University of Szeged

Doctoral School of Pharmaceutical Sciences

PhD programme: Pharmaceutical Chemistry and Drug Research

Programme director: Prof. Dr. Ferenc Fülöp

Institute: Department of Medical Chemistry

Supervisor: Prof. Dr. Tamás Martinek

Éva Karolina Bartus

Bottom-up design of foldamers for protein surface recognition

Final examination committee:

Chairman: Prof. Dr. Ferenc Fülöp

Members: Prof. Dr. György Dombi

Dr. Pál Szabó

Reviewer Committee:

Chairman: Prof. Dr. Loránd Kiss

Reviewers: Dr. Péter Kele

Dr. József Kardos

Members: Dr. Ferenc Ötvös

Dr. Attila Hunyadi

A. Introduction and aims

Molecular recognition events are controlled by specific noncovalent interactions and they play key role in almost all biological processes. Intermolecular contacts are affected by many different factors including shape and electrostatic complementarity of the molecular surfaces. Focusing on protein–protein complexes, the free energy of the binding is directly related to the buried mostly flat surface area, which lacks deep binding pockets shielded from water. These interactions are therefore coined "undruggable" for a small-molecule drug candidate. In order to modulate the function of such protein complexes, extended molecular surface mimetics are required with a specific arrangement of the hot-spot residues. This can be achieved by synthesizing rigid scaffolds via introducing cyclic constraints into the molecules; thus, the interacting surface is a direct product of the chemical synthesis. The well-defined shape of the molecule can be created in a subsequent stage through a folding procedure depending on the solution conditions and interacting partners. Typically, biopolymers and their structural mimetics can self-organize into these kinds of hierarchical structures. In terms of molecular recognition, foldamers, i.e., non-natural oligomers with distinct conformational preferences, are interesting, due to the ability to mimic structure and functions of biopolymers. Predictable three-dimensional structures can be generated from monomeric modules leading to large exposed surface patches, which make foldamers attractive tools for high-affinity, specific recognition of protein interfaces. Although ribosomal incorporation of foldameric building blocks has been achieved in special cases, foldamers are fundamentally evolution-free chemical entities. *De novo* or bottom-up design strategies are therefore particularly difficult for foldamer sequences in cases, where limited structural information is available about the target surface.

Our main goal was to design foldameric binders for the recognition of difficult protein target surfaces by applying bottom-up design approaches. One of our molecular targets was the soluble β-amyloid oligomer. Its level in cerebrospinal fluid correlates with the cognitive impairment in Alzheimer's disease, which makes it an attractive biomarker for monitoring the progression of the disorder. Application of the foldamer methodology and the fragment-based approach resulted in the development of high-affinity interacting partner for β-amyloid. Therefore, by exploiting this interaction, the aim was to optimize a sandwich-type affinity assay using multivalent foldamer conjugate as a capture antibody mimetic. In order to develop a specific and selective assay, structural optimization of the capture molecule and increasing the efficiency of the detection system were necessary.

As another target, a model protein calmodulin (CaM) having two separate hot-spot pockets was chosen for modular bottom-up design of a foldameric inhibitor. In order to find weakly interacting partners of the protein, a surface-patch mimetic library containing short foldameric fragments was screened and the simultaneous optimization of the two binding hot spot fragments was achieved by ligating the hits via dynamic combinatorial chemistry.

B. Methods

Enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISA assay was optimized for quantification of β-amyloid oligomers. Foldameric capture molecule was immobilized on a surface of a 96-well avidin coated ELISA plate. Amyloid samples were applied in the concentration range: 10^{-6}-1μM. Monoclonal anti β-amyloid 6E10 antibody was used as detection antibody and the secondary antibodies were Histols-M and HRP-tagged anti-mouse IgG. Development was carried out with 3,3',5,5'-tetramethylbenzidine (TMB) solution pipetted into each well and the absorbance was measured with a plate reader at 370 nm (NOVOstar OPTIMA, BMG Labtech, Offenburg, Germany). For validation of the foldamer-based sandwich assay, Innotest® β-amyloid (1-42) (Innogenetics, Gent, Belgium) assay was performed according to the manufacturer's instructions.

Pulldown assay and DCL experiments

Screening of the 256-membered fragment library with CaM was carried out by pulldown assay. The protein was immobilized on affinity resin and incubated with the foldamer library at pH 7.4. The amount of the unbound fragments was determined by quantitative evaluation of HPLC-MS chromatograms of the supernatant.

