PhD Thesis

Investigation of protein–ligand interactions of targets with solvent-exposed binding sites

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A. Introduction and aims

The goal of pharmaceutical research is the specific and effective manipulation of disease-modifying targets, which is ideally achieved by small molecules binding to the macromolecule with high affinity. However, it is still a challenging task to inhibit molecular interactions where large surface areas or solvent-exposed binding sites are involved. Macromolecules which could not be efficiently modulated by drug-like small molecules are designated as undruggable proteins. For the inhibition of these targets, a potential solution might be to exploit designed non-natural folded polymers (foldamers), which have emerged as promising materials for biomolecule recognition. We have investigated two medicinally relevant targets: the immunosuppressant, angiogenic and tumour-nursing Galectin-1 (Gal-1) homodimer, whose inhibition might result in antimetastatic and antiangiogenic drugs; and the synaptotoxic Amyloid β1-42 (Aβ1-42) fibrils and oligomers, which are among the major causative agents of Alzheimer's disease. The inhibition of Gal-1 and Aβ1-42 aggregates is difficult due to their solvent-exposed binding site and the absence of distinct binding pockets.

For studies of the protein–ligand systems, we have used primarily nuclear magnetic resonance (NMR) spectroscopy, which has become a powerful and versatile technique for the characterization of biomolecules and for the detection of molecular interactions. NMR can be exploited in the process of preclinical drug discovery by finding initial hits through screening, supporting lead optimization, fragment-based drug design and obtaining structure-activity relationships (SAR). As opposed to its complementary method X-ray crystallography, NMR can be used to investigate protein–ligand systems in a biologically more relevant medium, solution. Thus, molecular motions, which are confined to the solution phase, can also be captured. The atomic-level structural and dynamic information gained on biomolecules and their complexes from NMR studies can facilitate an understanding of their functions and mechanisms of action and can be utilized in structure-based drug design for the rational improvement of ligands.

Our primary aim was to gain information about our targets by means of NMR spectroscopic techniques, which can promote the structure-based design of new Gal-1-binding compounds and Aβ1-42 inhibitors. We aimed to optimize, validate and improve binding tests which can be efficiently used in an NMR screening process for new inhibitors. In the case of Gal-1, the NMR signal assignment of the labelled protein was a prerequisite for the experiments, so we set out to assign the backbone resonances of $^{15}N/^{13}C$-labelled Gal-1. As a first step, the investigation of the proteins

alone and/or in the presence of their natural or literature peptide ligands was targeted. The goal was to characterize their interactions and to obtain relevant information about the mode of action of the ligands. Finally, we planned to utilize the techniques and structural/dynamic information in drug discovery, most favourably, for the design of foldamer type ligands of the proteins. In order to support and explain the NMR results and test new compounds, the measurements were supplemented with other biophysical techniques and with biological experiments, such as isothermal titration calorimetry (ITC), particle size measurements and enzyme-linked immunosorbent assay (ELISA).

B. Methods

Ligand-detected NMR experiments

The interactions of Gal-1, asialofetuin (ASF) and Aβ1-42 and their ligands were characterized via ligand-observed NMR methods, which included saturation transfer difference (STD), transferred nuclear Overhauser effect (trNOE) and signal quenching experiments. For conventional ^{1}H STD experiments, the selective saturation of the protein was achieved via Gaussian-shaped pulses. For the trNOE experiments, 2D NOESY measurements were performed with and without the protein. The techniques were used for the following samples:

(i) Gal-1 or ASF and literature with Tyr-Xxx-Tyr type peptide ligands (TYDYF-NH_2, WYKYW-NH_2, TYDYFR-NH_2 and TYPYFR-NH_2) or lactose,

(ii) fibrillar Aβ1-42 with neuroprotective pentapeptides (LPFFD, LPYFD-NH_2, FRHDS-NH_2 and RIIGL-NH_2) and/or Thioflavin T (ThT),

(iii) Aβ1-42 oligomers with foldamer peptides.

With the aim of improving the methodology, different excitation schemes were probed for STD experiments. In the group-selective STD (GS-STD), the samples contained lactose complexed with ^{15}N/^{13}C-labelled Gal-1. For the selective saturation of the protein, a train of BIRD^{d} pulses was applied. For the aliphatic/aromatic ^{13}C GS-STD spectra, the band-selective inversion of carbon was obtained with a Q3 Gaussian cascade of 256 ms within the BIRD^{d} cycle.

Protein-detected NMR experiments

Protein-detected experiments were used for ^{15}N/^{13}C-Gal-1. For the resonance assignment of the protein, 3D HNCO, HNCA, HN(CO)CA, HN(CA)CO, CBCA(CO)NH, HBHA(CO)NH and ^{15}N-NOESY-HSQC experiments were recorded and analyzed. In the ^{15}N Heteronuclear Single Quantum Coherence (HSQC) titration experiments, ^{15}N HSQC spectra of ^{15}N-labelled Gal-1 were recorded for the protein alone and with increasing ligand (lactose or Tyr-Xxx-Tyr peptides) concentrations. In

order to explore the dynamic behaviour of Gal-1 in solution, T_1, T_2 relaxation and heteronuclear NOE experiments were recorded for samples of Gal-1 with and without lactose, and model-free analysis was carried out.

