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Design strategies for protein-protein interaction inhibitors using non-natural amino acids

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A. Introduction and aims

Proteins are the key elements of life. They govern various cellular functions, serve as molecular skeletons and catalyze chemical reactions. A complicated network of protein-protein interactions is involved in maintaining homeostasis in every living being. Proteins interact with each other through complex interfaces varying in area, size, and chemical characteristics. These interfaces lack deep binding pockets, which could be targeted with small molecules, thus considered „undruggable" or „hard-to-drug" using classical pharmaceutical chemistry approaches. Large molecules e.g., peptides, peptidomimetics, and proteins, are structurally better suited for this purpose. Skolnick and his colleagues discovered that despite having different overall structures, different proteins could have similar side chain arrangements at their protein-protein interfaces. This discovery led to the question of whether it is possible to design short, biomimetic molecules with secondary structures that are mimetics of these surface segments and have enough surface area to bind to a target protein. A large enough library containing these small surface fragments with different amino acid side chains could be used to probe a target protein. Tight binders could then be assembled into larger peptidomimetic molecules that can bind with high affinity and specificity.

An interesting phenomenon in structural biology is the interaction of proteins where one or both interacting partners are disordered; they do not adopt a well-defined structure under native conditions. In some cases, an IDP folds into a binding conformation when forming contact with its partner. Other disordered interfaces can only be described using multiple conformational states, hence the name fuzzy complex. The latter is an incredibly challenging interface for drug development; the binding surface on the receptor is shallow and diffuse, and the fuzzy partner is usually highly hydrophilic, lacking well-defined hot-spot residues. The amino acid composition in intrinsically disordered regions contains mostly hydrophilic residues, rendering the top-down design of IDP mimetics rather difficult. Extending the available side chains space with the incorporation of non-canonical amino acids opens up many possibilities in IDP mimetics drug design.

Our objective was to demonstrate that using surface fragments with a fragment-based approach can target protein-protein interfaces that were previously considered undruggable. We focused on short foldameric helices, which have a surface area large enough to interact with other helices or hot spots on proteins. These helices are made up of β-amino acids, which have a constrained backbone that stabilizes the helical structure. Our aim was to show that these helices can mimic local surfaces and be used as building blocks in a bottom-up design approach. Finally, our plan was to examine how an intrinsically disordered segment, SSB-Ct, interacts with its partner proteins, ExoI and RecO. Our aim was to determine whether we could enhance the weak binding affinity of a conserved and disordered

peptide by incorporating non-natural amino acids. We wanted to stabilize the fuzzy complex in a single binding conformation by making modifications that promote enthalpically driven binding at the expense of conformational entropy.

B. Methods

Pulldown assay experiments

Screening of the 512-membered fragment library with the five target protein was carried out in a pulldown assay. Proteins were immobilized on affinity resin and incubated with the foldamer library in an equimolar setup ($n_{foldamer}$:$n_{protein}$ = 1:1). The unbound fraction was quantified using HPLC-MS.

Fluorescence anisotropy experiments

The binding properties of SSB-Ct variant peptides were assessed in fluorescence anisotropy experiments using CLARIOstar® Plus Multi-Mode Microplate Reader spectrophotometer. Direct titrations of carboxyfluorescein-labeled peptides and competition titrations were also performed. Experiments were executed in three technical repeats, and the average fluorescence polarization signals were analyzed in Origin Pro 9.5. IC50/EC50 values were determined by Logistics Nonlinear fit function.

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were performed using a MicroCal VP-ITC titration microcalorimeter at 30 °C. 300 rpm stirring was used. Ligand solution was titrated to the protein-containing cell using 20 μL volumes over 300 seconds of injection time. The raw data were integrated using NITPIC. The binding parameters, association equilibrium constant (K_A), binding enthalpy (ΔH), and binding entropy (ΔS) were obtained by fitting the titration curves to a model of A + B = C in SEDPHAT. Stoichiometry was fixed to 1:1, and incompetent fraction was fitted for protein concentration.