Dynamic combinatorial library (DCL) was generated from 12 thiol-functionalized foldameric fragments in glutathione redox buffer via disulfide exchange reaction. Product distribution was determined after 4 days incubation at 37°C in the presence of CaM and without the protein. Amplification factor was calculated for each library member as its concentration ratio with and without CaM.

Isothermal titration calorimetry (ITC)

Isothermal titration calorimetric experiments were performed with foldameric ligands and their targets: β-amyloid and CaM using a Microcal VP-ITC calorimeter. Foldameric ligands were titrated to pre-aggregated β-amyloid at pH 7.4, 285 K using 100 μM protein concentration.

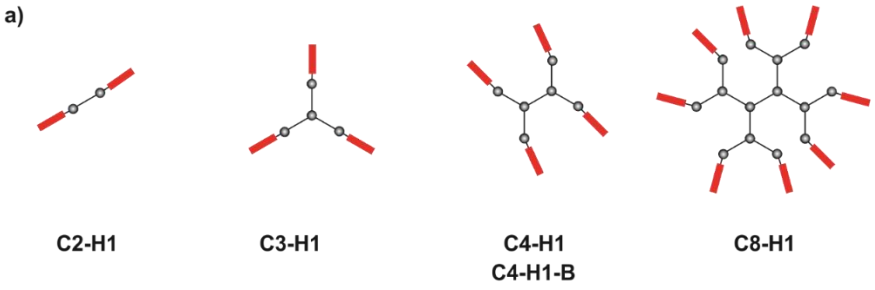
Selected fragments were titrated to calmodulin at pH 7.0, 298 K and the concentration of the protein in the cell varied between 3-7 μM.

C. Results and discussion

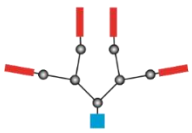
1. Development of foldamer-based sandwich ELISA assay

Utilizing the interaction between a previously studied antibody mimetic tetravalent foldamer–dendrimer conjugate and β-amyloid, a sandwich type analytical biochemistry assay was designed for detection and quantification of aggregated β-amyloid species in low nanomolar concentration.

1.1. 16 different conjugates were designed and produced by convergent synthesis to study the relationship between the structure of the foldameric conjugate and its binding affinity to β-amyloid.



b)



c)

F4-H1	**H1:** H-ACHC-β³R-ACHC-ACHC-β³D-ACHC-GGC-NH_2
B-F4-H1	**H1-B**: Biotinyl-ACHC-β³R-ACHC-ACHC-β³D-ACHC-GGC-NH_2
B-F4-H1-Ac	**H1-Ac**: Ac-ACHC-β³R-ACHC-ACHC-β³D-ACHC-GGC-NH_2
B-F4-H2	**H2**: H-ACHC-**β³K**-ACHC-ACHC-β³D-ACHC-GGC-NH_2
B-F4-H3	**H3:** H-ACHC-β³R-ACHC-ACHC-**β³E**-ACHC-GGC-NH_2
B-F4-H4	**H4:** H-ACHC-**R**-ACHC-ACHC-**D**-ACHC-GGC-NH_2
B-F4-H5	**H5**: H-ACHC-**ACHC-β³R**-ACHC-β³D-ACHC-GGC-NH_2
B-F4-H6	**H6**: H-**ABHC**-β³R-ACHC-ACHC-β³D-ACHC-GGC-NH_2
B-F4-H7	**H7**: H-ACHC-β³R-**ABHC**-ACHC-β³D-ACHC-GGC-NH_2
B-F4-H8	**H8**: H-ACHC-β³R-ACHC-**ABHC**-β³D-ACHC-GGC-NH_2
B-F4-H9	**H9**: H-ACHC-β³R-ACHC-ACHC-β³D-**ABHC**-GGC-NH_2

Figure 1. Designed foldameric conjugates for structure optimization. **a)** Schematic representation of di-, tri-, tetra- and octavalent conjugates having central symmetry, **b)** focally symmetric tetravalent conjugates containing different foldameric recognition segments, **c)** sequences of the recognition units. (ACHC: (1*S*,2*S*) -2-aminocyclohexanecarboxylic acid, ABHC: (1*S*,2*S*,3*S*,5*R*)-3-amino-6,6-dimethylbicyclo[3.1.1]heptane-2-carboxylic acid), β^3X: β^3- homologs of the corresponding (X) α-amino acid.

