Proceedings of the 36th European Peptide Symposium
Michal Lebl (Editor)
European Peptide Society, 2022 *https://doi.org/10.17952/36EPS.2022.093*

Design and Synthesis of Selective Ion Channel Blocker Peptide Toxin Analogs

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Introduction

Voltage-gated ion channels are pore-forming transmembrane proteins, which are important mediators of electrical signals and maintain ion homeostasis in cells [1]. Due to their prime role in signal transduction and cellular regulations, malfunctions of the ion channels can lead to the development of several diseases involving the cardiac, neuronal, immune, and endocrine systems [2,3,4]. Venomous peptides of natural origin (from snakes, scorpions, spiders, frogs, and cone snails) act upon ion channels. Besides their therapeutic importance, these toxins can be used to elucidate the mechanism of action of the channels [2]. However, their application is restricted by the limited selectivity between the numerous ion channels. We tried to investigate the possibility to design and synthesize more selective potassium channel blockers.

Results and Discussion

We have selected three peptides for our studies:

- Iberiotoxin (IbTx) has subnanomolar activity and it is fairly selective for the KCa1.1 channel,
- Agitoxin (AgTx2) has a similarly high affinity for both Kv1.1 and Kv1.3 channels,
- Kaliotoxin (KTX1) blocks Kv1.1 and Kv1.3 channels with nanomolar and KCa1.1 channels with micromolar affinity.

The disulfide bridge pattern of the 3 toxins is identical: abcabc. Our derivatives were synthesized by microwave-assisted solid phase peptide synthesis using N^{α}-Fmoc protected amino acids. Oxidation of the peptides were carried out on air at pH=7.5 (0.1 M NH_4OAc). We have examined the effect of the peptides on ion channels Kv1.1, Kv1.3, and KCa1.1 using patch clamp method. None of our analogs acted on the Kv1.1 channel.

First, a chimera of the three toxins was designed (ZFPK in Table 1). Some amino acids of the IbTx were exchanged with the corresponding residues of AgTx2 and KTX1. Namely, V9G, and L20A mutations were done, and the VDR block (between positions 23 and 25) and the C-terminal YQ were replaced with MRF and TPK, respectively.

The mutations resulted in the loss of activity toward the KCa1.1 channel, at the same time the ZFPK peptide blocked the Kv1.3 channel (Figure 1 and 2) So, although only a few amino acids were incorporated into the sequence of IbTx, the new analog rather acted as AgTx2. This result is in agreement with the literature [6 and references therein]: It is reported that Val at position 9 in IbTx is too bulky for the interaction with the turret of the S5-S6 loop of Kv1.3 and Shaker channels. Moreover, when Gly10 (note that position 10 in AgTx2 is corresponding to position 9 in IbTx) of AgTx2 was mutated to Val the affinity of the peptide was greatly decreased toward the Shaker channel. It was shown that Phe^{25} and Pro^{37} is an important determinant of the binding of AgTx2 to the Shaker channel. All of these 3 residues important for the binding of AgTx2 to Kv1.3 and Shaker were incorporated into these IbTx-based ZFPK chimeras.

In the next analog (ZFYQ) the Val at position 10 and the C-terminal of the IbTx were restored (Table 1). These changes were enough to gain back the activity toward the KCa1.1 channel, but the response of the Kv1.3 channel for ZFYQ was markedly less than that of ZFPK (Figure 1 and 2). It was noticed that the Arg between the 3rd and 4th Cys is shifted by one position in our chimeras (ZFYQ and ZFPK) compared to AgTx2 and KTX1. To check if this affects the interaction with Kv1.3 channel Phe^{21} was omitted from the sequence (Table 1, $desF^{21}$-ZFYQ). As it is seen in Figure 1 and 2, this slight modification altered the behavior of the peptide: the effect on Kv1.3 and KCa1.1 channels were

Table 1. Sequence alignment of the parent and the synthesized peptides. Z=pyroglutamic acid, the altered residues of IbTx analogs are marked with boldface characters.