C. Results and discussion

1. Local surface mimetics

We proved that foldameric local surface mimetics (LSMs) exhibit large enough surface area to have around 1-500 μM affinity toward proteins, can recognize orthosteric and non-orthosteric hot spots on protein surfaces, and are able to differentiate proteins by their functions. The total amount of foldamers bound to the proteins correlates with the number of protein-protein interaction partners a protein has. The preferred side chains are similar to the side chains of interacting partners. The side chain enrichments of foldamer-protein interfaces are similar to the enrichment of protein-protein interfaces.

1.1. Proteins mapped using LSMs

Five proteins were selected based on their structural homology, types of interactions, and the number of interacting protein partners. The reference protein was CaM, which plays a vital role in eukaryotic cells, and two other EF-hand proteins, S100A4 and S100B, were selected from the S100 family. Galectin-1 (Gal-1) and the winged helix domain of RecQ helicase (RecQ-WH) were used as target models with a low tendency to form protein-protein interactions.

1.2. Design of local surface mimetic library

Peptide sequences made from β-amino acids have a strong tendency to fold into secondary structures at short chain lengths, with specific geometry controlled by the backbone stereochemistry pattern and side chain topology. We utilized H14 and H12 helices, which have proteogenic side chains on the same face and comparable side chain distances to native PPI interfaces. Sixteen different β^3-amino acids were substituted at two positions, generating 512 local surface mimetics (LSMs) screened as sublibraries (L1-L4). LSMs are named using letter codes in the R1 and R2 positions, with four amino acids excluded from the pool.

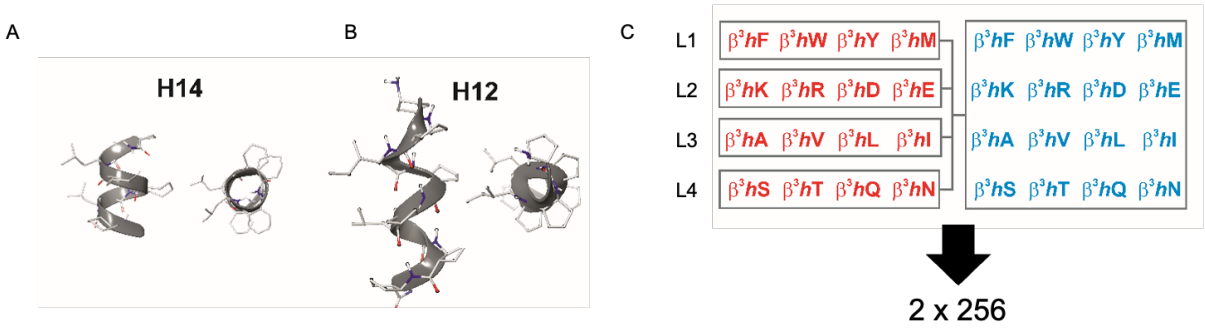

Figure 1. Design of local surface mimetic probe libraries using short foldamers. Scaffolds and structures of H14 (A) and H12 helices (B) and library composition showing sublibrary grouping (C).

1.3. LSM affinity patterns indicate secondary structure-dependent structural compatibility

Local surface mimetics (LSMs) were tested in pull-down assays. We found that the binding of LSM probes to their targets is not solely driven by hydrophobic and cyclic β-amino acid residues, as there were variations in binding patterns. The binding affinity of identified hits was calculated from bound fractions. The affinity limit of LSM was set to K_D of 150 μM. Many hits were obtained across all proteins, indicating that LSMs can interact with their targets. The H14 helical probes were found to have an overall ability to act as a local probe and adapt to different protein surfaces, while the H12 probes were less favored and yielded fewer hits. The orientation of the R1 and R2 proteogenic side chains had only a minor impact on binding.