1.2. A rising trend in efficiency was observed by increasing the number of arms of the conjugate, but the low nanomolar affinity could not be further enhanced by introducing an

octavalent template instead of a tetravalent. This finding confirmed that the high-affinity interaction does not require more than four foldameric capture segments.

1.3. Linking the designed 14-helix foldamers (Figure 1c) to a tetravalent oligo-L-lysine dendron scaffold resulted in a branched conjugate that binds to β-amyloid with high affinity similar to foldamer-dendrimer. The immobilization occurs through the root of the focal symmetry template (Figure 2b), in which the recognition units may freely point away from the surface.

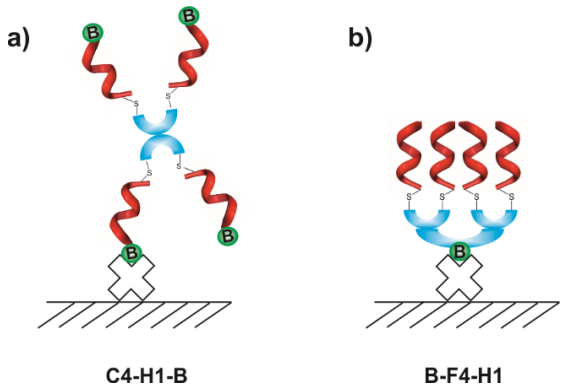


Figure 2. Schematic representation of the **a)** centrally symmetric **C4-H1-B** conjugate immobilized on a surface and **b)** dendron having a focal symmetry and arrangement of the foldamer helices of **B-F4-H1** immobilized on a surface.

1.4. Tetravalent dendron conjugate containing **H1** foldameric fragments (H-ACHC-β^3R-ACHC-ACHC-β^3D-ACHC-GGC-NH_2) was found as the most efficient in amyloid recognition and it was successfully used as a capture element in a sandwich ELISA experiment.

1.5. Utilizing the low nanomolar binding affinity of the foldameric conjugate, a highly sensitive ELISA assay was developed for quantification of β-amyloid oligomer. The limit of detection was estimated to 5 pM and linear dependence of intensity on β-amyloid concentration was found over the concentration range 10-500 pM (Figure 3).

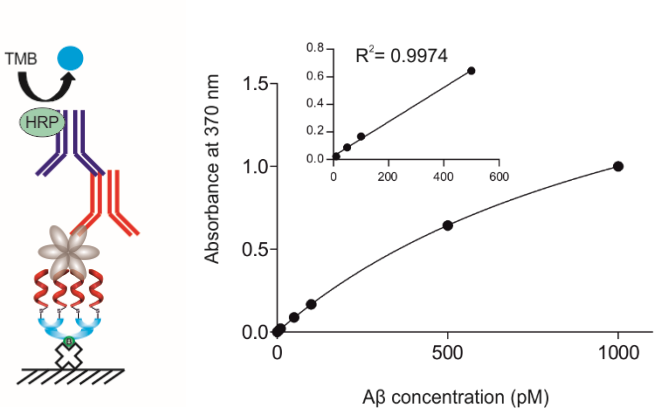


Figure 3. Schematic representation of the sandwich ELISA setup and illustration of the sensitivity of the assay.

1.6. The optimized assay was able to distinguish between β-amyloid monomers and associated forms with high selectivity toward soluble β-amyloid oligomer, a potential biomarker in early diagnosis of Alzheimer's disease.

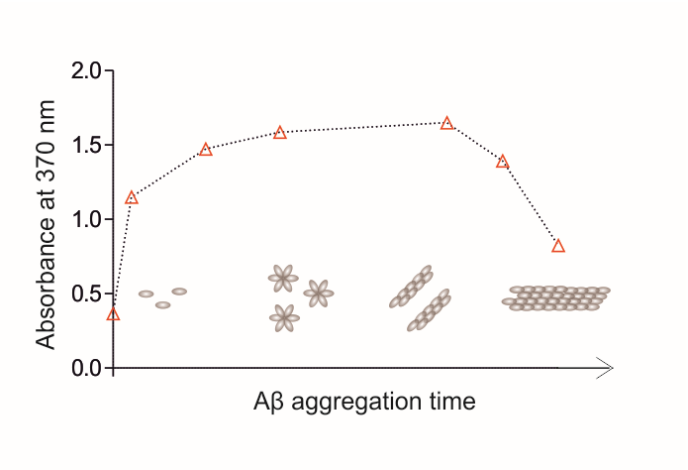


Figure 4. Monitoring time-dependent aggregation of β-amyloid represents the oligomer selectivity of the assay.

