 12000-fold decrease in affinity
15 of APEKTx1 for mutated $K_V1.1$ channels can be explained partially by the Y379H mutation. The same
16 mutation prevented the $K_V1.1$ binding of another sea anemone toxin BgK, which binds to wild type
17 channels with an IC_{50} value of 0.7 nM [43-46]. The E353 also seems to be critical for interaction with
18 APEKTx1. *Hurst et al* have shown that each of the 3 mutations on its own decreased dendrotoxin potency
19 and that the removal of a negative charge at position 353 caused the greatest decrease in affinity [24].
20 However, simultaneous substitution of all 3 residues caused a much stronger decrease in DTX sensitivity
21 than the single mutations. Further site-directed mutagenesis studies or ideally co-crystallization studies
22 are required to elucidate if such subtle changes in sequence of the pore region are responsible for the
23 dramatically loss of sensitivity of $K_V1.1$ for this toxin.

24 Also shown in figure 7 are the basic residues which are conserved in DTX K and α -DTX. These residues
25 possibly contribute to the basic ring important for recognition, interaction and correct positioning of the
26 toxin on the channel. As mentioned above, previous work has shown that mutating these conserved
27 residues leads to affinity loss in DTX K or α -DTX [30]. However, this is only a hypothesis which requires
28 further conformation from site-directed mutagenesis studies, docking experiments and structure analysis.

29 The bovine pancreatic trypsin inhibitor belongs to the Kunitz-type inhibitors and is a potent inhibitor of
30 serine proteases. The crystal structures of BPTI with bovine trypsin and with bovine chymotrypsin have
31 been determined [47-49]. Analysis of these complexes have shown that there are 10-13 residues on the
32 inhibitor forming less than 4 Å contacts with 20-25 residues of the enzyme. The 13 key residues of the
33 inhibitor form a structural epitope seen as two extended and exposed loops. The first loop which is the
34 primary contact site is composed of the residues Thr11 to Ile19. The second loop is more C-terminal
35 situated and is formed by residues Gly36 to Arg39 [50,51]. The Lys15 has been pinpointed as a single
36 residue hot spot responsible for a major part of the enzyme-inhibitor contacts. The side chain of this basic
37 amino acid penetrates deeply into the specificity binding pocket of the protease where it electrostatically
38 interacts with the Asp189 of the protease. This Lys15-Asp189 interaction delivers the major contribution
39 to the association energy for the BPTI-trypsin complex. The role of Lys15 as a crucial player in the
40 energetics of the recognition was demonstrated by substituting this residue with Gly. The point mutation
41 reduced the total association energy with 70% leading to an association constant decreased by 9 orders of
42 magnitude. Structure determination of α -DTX provided an explanation why this toxin and its homologue
43 DTX I cannot inhibit proteases. The basic amino acid in BPTI has been replaced in α -DTX by a Tyr
44 incapable of interacting effectively with binding pocket of trypsin [52-54]. Further explanation for the
45 lack of protease inhibition by the dendrotoxins can be found in the presence of Asp17 in α -DTX, Glu17 in
46 DTX I and Arg16 in DTX K. The existence of these longer amino acid residues in dendrotoxins instead of
47 the smaller alanine in BPTI causes a most unfavorable complex with the trypsin binding site. Moreover,
48 the Pro19 in DTX K and Pro21 in DTX I and α -DTX instead of the Ile19 in BPTI greatly impedes a good
49 interaction with the side chain of the Tyr39 of trypsin. The residues essential for kunitz-type inhibition in
50 the primary contact site (Gly12, Cys14, Lys15 and Ala16) are all conserved in APEKTx1 except for the
51 Arg15 (fig. 7). However, previous residue-specific modification studies have shown that mutating the Lys
52 15 to Arg did not alter the association constant. The lower protease inhibition activity of APEKTx1
53 compared to BPTI (fig.5) can be explained by the presence of Phe13 and Pro19. A double BPTI mutant
54 with a phenylalanine at position 13 and an arginine at position 15, exactly as in APEKTx1, resulted in a
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4 100-fold decreased association constant compared to the wild type [54]. The unfavorable interaction of
5 Pro19 with trypsin has been described above.
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7 5. Conclusion

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9 We have demonstrated the unique dual function of APEKTx1, which is a competitive Kunitz-type
10 protease inhibitor and a very potent and selective $K_V1.1$ channel blocker. Moreover, several amino acid
11 residues have been suggested to play a functionally critical role in the high potency and unique selectivity
12 of this toxin. Further structure-function analysis involving site-directed mutagenesis studies, docking
13 experiments and NMR are required in order to confirm their role. Because of its high affinity and
14 selectivity, APEKTx1 might be a very interesting probe for further characterization of the binding sites of
15 $K_V1.1$ channels. Finally, APEKTx1 represents a valuable tool to examine the exact role of $K_V1.1$ in
16 various channelopathies, and might serve as a lead compound in the development of novel therapeutical
17 agents for these diseases.
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37 Reference

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Footnotes

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39 § To whom correspondence should be addressed. Tel.: +32 1632 34 03; Fax: +32 16 32 34 05; E-mail:
40 jan.tytgat@pharm.kuleuven.be

41 The abbreviations used are: APEKTx1, Anthopleura elegantissima potassium channel toxin 1; MALDI
42 TOF, matrix-assisted laser desorption-ionization time of flight; RP-HPLC, reversed-phase high
43 performance liquid chromatography; TFA, trifluoroacetic acid; K_v channel, voltage-gated potassium
44 channel; DTX; dendrotoxin; BPTI, bovine pancreatic trypsin inhibitor.
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Figure Legends

Fig. 1: Purification of APEKTx1.