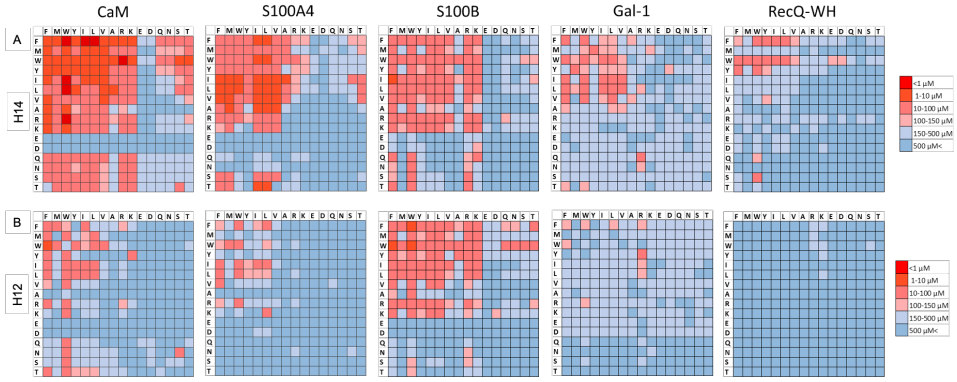

Figure 2. Binding patterns of foldameric LSM probes are represented as heat maps in the K_D dimension (in μM)..

1.4. Foldameric LSMs detect orthosteric and non-orthosteric spots

Pull-down assays were performed using native ligands of target proteins to assess the drug-like behavior of the H14 helical LSMs. Native ligands changed the LSM affinity patterns for all proteins. However, only a few LSM probes displayed replacement for Gal-1, likely due to its glycan selective native recognition domain. The replacement pattern for a single probe can be complex, as it can bind to both orthosteric and non-orthosteric spots, which limits overall displacement levels. To avoid overlooking interesting orthosteric weak binders, direct replacement levels were assessed and marked in Figure 3.

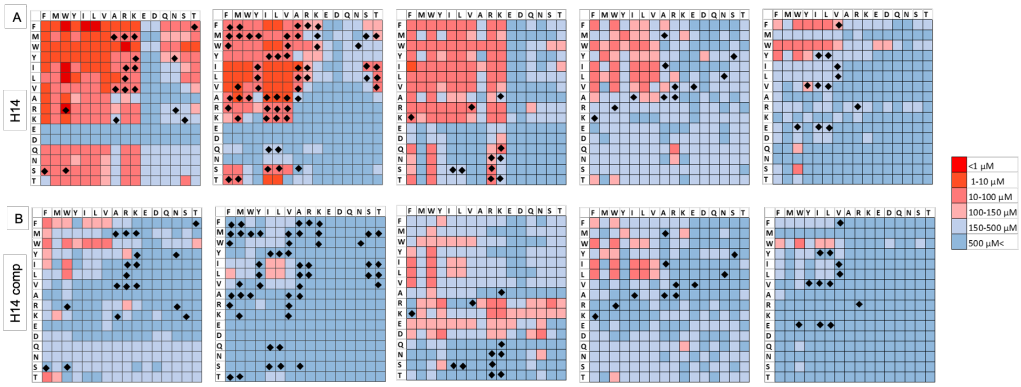

Figure 3. Competition pull-down experiments with the H14 LSM library. K_D heat maps show side chain preferences using H14 helices alone and in the presence of native ligands as competitors.

1.5. Affinity patterns of foldameric LSMs are characteristic of the target proteins

Next, we investigated if LSM probes can differentiate proteins based on their PPI interfaces. The correlation between the promiscuity of each protein towards LSM probes and their natural interactome was assessed using PPI databases. The results showed a correlation between the number of database PPI partners and the average bound fraction values of H14 libraries. Pairwise covariance values were calculated from the bound fraction values for each protein pair, and we found that non-orthosteric binders exhibit promiscuous behavior. The scaled covariances for orthosteric binders were between 13-20% for proteins with high structural homology, suggesting that the foldameric probes detected a low level of interface similarity.