2. *De novo* development of high affinity foldameric ligand

A modular bottom-up approach was used to create foldameric protein–protein interaction inhibitor for separate hot spots.

2.1. A 256-membered foldamer library was designed and synthesized containing diverse surface-patch mimetic 14-helix fragments (Figure 5.). On the basis of the characteristics of β^3-amino acid at the 2nd position of the hexamer, the sublibraries have aromatic (L1), charged (L2), aliphatic (L3), and polar (L4) side chains.

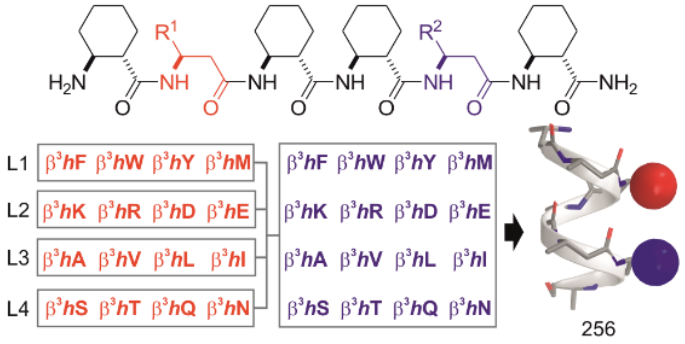


Figure 5. General structural formula and schematic representation of folded segments and the composition of the foldamer sublibraries.

2.2. The binding of the library members towards the model protein CaM with separate hot spots was tested in a pulldown assay, and hits were determined having a high propensity to form a complex with the protein.

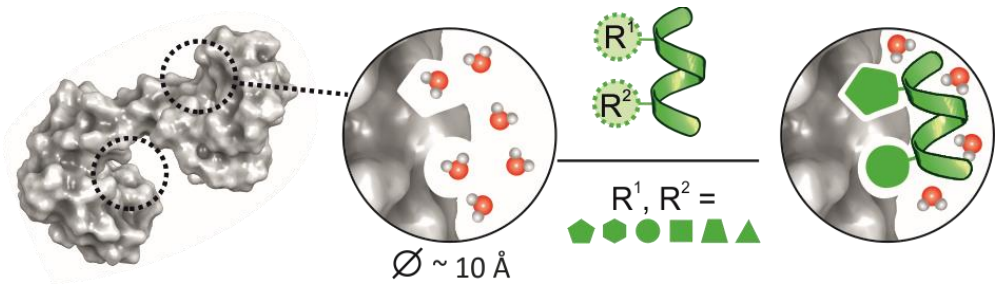


Figure 6. Mapping of the protein surface by a short folded segment library with protruding proteinogenic side chains and local solvent shielding.

2.3. The role of the ordered secondary structure and binding efficiencies of the four selected hits (**WF, RW, LW, TW**; according to the standard one-letter codes of the proteinogenic side chains presented on a 14-helix template) were investigated. Interactions of the helical fragments with CaM were found in the low micromolar to submicromolar range but there was no binding between the non-helical derivative of **WF** (**rrWF**) and CaM. This finding

confirmed the importance of the bulky, ordered secondary structure in the recognition of the protein surface.

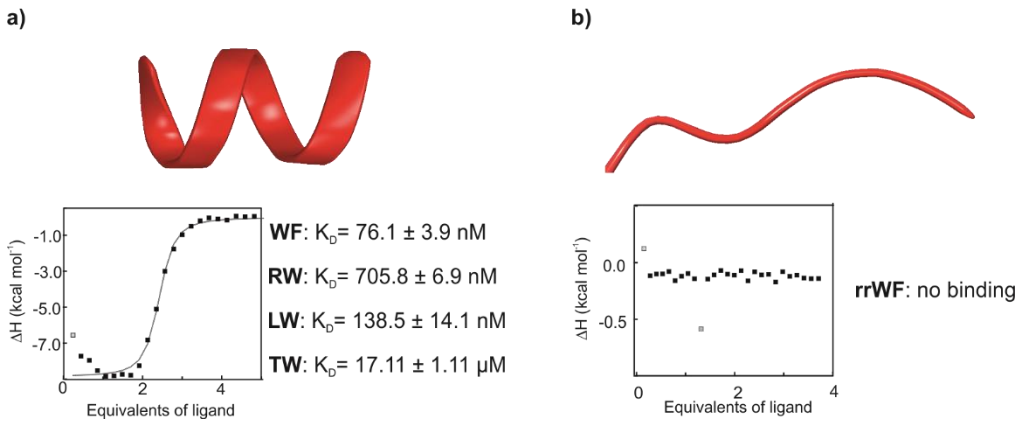


Figure 7. Representative ITC titration curves and determined K_D values for ordered fragments (**a**) and non-helical analog (**b**) confirming the necessity of the compact and bulky structure.