Code	*Sequence*
IbTx	ZFTDVDCSVSKECWSVCKDLFGVDRGKCMGKKCRCYQ
AgTx2	GVEINVKCSGSPQCLKPCKDAGMRFGKCMNRKCHCTPK
KTX1	GVPINVSCTGSPQCIKPCKDAGMRFGKCMNRKCHCTPK
ZFPK	ZFTDVDCSGSKECWSVCKD**A**FG**MRF**GKCMGKKCRC**TPK**
ZFYQ	ZFTDVDCSVSKECWSVCKD**A**FG**MRF**GKCMGKKCRCYQ
desF21-ZFYQ	ZFTDVDCSVSKECWSVCKD**A**G**MRF**GKCMGKKCRCYQ
ZFYQ-RRR	ZFTDVDCSVSKECWSVCKD**A**FG**RRR**GKCMGKKCRCYQ
ZFYQ-MRR	ZFTDVDCSVSKECWSVCKD**A**FG**MRR**GKCMGKKCRCYQ
ZFYQ-RWR	ZFTDVDCSVSKECWSVCKD**A**FG**RWR**GKCMGKKCRCYQ
ZFYQ-DWR	ZFTDVDCSVSKECWSVCKD**A**FG**DWR**GKCMGKKCRCYQ
IbTx-R25Cit	ZFTDVDCSVSKECWSVCKDLFGVD**X**GKCMGKKCRCYQ **X**=citrulline
IbTx-G30Dab	ZFTDVDCSVSKECWSVCKDLFGVDRGKCM**X**KKCRCYQ **X**=2,4-diaminobutyric acid
IbTx-R34E	ZFTDVDCSVSKECWSVCKDLFGVDRGKCMGKKC**E**CYQ

restored and diminished, respectively. (Note that in this study only 100 nM concentration of the peptides were used.)

Homology model on the binding of the IbTx to its ion channel and *in silico* residue scanning experiments on positions 23-25 revealed that the exchange of these residues to RRR, MRR, DWR, or RWR might result in a stronger interaction with the ion channels. Thus, the corresponding analogs were synthesized and tested. The result shows that only ZFYQ-RWR-b and ZFYQ-RRR affected the blockade of the Kv1.3 ion channel. None of the new analogs act on the KCa1.1 channel. (a and b indexes in the names designate peptides with the same sequences having different disulfide bridge patterns.)

It seems that the analogs with an Arg residue at position 23 (desF^{21}-ZFYQ, ZFYQ-RWR-b, ZFYQ-RRR) can close – at least partially - Kv1.3 channel and an aromatic residue at position 24 is beneficial (desF^{21}-ZFYQ and ZFYQ-RWR). Indeed, this position corresponds to position 25 in AgTx2, which contains a Phe here.

The homology model elucidated that Arg^{25} of IbTx forms a salt bridge with Asp^{357} of the KCa1.1 channel. To test this hypothesis this residue was changed to citrulline, which has a similar size to Arg, and can form H-bond, but cannot form a salt bridge. R25Cit mutant has no affinity to the KCa1.1 channel (Figure 2.). This is in agreement with our previous measurements: Only peptides with Arg at position 25 (ZFYQ-RWR-a, ZFYQ-RRR, ZFYQ-MRR) blocked KCa1.1 – at least to some extent.

Gly^{30} has a key role in the insensitivity of the IbTx toward Kv1.3. Other toxins (charybdotoxin, AgTx2, KTX1) contain an Asn in this position, which forms an H-bond with Asp^{381} of the channel. It was shown earlier that IbTx G30N has an affinity to Kv1.3 [7]. We wanted to see what will be the effect of the incorporation of 2,4-diaminobutyric acid into position 30. This residue would be able to form not only an H-bond but a salt bridge with Asp^{381}. This mutation did not increase the affinity to Kv1.3, but it did to the KCa1.1 channel.

Vm24 (α-KTx 23.1) is a 36-residue Kv1.3-blocker peptide from the venom of the scorpion *Vaejovis mexicanus smithi*. Vm24 blocks the Kv1.3 channel with high affinity (Kd = 2.9 pM) and displays excellent selectivity against several ion channels. However, at high peptide concentrations (over 10 nM) it also blocks other ion channels including Kv1.1, Kv1.2, and KCa3.1. A natural analog of Vm24 called sVmKTx differ from the parent peptide only in a single residue: namely Vm24 contains lysine at position 32 and sVmKTx has glutamate here. Although the affinity of sVmKTx decreased to 770 pM concerning the Kv1.3 channel, it does not block any other ion channel even at 100 nM (and in some cases at 2.5 μM) concentrations. Molecular dynamics simulations showed that K32E mutation led to the decreased structural fluctuation of the N-terminal segment of the mutant peptide. This difference was preserved after their binding to Kv1.3 [5].

