Syntheses and investigations of modified analogues of biologically active peptides

Doctoral thesis

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1. Introduction

After decoding the human genom, biomolecular chemical research has brought in focus proteomics. The expansion of the wide range of proteins discovered is still in full blast nowadays. Understanding the function and role of these biologically active proteins in the organism can be made on the basis of smaller peptides. With these informations derived from structure-function relationships, the necessary elements for the activity of a protein become recognizable. The primary preparative tool for the synthesis of model peptides, short protein fragments, biologically active peptides, peptidomimetics is the solid-phase peptide chemistry. Since its introduction, the solid-phase peptide chemistry has showed major improvements, and still there are sequences that preparation with standard methods is problematic, especially those biologically active peptides that contain post translational modifications.

During my doctoral work, I focused on the preparation of synthetic peptides – mostly modified peptides – that can influence some biological processes on a molecular level, and the folding processes of proteins also can be studied by these modified peptides.

2. Aims

The goal of my doctoral work was:

- The synthesis of urocortin 3 (Ucn 3) neuropeptide – that effectively regulates the stress response – and its fragments obtained with targeted deletion of amino acids to localize the active center of the molecule and testing of these fragments in behavioural experiments.
- On the basis of the active center of the molecule, design and synthesis of analogues that keep or even improve the antidepressive and anxiolitic effect of the longer Ucn 3 peptide and have better pharmacokinetic properties, hereby could serve as potential pharmacons in the treatment of depression and anxiety.
- Optimalization of the synthesis of neuropeptide orexin A and its testing on water metabolism and secretion of vasopressin in rats.
- The synthesis of peptides that can selectively act in the signal transduction pathways between the innate and adaptive immune system and could serve as drug candidates in the treatment of disorders of the immune system:
  - Synthesis of the native scorpion toxins anuroctoxin and Tc32 that were isolated from the venom of the mexican scorpion *Anuroctonus phaiodactylus* and the venom of the brazilian scorpion *Tityus cambridgei*, respectively as potential blockers of the Kv1.3 ion channels.
  - Synthesis and testing of point mutational analogues of these native scorpion toxin peptides to design inhibitors of the Kv1.3 ion channels with increased selectivity that might be lead compounds for drugs that can treat autoimmune diseases.
  - Synthesis of the C3a-derived C3a9 symmetrical dimer to confirm the presumption that the active C3a9 sequence is present in its dimeric form

while inhibiting allergic reactions and is stabilized with two intermolecular disulfide bridges.

o Synthesis of Gab1-derived phosphopeptides and their modified analogues, their biotinylation and conjugation with cell-penetrating peptides to study the role of these adaptor protein fragments in the regulation of the B-cell signaling pathways.

• The synthesis and conformational studies of β-model peptides (SETE-peptides) and miniproteins (Betanova, Tc5b) that mimick the protein folding processes.

3. Experimental methods

For the preparation of the peptides and their derivatives, standard organic chemistry methods were used, mainly manual or microwave-assisted solid-phase peptide synthesis methods. The structures were proved by mass spectrometry analysis, mainly electro-spray ionization (ESI). Purification and further analysis of the products was made with reversed phase high performance liquid chromatography (RP-HPLC). For biological studies, both *in vitro* and *in vivo* methods were applied. Conformational analysis was made with the use of NMR- and CD-spectroscopic data.

4. Results and discussion

4.1. Corticotropin releasing factor (CRF) has an important role in the activation of stress mechanism. Our earlier research showed, that urocortin 3 – a 38 amino acid peptide – as member of the CRF family is suitable for designing lead compounds for treating depression and anxiety.

The urocortin 3 (Ucn 3) neuropeptide and its fragments with targeted deletion of amino acids were synthesized. These peptides were tested in elevated-plus-maze test (EPM) and modified forced swimming test

(FST). The active center of the molecule that preserves the antidepressive and anxiolytic action of the longer sequence was found to be the *C*-terminal Ala-Gln-Ile-NH_2 tripeptide. On the basis of this Ucn 3 (36-38) sequence, I designed and synthesized more than 20, mainly peptidomimetic analogues. Most of these analogues have been investigated in biological tests and some of them showed to be suitable even for oral administration.

The preparation and the potential use in therapy of depression and anxiety of the Ucn 3 fragments and analogues are protected by our Hungarian and international patents.