2.4. The best 12 fragments of the library were synthesized individually with free thiol functionality and a dynamic combinatorial library was generated in a glutathione redox buffer using calmodulin as template. Under this experimental condition, the best binding ligand was the thermodynamically most favored species, because the selection of the products is based on their position in the free energy landscape.

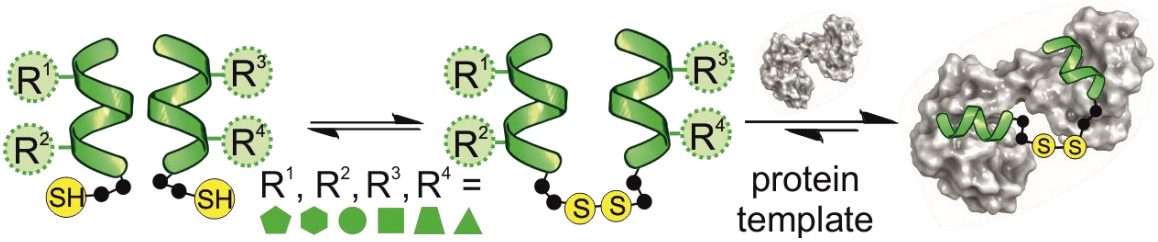


Figure 8. Self-sorting of the folded segments in the presence of the protein target using a dynamic combinatorial library based on a disulfide-exchange reaction, and selection of the highest-affinity ligand.

2.5. Product distribution of the 102-membered DCLs was analyzed and amplification factor was determined for each library member (Figure 9a). The most amplified dimeric helix (**LW-SS-RW**) considered to be the best ligand for calmodulin was selected for further characterization.

2.6. **LW-SS-RW** was re-synthesized via linking the fragments in appropriate orientation by the thioether linkage (**LW-S-RW,** Figure 9b), that showed two orders of magnitude lower dissociation constants than that of the monomeric fragments.

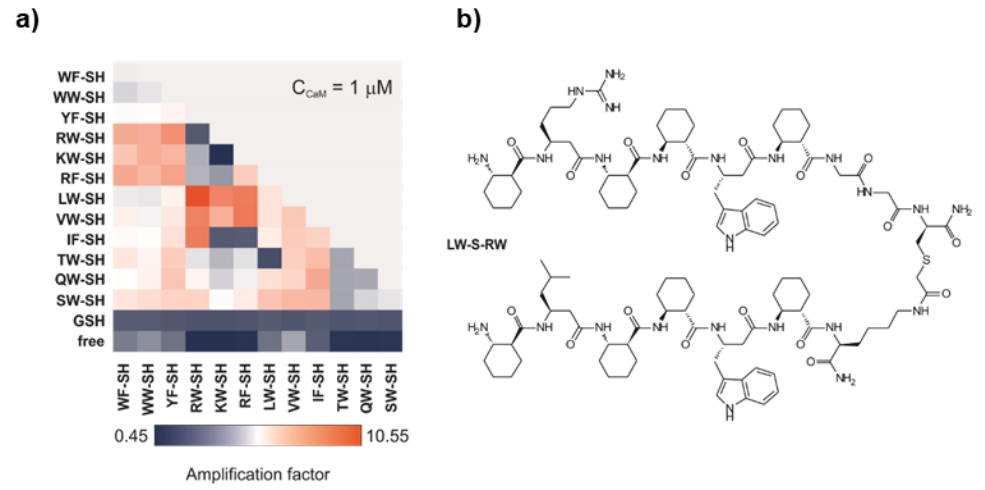


Figure 9. Amplification factors of the members of the dynamic combinatorial library (**a**) The amplification factor was defined as the enhancement of a given molecule in the presence of the template. Codes of the monomeric foldamers are on the horizontal and vertical axes. Color scales from blue to red indicate the lowest to highest amplification factor, **b)** structure of the most amplified dimer synthesized via thioether linkage.

2.7. Competitive experiments confirmed that the foldameric ligand bound to the same binding site as the native ligand (C-terminal of the vanilloid receptor, $TRPV1_{784-798}$) and the foldamer can function as a protein-protein interaction inhibitor.