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49 A, the crude fraction found to be active in the electrophysiological screening was loaded on a C18 column
50 with a linear gradient of 0.085% TFA in acetonitrile. Only the fraction containing the largest peak
51 indicated by the arrow was active on K_v1.1 channels. B, this active fraction was further purified using the
52 same conditions as in A.
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Fig. 2: Alignment.

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55 Amino acid sequence of APEKTx1 and alignment with the other members of the type 2 sea anemone
56 toxins, dendrotoxins, protease inhibitors from sea anemones and BPTI. Amino acid residues identical
57 with APEKTx1 are shown on grey background, cysteine residues are printed in red. Disulfide bridge
58 pattern based on AsKC3 are indicated. AsKC1-3 from *Anemonia sulcata*, AFAPI-I and AFAPI-III from
59 *Anthopleura fuscoviridis*, AXPI-II from *Anthopleura xanthogrammica*, DTX E from *Dendroaspis*
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4 *polylepis polylepis*, SHTX III from *Stichodactyla haddoni*, DTX K from *Dendroaspis polylepis*, α -DTX
5 from and BPTI from *Bos taurus*.

6 **Fig. 3: Activity of APEKTx1 on K_V channels.**

7 **A**, Differential effects of APEKTx1 on several cloned voltage gated potassium and sodium channel
8 isoforms expressed in *X. laevis* oocytes. Representative whole-cell current traces in control and toxin
9 conditions are shown. The dotted line indicates the zero-current level. The asterisk (*) marks steady-state
10 current traces after application of 1 μ M of APEKTx1. This screening on a large number of K_V channel
11 isoforms belonging to different subfamilies of the voltage gated potassium channel family shows that
12 APEKTx1 selectively blocks $K_V1.1$ channels at a concentration of 1 μ M. Traces shown are representative
13 traces of at least 3 independent experiments ($n \geq 3$).

14 **B**, Original traces for wild type $K_V1.1$ channels with application of 0.3, 1 and 3 nM APEKTx1. For
15 mutant $K_V1.1$ channels original traces with application of 100 μ M APEKTx1 are also shown.

16 **Fig. 4: Effects on $K_V1.1$ channel gating.**

17 **A**, dose-response curve on wild-type (closed symbols) and triple mutant (open symbols) $K_V1.1$ channels
18 obtained by plotting the percentage blocked current as a function of increasing toxin concentrations. For
19 APEKTx1 (triangles), IC_{50} values yielded 0.9 ± 0.1 nM and 10.8 ± 0.6 μ M for wild type and mutant
20 channels, respectively. For DTX K (squares), IC_{50} values were determined at 0.51 ± 0.064 nM and $5.28 \pm$
21 0.2 nM for wild-type and mutant channels, respectively.

22 **B**, the percentage of residual current after application of 10 nM APEKTx1 as a function of membrane
23 potential. In a range of test potentials from -30 mV to +40 mV, no difference in the degree of APEKTx1
24 induced block could be observed.

25 **C and D**, show *IV* and *gV* curves in ND96 and HK respectively. APEKTx1 does not act as a gating
26 modifier. In ND96, the $V_{1/2}$ in control (open circles) and in the presence of 10 nM APEKTx1 (closed
27 circles) was characterized by a value of -15.7 ± 1.23 mV ($n=5$) and -12.71 ± 0.5 mV ($n=6$) respectively. In
28 HK, the $V_{1/2}$ yielded respectively, -25.84 ± 2.53 mV and -19.89 ± 3.2 mV. No significant shift was
29 observed. Moreover, APEKTx1 did not significantly alter the reversal potential.

30 **E**, fast kinetics of inhibition of $K_V1.1$ channels and reversibility of the inhibition upon washout. Control
31 (open triangles), wash-in, (open triangles+ black bar), wash-out (open circles). Values for τ_{on} and τ_{off} in
32 this experiment were 92.7 and 47s, respectively.

33 **F**, APEKTx1 blockage followed the kinetic behavior of a bimolecular reaction as proposed by Tytgat *et*
34 *al.* Displayed are the effects of increasing concentrations APEKTx1 on a $K_V1.1$ channel. The apparent
35 first order association rate constant k_{on} (open circles) increased linearly with the toxin concentration. The
36 second order dissociation constant k_{off} (closed circles) remained constant. Both these values are in
37 correlation with the scheme of a bimolecular reaction.