4.2. The oxidative folding conditions for the synthesis of orexin A were optimized. The native isomer can be isolated as the major reaction product by using a polymer-supported oxidant the Clear-ox™ resin within two hours. The effect of Orexin A was tested on water metabolism *in vivo* and on vasopressin secretion *in vitro*. It was found that intracerebroventricular (i.c.v.) administration of orexin A enhanced the food- and water-intake and urination of rats. According to our results, polidipsia and poliuria presumably are independent of the vasopressin-system. Results of *in vitro* experiments suggested that moderating the effect of compounds that increase vasopressin secretion with orexins could have a role in development of polidipsia, poliuria.

4.3. Peptides that can selectively act in the signal transduction pathways between the innate and adaptive immune system were synthesized. They can serve as potential drug candidates in treating different immunological diseases:

4.3.1. Several studies *in vivo* and *in vitro* underlie the essential role of K^+ channel blockers in prevention of T-cell activation and proliferation

through the Ca^{2+} signal. In my work, I focused on the synthesis of anuroctoxin (AnTx) and Tc32.

4.3.1.1. The AnTx was synthesized with high selectivity, and the folding conditions were optimized. The majority of the linear peptide folds in cyclic form with all four disulfide bonds in the correct form within 1 hour. The regioselectivity was verified with measurements that prove the identity of the electrophysiological properties of the native and synthetic toxin. The AnTx blocks the Kv1.3 channel in 730 pM concentration, although AnTx is not selective enough since it also blocks the Kv1.2 channels with smaller affinity than Kv1.3 channel.

Analogues of AnTx were designed to increase the inhibition selectivity for the Kv1.3 ion channels. Results of biological measurements of analogues with single or double point mutation show that selectivity of all three toxins (AnTx F32T, AnTx F32TK16D, AnTx F32T N17A) are increased compared to the wild-type toxin, and their affinity to the Kv1.3 channels slightly decreased. AnTx F32T N17A, the most effective toxin blocks 50% of the Kv1.3 channels in 1 nM concentration, while it has no effect on the Kv1.2 channels in 100 nM concentration.

Further measurements are needed to prove that the double, triple, and multiple mutants of the prepared analogues surpass both the affinity and selectivity of the native toxin, and accordingly, they enhance the possibility of their therapeutical use in treating autoimmune diseases.

4.3.1.2. The preparation of Tc32 with oxidative folding and convergent synthesis did not resulted in peptides with the desired disulfide bridges. The product of the stepwise, regioselective cysteine pairings method did not show the selectivity, because one of the chosen protecting groups did not fulfiled the required orthogonality. The cleavage of the S*t*Bu group failed in solid-phase with the methods known from the literature. The

cleavage of the S*t*Bu group in liquid phase with compounds having free tiol group could have caused a scrambling of the disulfide bridges. According to the biological results, the different synthetic methods resulted in the same inactive isomer as main product. This points to presumably innacurate previous determination of the disulfide bridge connections in the toxin Tc32. Henceforward, we plan another protecting group scheme with stepwise regioselective cysteine pairings method to prove our presumption.

4.3.2. The C3a peptide blocks the activation of the mucosal type of mast cells and the release of the content of the granules such as histamine that cause immediate hypersensitivity (allergic reaction).

In order to prove the presumption, that the dimeric form of C3a9 stabilized by intermolecular disulfide bridges is responsible for the inhibition of allergic reactions, the symmetrical dimer of the C3a9 peptide was synthesized with the stepwise regioselective cysteine pairings method in high yield. The testing of the dimer compound in biological systems is in progress.

4.3.3. The SHP-2 phosphatase has positive regulatory function in cell growth and hence this enzyme might be attractive target in the therapy of malignant cell growth. Our main goal was to synthesize cell membrane permeable phosphopeptides („baits”) that could inhibit the growth of tumors.

The GDLDpe (621-633) peptide that is derived from the Gab1 adaptor protein and its shorter fragments and modified sequences were prepared. Biotinylated peptides were used for measurements of the phosphatase activity. In order to transport the chosen Gab1 derived peptides into the living cells, they were conjugated with membrane permeable carriers through a linker (octanoyl-R8C). Our results suggests that the cell

penetrating phosphopeptides may regulate the Gab1/SHP-2 and Gab1/PI3-K interactions and modulate intracellular signaling that lead to cell proliferation and cell survival.

4.4. β-model peptides (SETE-peptides) and miniproteins (Betanova, some glyco- and phospho-analogues of Tc5b) were also synthesized. We found that these peptides still show significant mobility. Accordingly, further modification is needed in order to obtain a more stable structure. A single structure can not correctly describe these model peptides, however we managed to restrict the number of possibilities for the SETE-S3E peptide. This peptide could serve hereafter as a useful model for β-hairpin studies.