Isothermal titration calorimetry for Aβ1-42 oligomers and foldamers

Isothermal titrations were performed for Aβ1-42 oligomers and foldamers with a Microcal VP-ITC microcalorimeter. The experimental data were fitted to the two-independent-site binding model.

Other methods

The binding of Tyr-Xxx-Tyr peptides to Gal-1 was investigated by ELISA test for the binding of Gal-1 to ASF in the presence of competitors and by flow cytometric analysis of Gal-1 binding to cells. Time-dependent signal intensity loss and quantitative NMR spectroscopic binding tests for the Aβ1-42 fibril samples were complemented with Dynamic Light Scattering (DLS), Transmission Electron Microscopy (TEM) and ζ-potential measurements. The interaction of the multivalent foldamer and Aβ1-42 oligomers was characterized by several biophysical methods.

C. Results and discussion

1. The solution-phase behaviour of the tumour-nursing protein Gal-1 was characterized by means of NMR spectroscopy in the absence and in the presence of its natural ligand lactose.
   1.1. The backbone chemical shifts of $^{15}N/^{13}C$-labelled Gal-1 could be successfully assigned at physiological pH 7.4 and the great majority of the CB and HB resonances were also identified.
   1.2. ^{15}N HSQC titrations showed that lactose binding affects the structure of Gal-1 even remote from the CRD, on the opposite side of the protein (Figure 1).

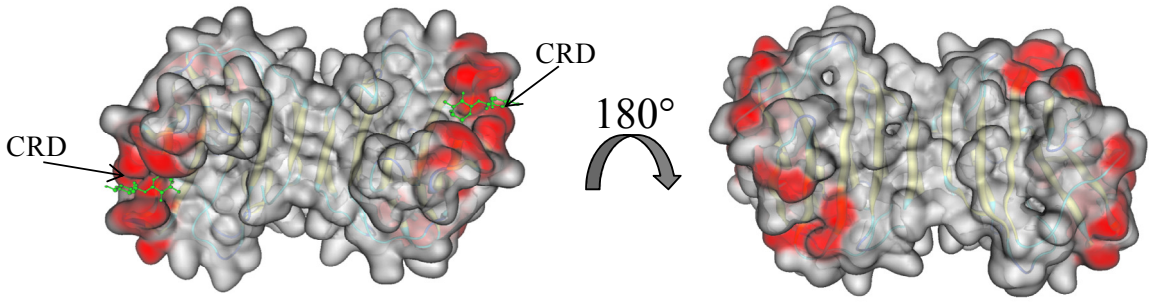

Figure 1. Results of the ^{15}N HSQC titration of Gal-1 with lactose at pH 7.4. Top 15 shifted residues are mapped (red) on the surface of the Gal-1 homodimer.

1.3. Backbone relaxation experiments and model-free analyses revealed that lactose binding not only affects the dynamic behaviour of the CRD, but these changes propagate throughout the whole protein. The outstanding flexibility of the loop involved in lactose binding deserves attention.

1.4. Lactose-complexed Gal-1 was used as a model system to study the performance of GS-STD experiments. The $^{15}N/^{13}C$ variants of GS-STD experiments provided a powerful approach for the binding epitope mapping of lactose. The STD spectra obtained in four different experimental setups (selective ^{1}H STD, ^{15}N GS-STD, $^{13}C_{Ar}$ and $^{13}C_{aliphatic}$ GS-STD approaches) revealed that the signal-intensity patterns of the difference spectra are affected by both the type and the spatial distribution of the excited 'transmitter' atoms, and also by the efficiency of the spin-diffusion-mediated magnetization transfer (Figure 2).