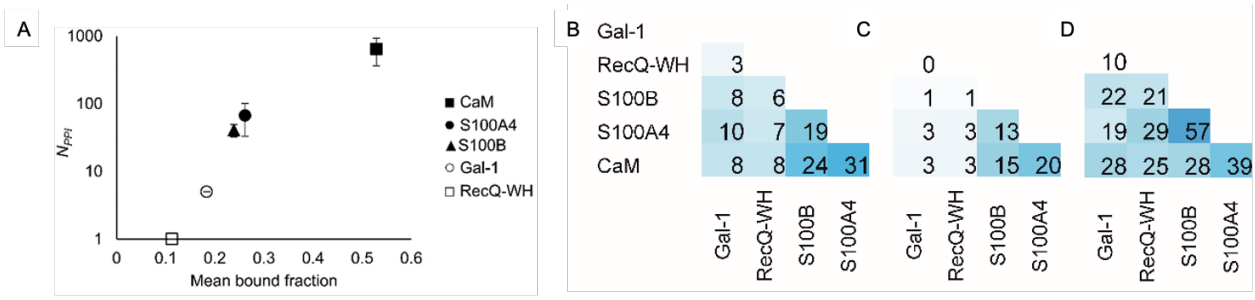

Figure 4. Average bound fractions for the H14 helical LSM library compared with the mean number of PPIs found in databases (A). Pairwise scaled covariances (%, maximum similarity: 100%, zero covariance: 0%) of H14 LSM fraction bound (FB) patterns calculated for total (B) and the orthosteric (C) FB values. %. The sequence homologies of proteins are given in panel (D).

1.6. Side chain binding propensities are biomimetic

Normalized frequencies were calculated for each residue to compare side chain enrichment on the foldamer-protein interface to naturally occurring protein-protein interaction interfaces. We found that the side chains selected from the LSM libraries are similar to the selection of natural interacting partners. The affinity patterns observed closely match the natural side chain preferences of each protein tested. We could conclude that the foldameric probes are biomimetic and may serve as minimal surface mimetic motifs for drug design.

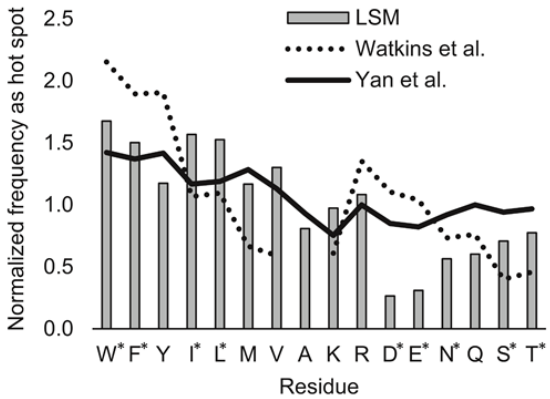

Figure 5. Normalized frequencies were obtained from literature data and calculated for the H14 LSM library. Values from Watkins et al. and Yan et al. are based on computational Ala scanning and PPI interface analysis, respectively.

2. Designing bacterial single-stranded DNA-binding protein (SSB) mimetic peptides to inhibit its interactions with DNA metabolizing enzymes

We could modify SSB-Ct, a highly conserved and flexible sequence, to increase its affinity toward its interacting partners. Combining modifications created high-affinity binders for ExoI and RecO, with lower overall conformational flexibility of the octapeptide. Molecular dynamics simulations were used to assess the reduced conformational flexibility of the designer peptides. We propose a two hot spot binding model of SSB-Ct octapeptide to interacting partners and suggest that these results could lead to the design of effective SSB mimetic antibiotics.

2.1. The role of the proximal segment of SSB in binding to its interacting partners

Crystal structures of SSB-Ct octapeptide (DFDDDIPF) in complex with interacting partners contain only the 3-4 C-terminal residues (proximal segment). However, literature data suggest the importance of the rest of the peptide - the distal segment - in binding. Our molecular dynamics simulations are in accord with the literature and showed increased flexibility of SSB-Ct with the C-terminal end being more fixed in position than the proximal end. We conducted competitive fluorescence anisotropy assays with synthetic half octapeptides to determine the importance of different segments of SSB-Ct in binding. We could conclude that the proximal segment of SSB-Ct can have transient stabilizing contacts with the secondary hot spots on ExoI and RecO and that both segments are important in the binding of SSB-Ct to these proteins.