38 **Fig. 5: Alignment of the pore regions of K_V1 family.**

39 Sequence alignment of the pore region of $K_V1.1$ - $K_V1.6$ and the $K_V1.1$ mutant. The 3 mutated residues
40 (A352P, E353S and Y379H) in the $K_V1.1$ mutant are shown in red. Asp377, important for stabilizing the
41 side chain of the basic residue of the dyad is shown in blue while the residue from the aromatic cluster
42 (Phe356, Trp364, Trp365, Tyr375) which interacts with the hydrophobic residue of the dyad are shown in
43 green.

44 **Fig. 6: Kunitz-type protease inhibition.**

45 The figure shows the concentration dependence of trypsin inhibition by different concentrations of BPTI,
46 closed circles; APEKTx1, open circles; DTX I, open triangles and DTX K, open squares. A 3-fold
47 molecular excess of APEKTx1 over trypsin inhibited the paranitroanaline release completely

48 **Fig. 7: Spatial structure models of α -DTX, DTX K, BPTI and APEKTx1.**

49 Structural models for α -DTX (PDB accession code 1DTX), DTX K (1DTK), BPTI (1PIT) and APEKTx1.
50 The model of APEKTx1 was built on the basis of the structure of the homologue snake protease inhibitor
51 textilin (3BYB). Residues known to be important for K_V channel blockade (α -DTX, DTX K,
52 APEKTx1) are indicated in red. The model for APEKTx1 shows the residues possibly forming a dyad in
53 red, the residues possibly contributing to the basic ring are shown in green. The residues known to be
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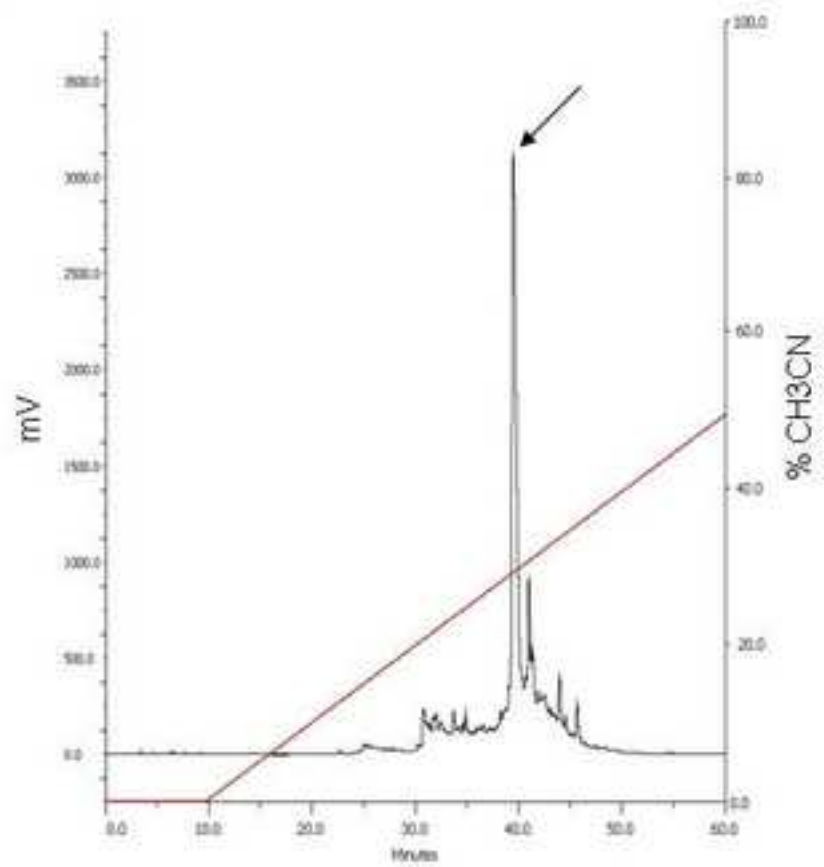
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4 important for protease inhibition (BPTI and APEKTx1) are colored in blue (primary contact site) and in
5 yellow (secondary contact site).
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Figure 1