If we compare the sequence of Vm24 (AAAISCVGSPECPPKCRAQGCKNGKCMNRKCKCYYC) and IbTx (Table 1), it is visible that Lys^{32} in Vm24 corresponds to Arg^{34} in IbTx. As a consequence of that IbTx-R34E was prepared and examined. The mutated peptide was able to act on the Kv1.3 channel with a small affinity, but with higher selectivity, since it lost its activity on the KCa1.1 channel.

Conclusion

The selectivity of IbTx can be altered by the mutation of a couple of residues. Incorporation of Gly at position 9, the exchange of the C-terminal residues of IbTx to that of AgTx2, and the presence of Arg and an aromatic residue at positions 23 and 24, respectively, resulted in an increase in affinity and sometimes in selectivity toward the Kv1.3 ion channel. Arg^{25} is an important determinant of the binding to KCa1.1. Replacement of Gly^{30} with 2,4-diaminobutyric acid did not increase the affinity toward Kv1.3, so a salt bridge between this residue and Asp^{381} of Kv1.3 was not formed, probably. R34E mutation might have some beneficial effects by increasing the selectivity of the chimera.

In the future, we will check the additivity of the findings listed above by synthesizing such analogs of desF^{21}-ZFYQ and ZFYQ-RWR which have a Gly at position 9. Further increase in affinity and selectivity can be expected by incorporating again the *C*-terminal residues of AgTx2 and the effect of R34E replacement should be studied more.

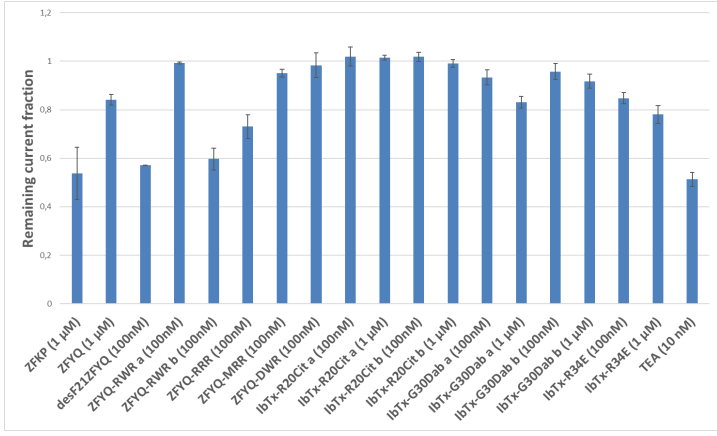

Fig. 1. The electrophysiological effect of analogs on Kv1.3 channel.

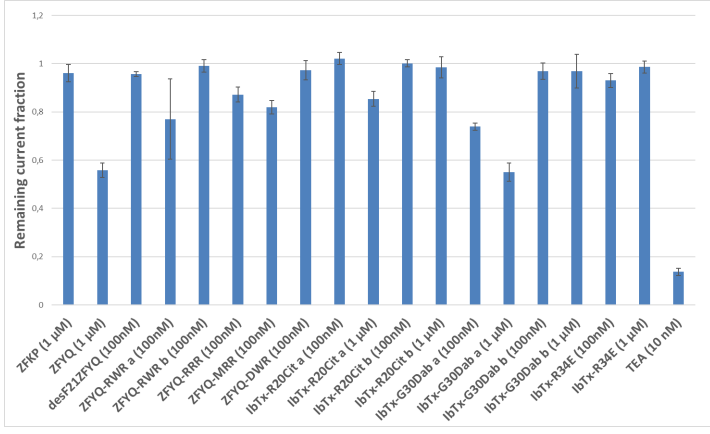

Fig. 2. The electrophysiological effect of analogs on KCa1.1 channel.

Acknowledgments

This work was supported by the following grants: Ministry of Human Capacities, Hungary, grant EFOP-3.6.2-16-2017-00006.

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