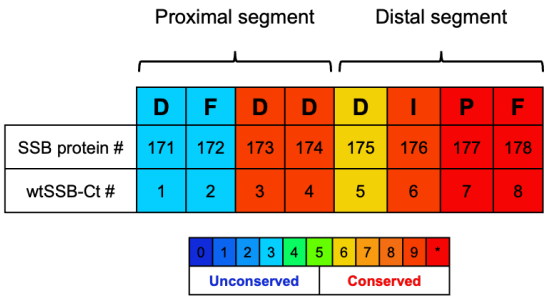

Figure 11. Sequence and conservation of SSB C-t.

2.2. Synthesis and screening of the single mutant SSB-Ct library

We generated a library of modified SSB-Ct peptides to investigate the effect of chemical modifications on affinity. The use of non-natural amino acids enabled us to study the importance of side-chain chemistry. The library members were screened using a fluorescence anisotropy-based competition assay. We could identify favorable, tolerated, and detrimental substitutions.

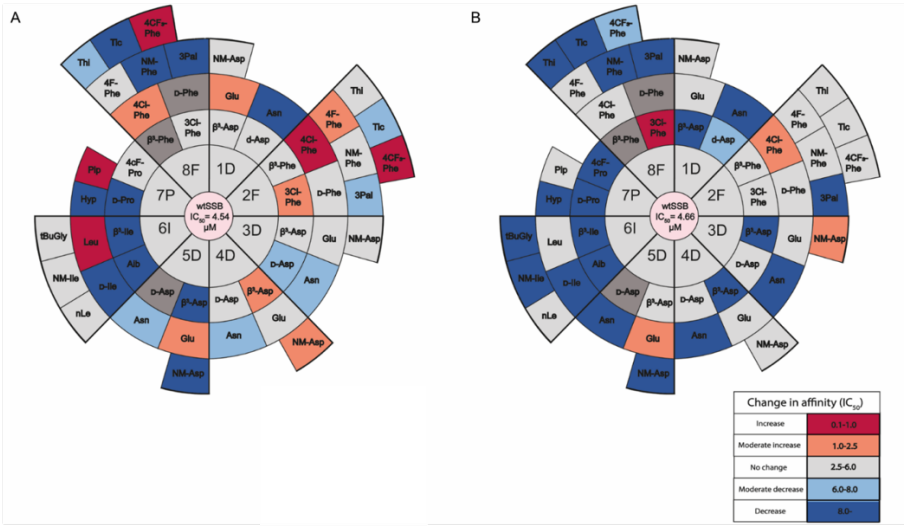

Figure 11. Screening and design of the mSSB-Ct library. Radial heat map showing competitive fluorescent anisotropy screening data of mSSBs on A) ExoI and on B) RecO. IC_{50} values lower than 1 μM are highlighted in red. Moderate decreases in IC_{50} (1.0 – 2.5 μM) are shown in orange.

2.3. Modifications both in the proximal DFDD and the distal DIPF segments increase affinity

We found that certain modifications at positions 2 and 8 of the peptide significantly increased the affinity towards ExoI and RecO, respectively. We also observed that the behavior of the two proteins tested is not uniform in interacting with Phe2. The results suggest that appropriate chemical modifications in the side chain of Phe2 can increase affinity to the targets even if the native binding mode of SSB-Ct does not directly depend on the presence of Phe2.

2.4. Combined modifications yield high-affinity ligands

We tested the combination of favorable target-specific single mutations to increase affinity. Modified peptides E1-sSSB-Ct and E2-sSSB-Ct yielded further improvement in binding to ExoI.