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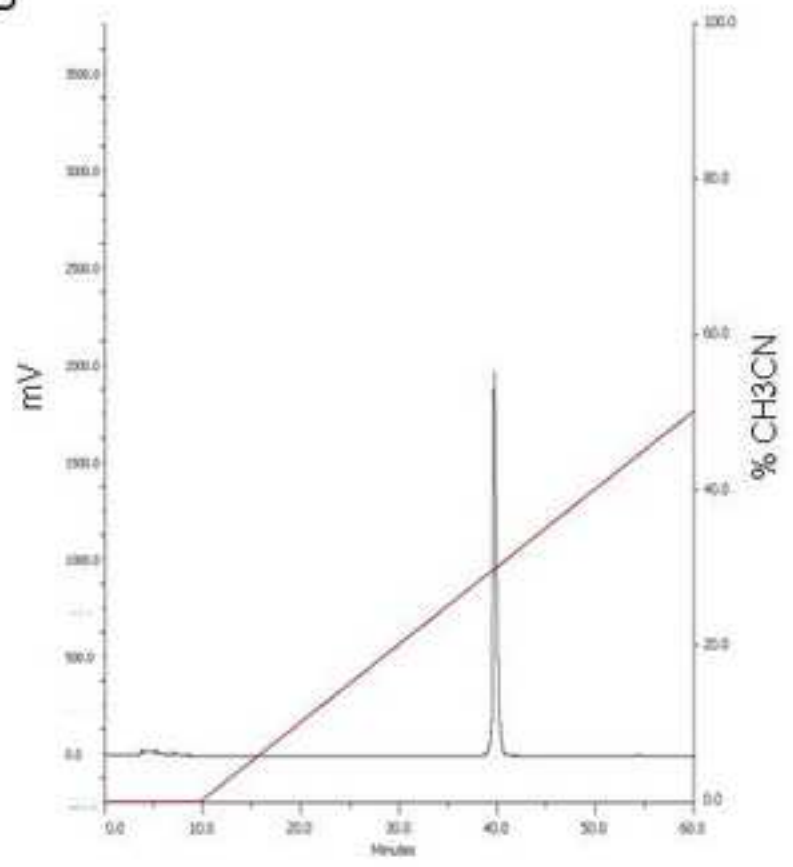