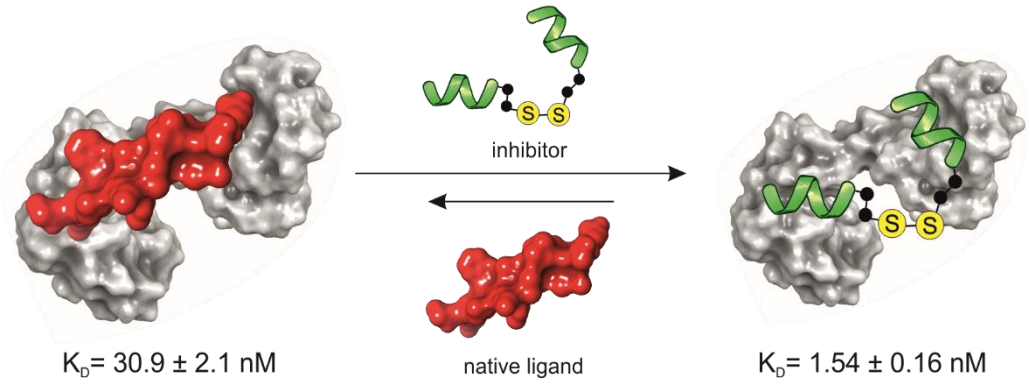


Figure 10. Schematic representation of inhibitory potential of **LW-S-RW** and calculated K_D values for foldameric binder and native ligand (C-terminal fragment of vanilloid receptor; $TRPV1_{784-798}$) of CaM.

1. Full papers related to the thesis

I. É. Bartus, G. Olajos, I. Schuster, Zs. Bozsó, M. A. Deli, Sz.Veszelka, F. R. Walter, Zs. Datki, Zs. Szakonyi, T. A. Martinek, L. Fülöp (2018). Structural Optimization of Foldamer-Dendrimer Conjugates as Multivalent Agents against the Toxic Effects of Amyloid Beta Oligomers. *Molecules,* 23(10), 2523.

IF: 3.098

II. G. Olajos, É. Bartus, I. Schuster, G. Lautner, R. E. Gyurcsányi, T. Szögi, L. Fülöp, T. A. Martinek (2017). Multivalent foldamer-based affinity assay for selective recognition of Aβ oligomers. *Analytica Chimica Acta,* **960**, 131-137.
IF: 5.123

III. É. Bartus, Zs. Hegedüs, E. Wéber, B. Csipak, G. Szakony, T. A. Martinek (2017). De Novo Modular Development of a Foldameric Protein–Protein Interaction Inhibitor for Separate Hot Spots: A Dynamic Covalent Assembly Approach. *ChemistryOpen*, **6** (2), 236-241.
IF: 2.801

Other full papers

IV. A. Hetényi, L. Németh, E. Wéber, G. Szakonyi, Z. Winter, K. Jósvay, É. Bartus, Z. Oláh, T. A. Martinek. (2016). Competitive inhibition of TRPV1–calmodulin interaction by vanilloids. *FEBS Letters* **590**(16): 2768-2775.
IF: 3.623

Scientific lectures related to the thesis

1. Bartus É., Hegedüs Zs., Wéber E., Csipak B., Szakonyi G., Martinek T.
   Célfehérje által vezérelt ligandum tervezés rendezett fragmensek segítségével - Affinitás-növelés
   MTA Peptidkémiai Munkabizottság Tudományos Ülése
   Balatonszemes, 2016. május 30- június 1.

2. Bartus É., Hegedüs Zs., Wéber E., Csipak B., Szakonyi G., Martinek T.
   Célfehérje által vezérelt ligandum tervezés rendezett fragmensek segítségével
   Magyar Tudomány Ünnepe Előadóülés
   Szeged, 2016. november 8.

3. É. Bartus, Zs. Hegedüs, E. Wéber, B. Csipak, G. Szakonyi, T.A.Martinek
   Target directed synthesis of foldameric protein-protein interaction inhibitors
   Poster presentation
   Blue Danube Symposium on Heterocyclic Chemistry Conference
   Linz, August 30-September 2, 2017.

4. Bartus É., Mag B, Csipak B., Ecsédi P. Nyitray L. Martinek T. Proteinek felismerési mintázata a foldamer térben
   MTA Peptidkémiai Munkabizottság Tudományos Ülése
   Balatonszemes, 2018. május 28-30.