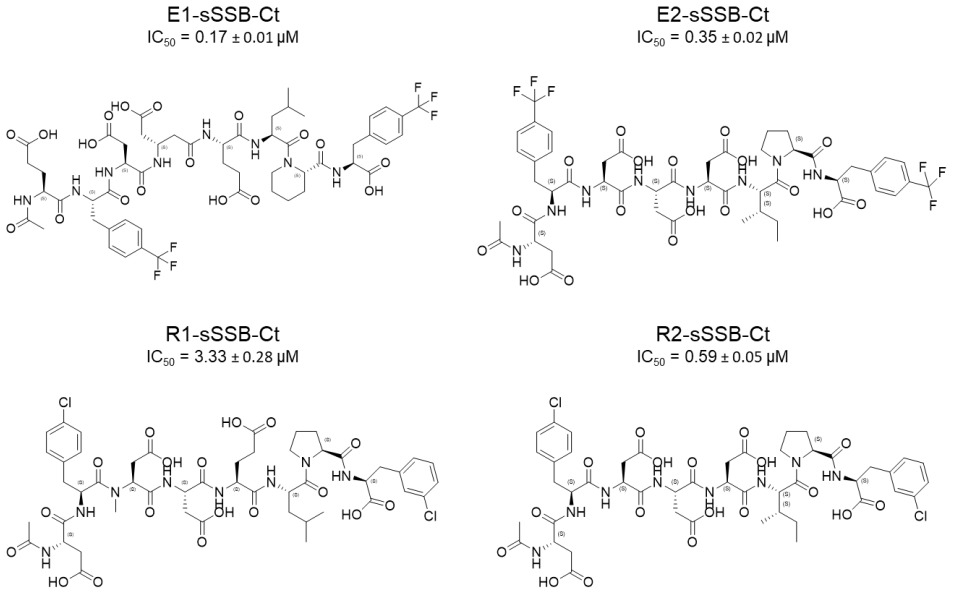

Figure 12. Combined modifications for SSB-Ct. E1-sSSB-Ct and E2-sSSB-Ct were tested on ExoI. R1-sSSB-Ct and R2-sSSB-Ct were tested on RecO. IC_{50} values show the ability of the sequences to compete with F-wtSSB-Ct in competitive fluorescent anisotropy assay.

Isothermal titration calorimetry measurements showed that the main enthalpy gain was due to the 4-CF3-Phe-Phe replacements at positions 2 and 8, supporting a two-hot-spot binding scenario. However, the additional modifications in E1-sSSB-Ct could only slightly improve binding affinity. For RecO, only R2-sSSB-Ct could improve affinity. For all peptides containing multiple favorable modifications, the affinity increase was enthalpy driven, with a marked enthalpy-entropy compensation effect.

2.5. Molecular dynamics simulations provide insight into the binding modes of E-sSSB-Cts and R-sSSB-Cts

We used replica-exchange solute tempering simulations to test the changes in residual flexibility of E-sSSB-Cts and R-sSSB-Cts. Both simulations showed reduced residual flexibility in the bound state, improving stability. E-sSSB-Cts were anchored to site B through $4CF_3$-Phe2 and formed stable contact with site A through 4CF3-Phe8, resulting in lower RMSF values. R-sSSB-Cts were connected to sites A and B through 4Cl-Phe2 and 3Cl-Phe8, respectively. The central segment of R-sSSB-Cts was the least flexible part compared to the terminals, and the RMSF values displayed an overall downward shift.