Our results prove that modified biologically active peptides with greater selectivity and improved activities of the native sequences can succesfully be synthesized by careful selection of the synthetic methods. Recent studies on market research show that the potential use of peptide therapeutics has increased, so the modified biologically active peptides can be valuable alternatives of the small synthetic drug candidates.

The thesis are based on the following publications:

I. **Kádár Kinga**, Tóth Gábor K., Telegdy Gyula, Tanaka Masaru: Urocortin 3 fragmensek és analógok, előállításuk és alkalmazásuk depresszió és szorongás kezelésében. *P0800527 hungarian patent* (2008)*;* Urocortin 3 fragments and analogues, their preparation and use as antidepressive and anxiolytic agents. *WO 2010/020825 international patent* (2010), (IF = 1).

II. **Rákosi Kinga**, Szolomájer-Csikós Orsolya, Kalmár László, Szurmai Zoltán, Kerékgyártó János, Tóth Gábor K.: Synthesis of *N*-glycopeptides applying glycoamino acid building blocks with a combined Fmoc/Boc strategy. *Protein & Peptide Letters* (2011), **18**(7), (IF = 1,755), (in press).

III. Telegdy Gyula, **Kádár Kinga**, Tóth Gábor K.: Anxiolytic action of urocortin 3 fragments in mice, *Behavioural Brain Research* (2011), (IF = 3,220), (in press), DOI: 10.1016/j.bbr.2011.03.047.

IV. Tanaka Masaru, **Kádár Kinga**, Tóth Gábor K., Telegdy Gyula: Antidepressant-like effects of urocortin 3 fragments. *Brain Research Bulletin* (2011), **84**, 414-418, (IF = 2.184).

V. Panyi György, Varga Zoltán, **Rákosi Kinga**, Tóth Gábor K.: Kv1.3 ioncsatorna gátló szerek fejlesztése immunológiai betegségek kezelésére, *hungarian patent in prepare.*

Total IF = 8,159.

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1. **Kádár Kinga**, Panyi György, Varga Zoltán, Tóth Gábor K.: Synthesis of cysteine-rich peptides, *Proceedings of the 30th European Peptide Symposium*, Helsinki, Finnland, Aug.31- Sept. 5., 2008,146-147.

2. Tóth Gábor K., **Kádár Kinga**, Hegyi Orsolya, Szolomájer-Csikós Orsolya, Kalmár László, Kerékgyártó János: Glycopeptides - a synthetic challenge, *Proceedings of the 30th European Peptide Symposium*, Helsinki, Finnland, Aug.31- Sept. 5., 2008,148-149.

3. Kádár Kinga, Panyi György, Varga Zoltán, Tóth Gábor K.: Cisztein gazdag peptidek szintézise, XIV. Nemzetközi Vegyészkonferencia, Cluj-Napoca, România, Nov. 13-15, 2008.

4. **Kádár Kinga**, Szolomájer-Csikós Orsolya, Hegyi Orsolya, Váradi Györgyi, Kalmár László, Kerékgyártó János, Tóth Gábor K.: Glikopeptidek szintézise – egy szintetikus kihívás, Cluj-Napoca, Romania, Nov. 13-15, 2008.

5. **Rákosi Kinga**, Szolomájer-Csikós Orsolya, Hegyi Orsolya, Kovács Anita, Váradi György, Kalmár László, Kerékgyártó János, Tóth Gábor K.: *O*-glikopeptidek szintézisének lehetőségei, XV. Nemzetközi Vegyészkonferencia, Târgu-Mureş, Romania, Nov. 12-15, 2009.

6. **Rákosi Kinga**, Tanaka Masaru, Telegdy Gyula, Tóth Gábor K.: Antidepressive action of human urocortin III fragments and analogues, *Proceedings of the 31st European Peptide Symposium*, Copenhagen, Denmark, Sept.5-9, 2010.

7. Szolomájer-Csikós Orsolya, **Rákosi Kinga**, Hegyi Orsolya, Kalmár László, Kerékgyártó János, Tóth Gábor K.: The application of the new

tin(IV) chloride deprotection for the preparation of glycosylated peptides, *Proceedings of the 31st European Peptide Symposium*, Copenhagen, Denmark, Sept.5-9, 2010.

8. Kis-Karcsú Gyöngyi, Gálfi Márta, Radács Mariann, **Kádár Kinga**, Molnár Zita, Molnár Andor H., László Ferenc, Varga Csaba, László Ferenc A.: Effects of orexin-monoaminergic interactions on vasopressin secretion in rat neurophyseal cell cultures, *Endocrine Abstracts*, Rotterdam, The Netherlands, Apr. 30-May 04, 2011.