Figure 2

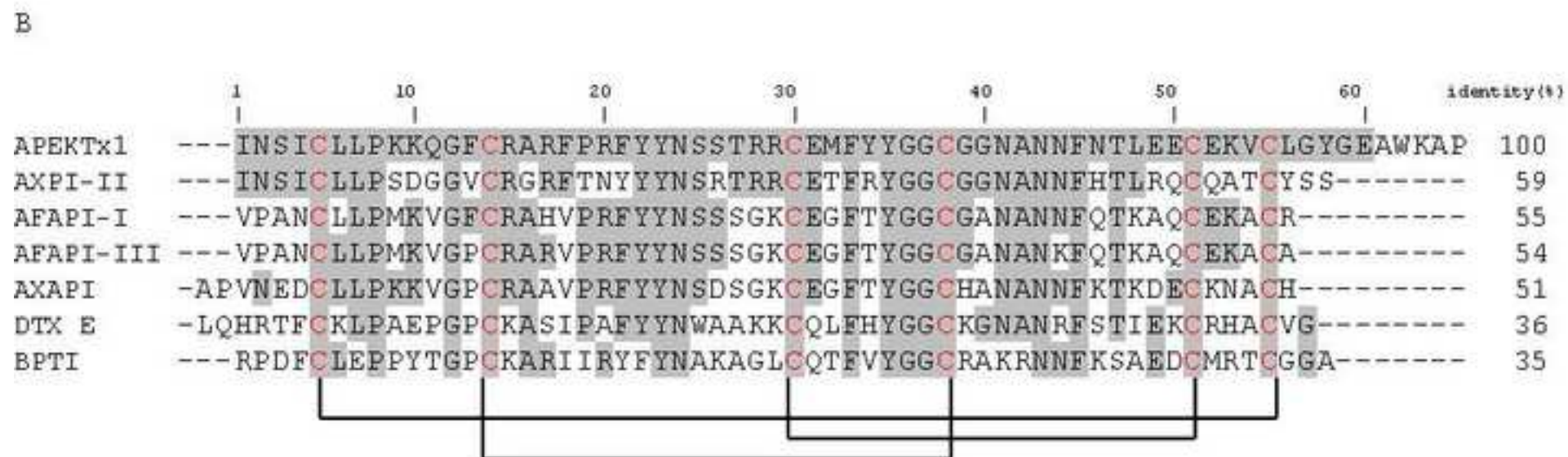
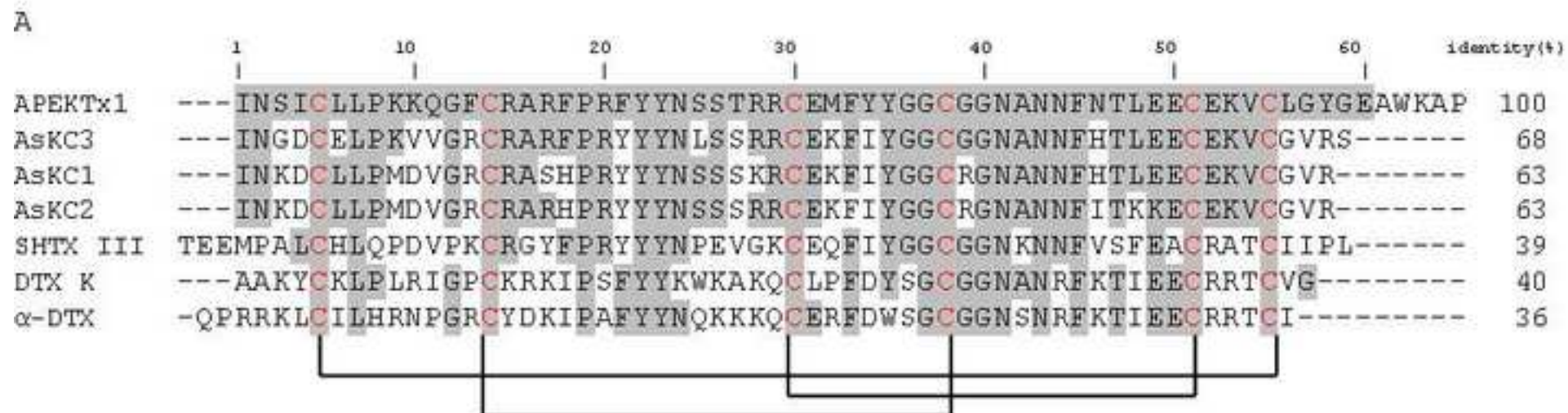