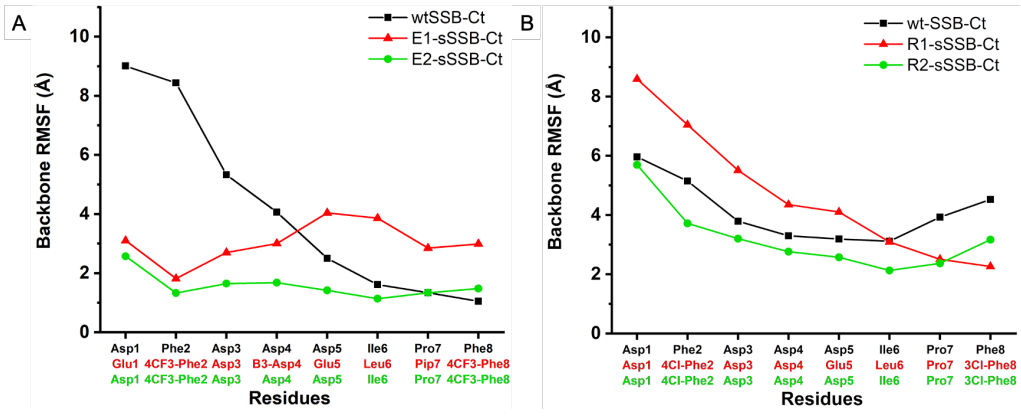

Figure 13. RMSF values of the backbone atom coordinates for wtSSB-Ct (black squares), E1-sSSB-Ct (red triangles), and E2-sSSB-Ct (green circles) in interaction with ExoI (A). RMSF values of the backbone atom coordinates displaying wtSSB-Ct (black squares), R1-sSSB-Ct (red triangles), and R2-sSSB-Ct (green circles) in interaction with RecO (B).

Full papers related to the thesis

I. A. Tököli, B. Mag, É. Bartus, E. Wéber, G. Szakonyi, M. A. Simon, Á. Czibula, É. Monostori, L. Nyitray, T. A. Martinek (2020). Proteomimetis surface fragments distinguish proteins by function. *Chemical Science*, 11, 10390-10398

II. A. Tököli, B. Bodnár, F. Bogár, G. Paragi, A. Hetényi, É. Bartus, E. Wéber, Z. Hegedüs, Z. Szabó, G. Kecskeméti, G. Szakonyi, T. A. Martinek (2023). Structural adaptation of the single-stranded DNA-binding protein C-terminal to DNA metabolizing partners guides inhibitor design. *Pharmaceutics*,

Other full papers

III. A. Hetenyi, E. Szabo, N. Imre, K. N. Bhaumik, A. Tököli, T. Füzesi, R. Hollandi, P. Horvath, Á. Czibula, É. Monostori, M. A. Deli, T. A. Martinek (2022). α/β-Peptides as Nanomolar Triggers of Lipid Raft-Mediated Endocytosis through GM1 Ganglioside Recognition. *Pharmaceutics*, *14*(3), 580

IV. É. Bartus, A. Tököli, B. Mag, Á. Bajcsi, G. Kecskeméti, E. Wéber, Z. Kele, G. Fenteany, T. A Martinek (2022). Light-fuelled primitive replication and selection in evolvable biomimetic chemical networks. *ChemRxiv*, under major revision, 10.26434/chemrxiv-2021-3dnjt-v4

Scientific lectures related to the thesis

1. A. Tököli, G. Szakonyi, T. Martinek. A bakteriális RecQ helikáz szárnyas doménjének karakterizálása, egy lehetséges antibakteriális célpont.
   MTA Peptidkémiai Munkabizottsági Ülés 2017. Balatonszemes – 2017. 05. 29.
2. A. Tököli, É. Bartus, G. Szakonyi, T. Martinek. Characterization of the bacterial winged-helix domain of RecQ helicase. A novel antibacterial target.
   7th BBBB International Conference on Pharmaceutical Sciences – Balatonfüred – 2017. 10. 06.
3. A. Tököli, B. Mag, É. Bartus, E. Wéber, G. Szakonyi, M. A. Simon, Á. Czibula, É. Monostori, L. Nyitray, Tamás A. Martinek. Protein affinity patterns of foldameric local surface mimetics: druggability and promiscuity.
   Department of Medicinal Chemistry, Institute Seminar. Szeged – 2020.11.27.
4. A. Tököli, B. Bodnár, F. Bogár[2], G. Paragi, A. Hetényi, É. Kovács-Bartus, E. Wéber, Z. Hegedüs, G. Szakonyi and T. A. Martinek. Two anchor point-binding of the SSB C-terminal to DNA metabolizing proteins facilitates the development of enhanced inhibitors.
   Peptide Chemistry and Chemical Biology Working Committees of the Hungarian Academy of Sciences – Symposium 2022. Balatonszemes – 2022. 05. 30.