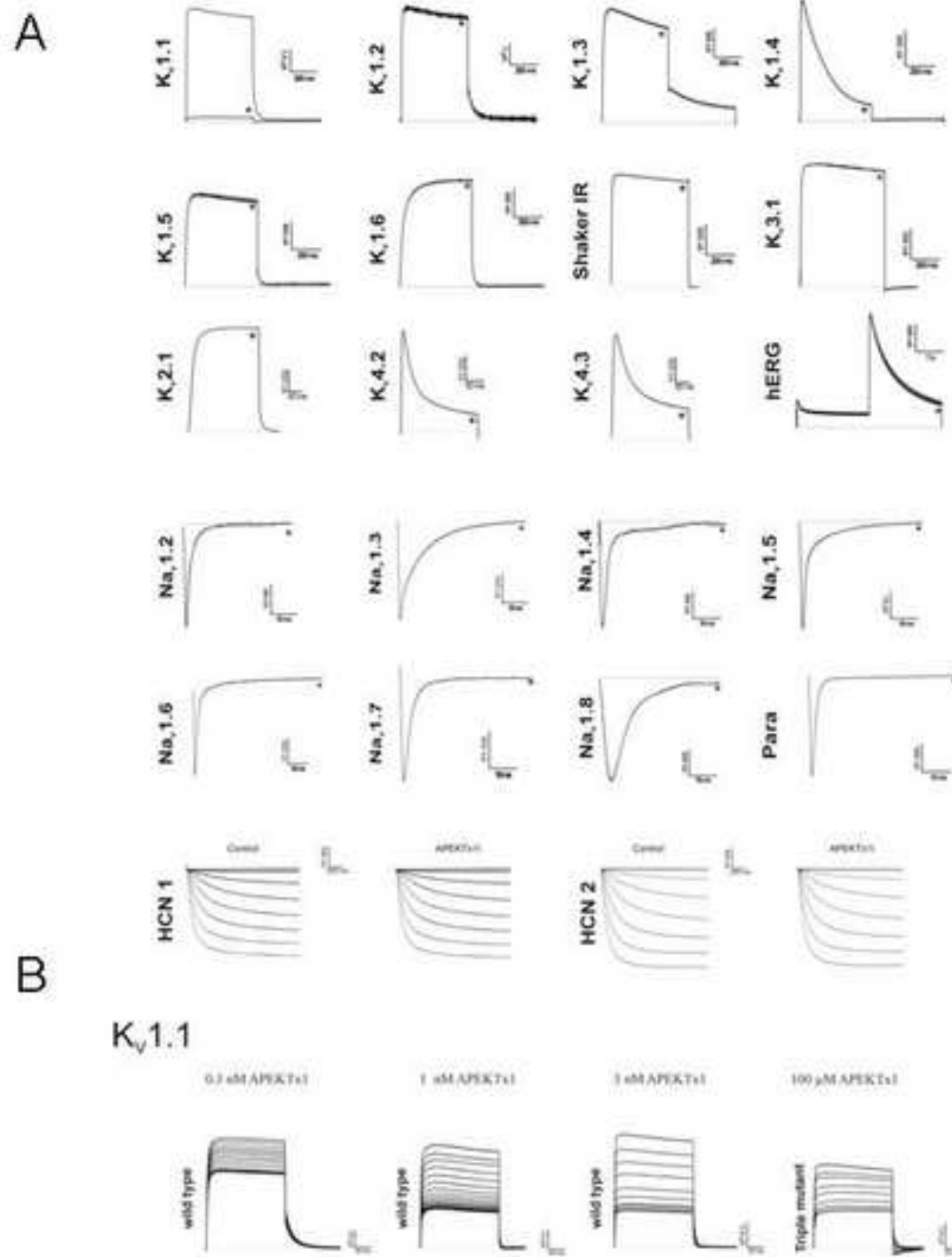
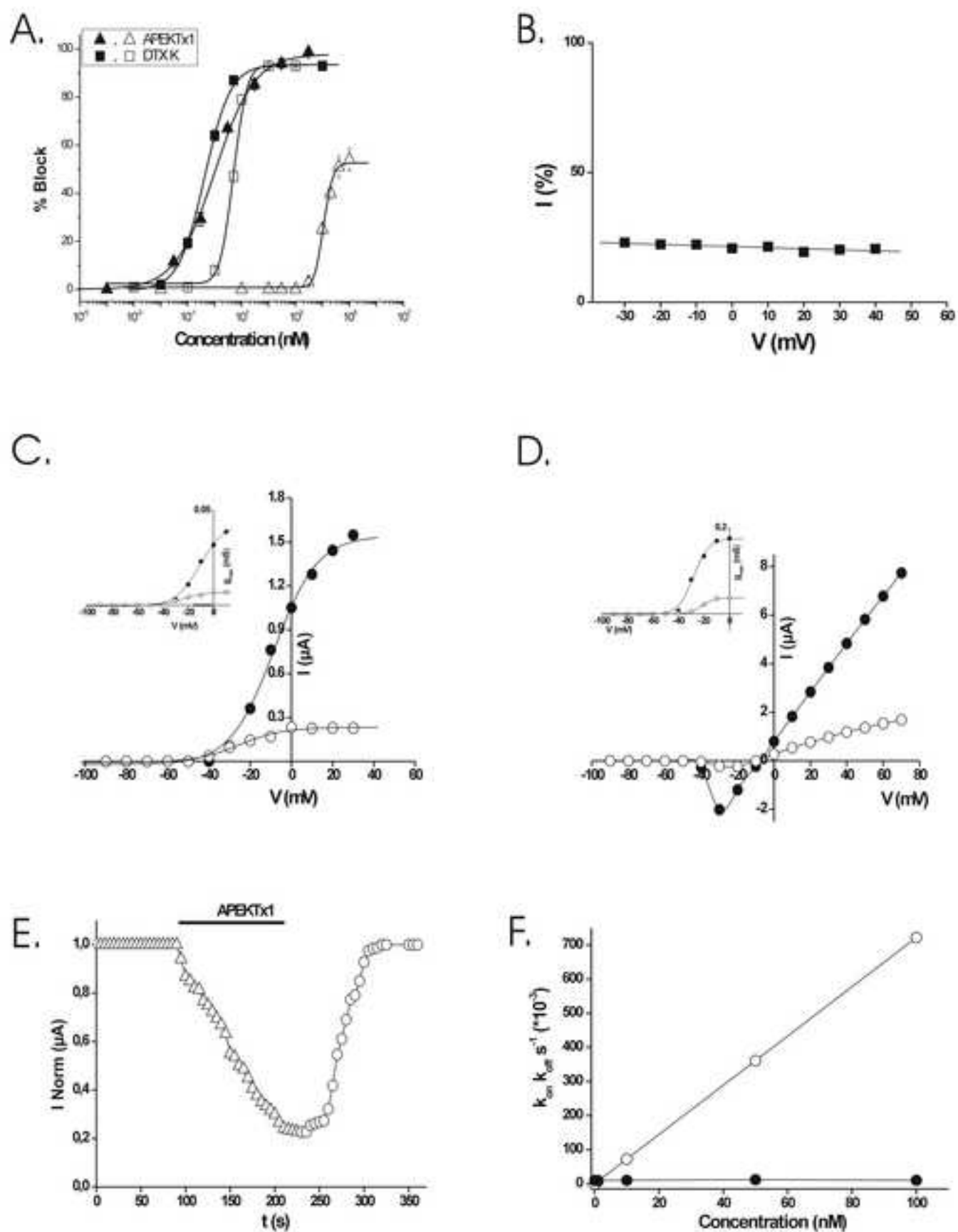


Figure 3





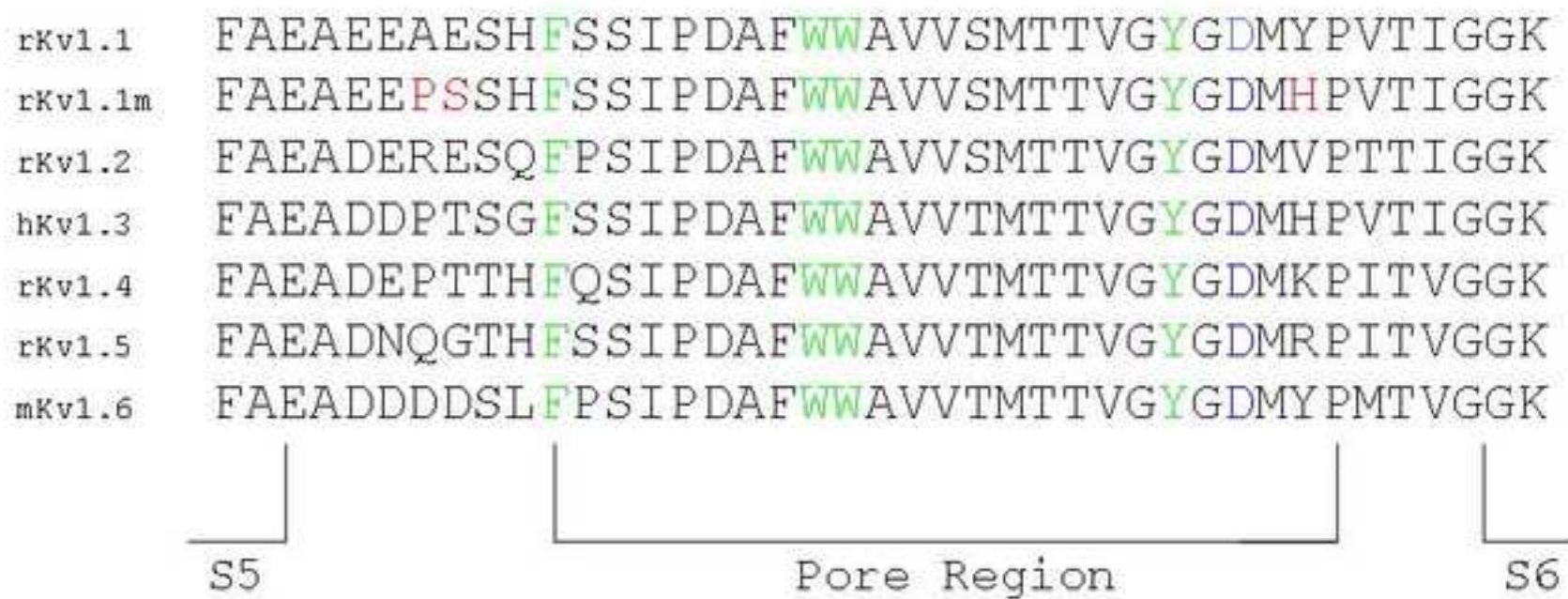


Figure 6

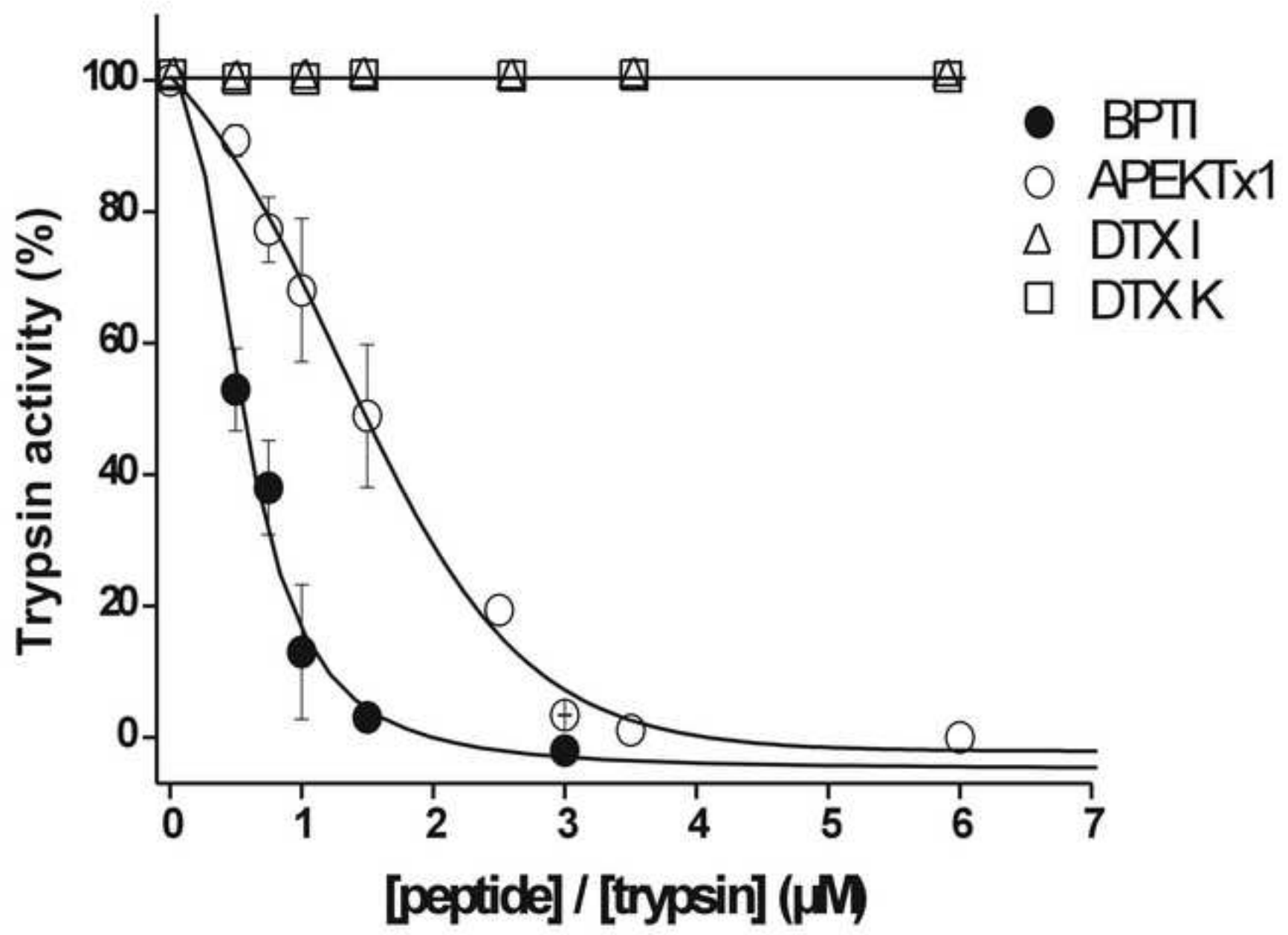
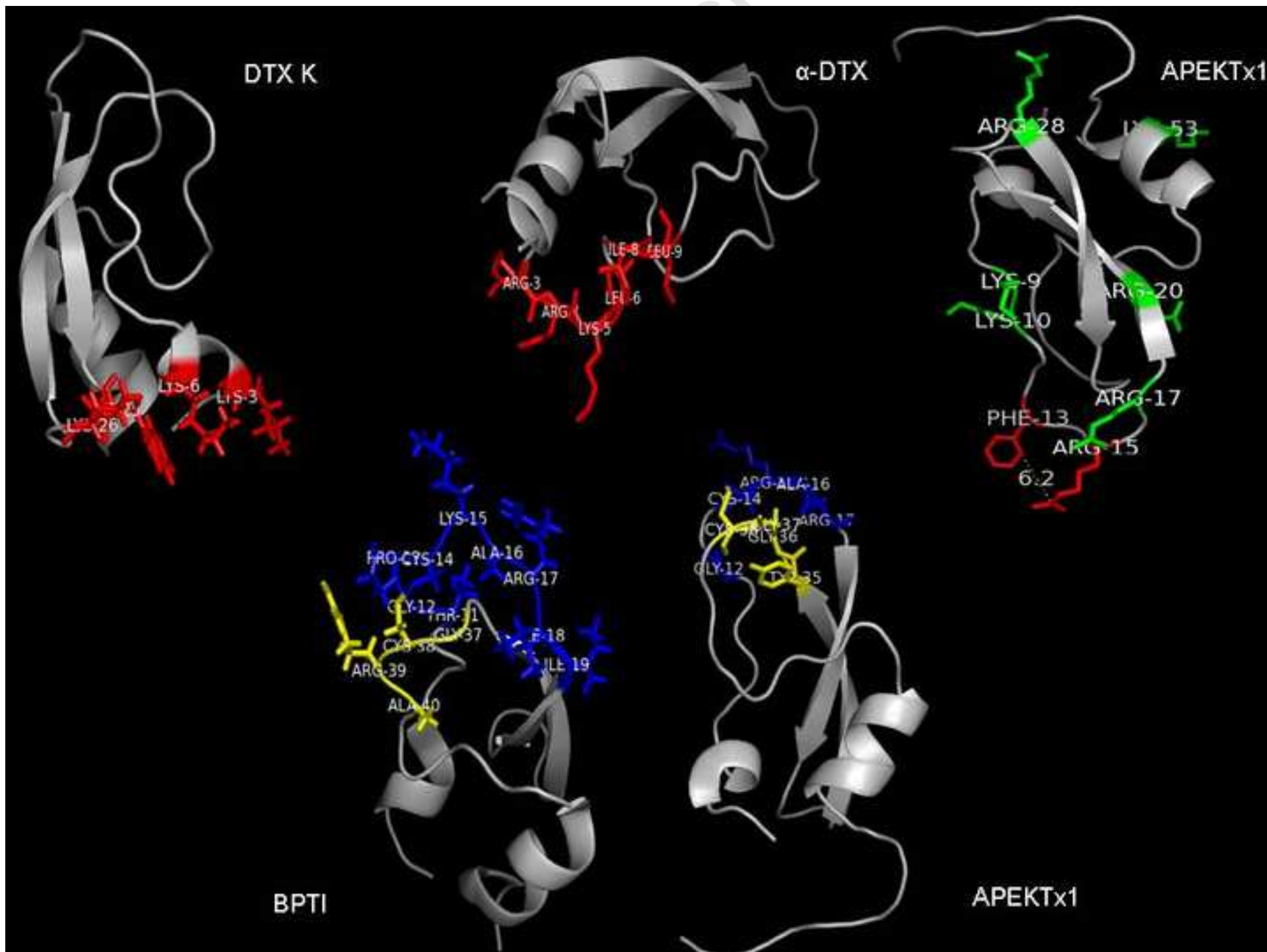
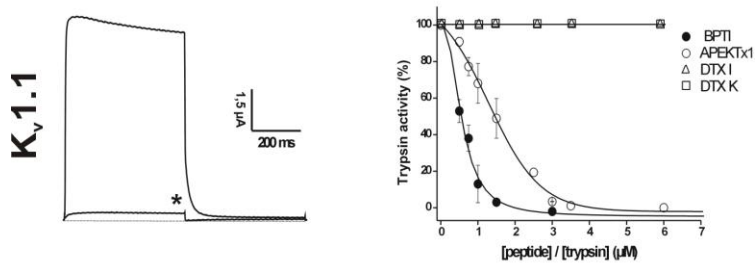


Figure 7



APEKTx1 is a potent and selective K_V blocker and protease inhibitor



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