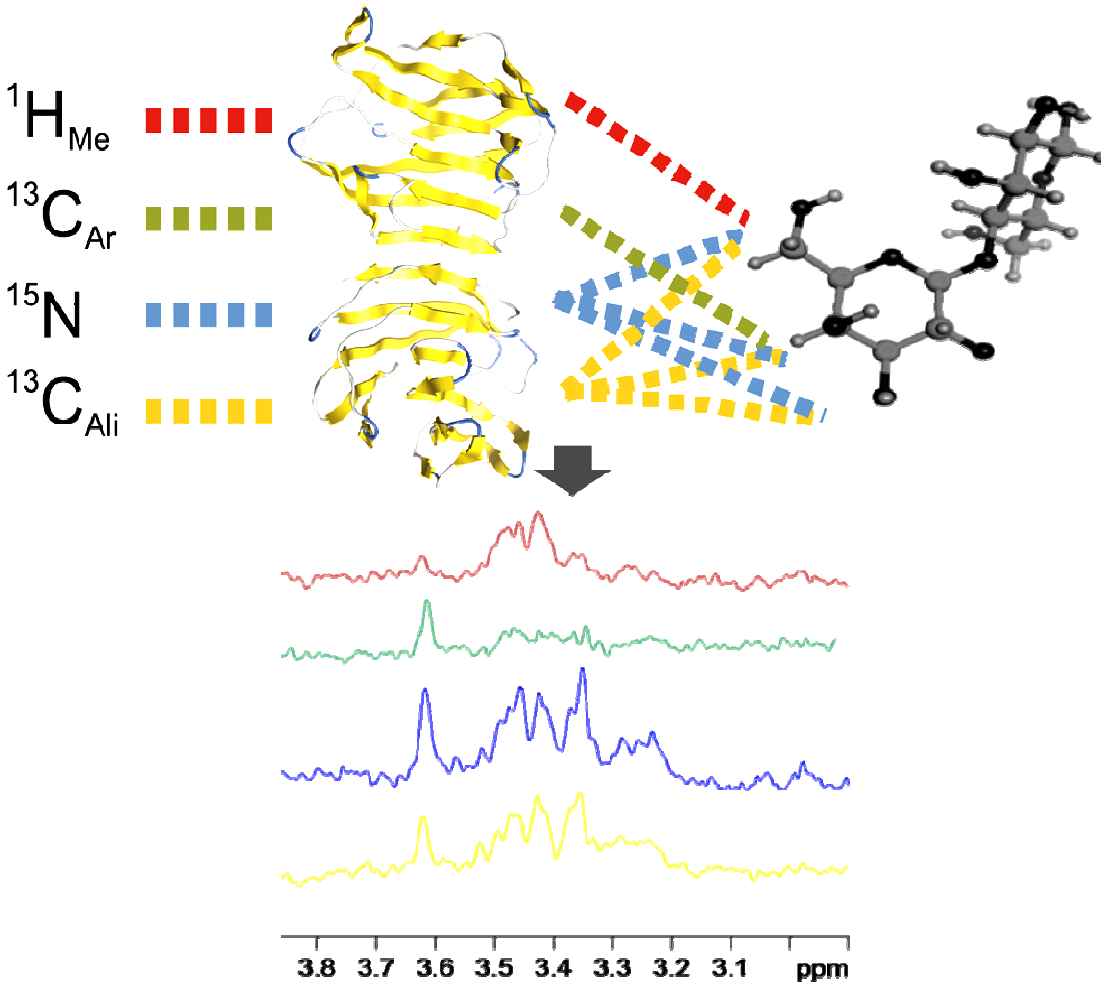

Figure 2. Different variants of ^{15}N and ^{13}C GS-STD experiments (indicated by different colours) allow the detection of weak host–guest interactions even in the presence of enhanced dynamics and in case of a solvent-exposed binding site. Due to the different spatial and chemical selectivities of the excitation schemes, local effects can be observed.

2. The Tyr-Xxx-Tyr peptide motif has been reported to be a glycomimetic sequence, mainly on the basis of its inhibitory effect on the Gal-1–ASF interaction. Our STD and trNOE NMR experiments revealed the following findings:
   2.1. The Tyr-Xxx-Tyr peptides studied did not bind to Gal-1. ^{15}N HSQC titrations with ^{15}N-labelled Gal-1 confirmed the absence of any peptide–Gal-1 interaction.
   2.2. The binding of Tyr-Xxx-Tyr peptides to ASF was clearly detected.
   2.3. The Tyr-Xxx-Tyr peptides proved effective in the competitive tests not because they were able to bind to Gal-1 and to replace ASF, but rather because of their interactions with the glycoprotein ASF (Figure 3). These data indicated that the Tyr-Xxx-Tyr peptides tested in this work are not glycomimetics as they interact with ASF via an unrevealed molecular linkage.

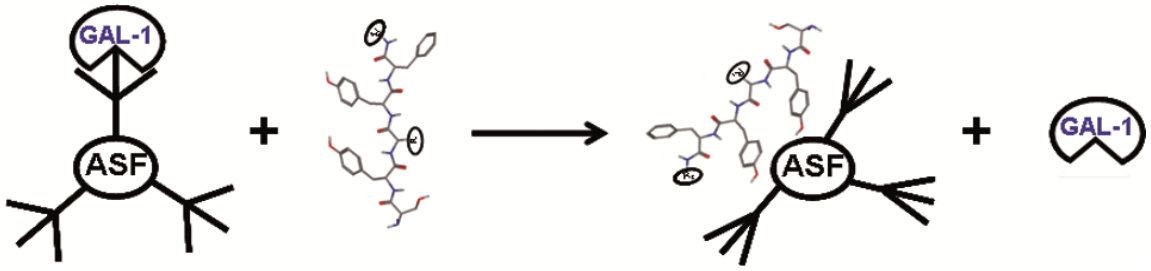

Figure 3. Proposed mechanism for inhibition of the Gal-1–ASF interaction by Tyr-Xxx-Tyr type peptides.

3. The NMR signal quenching of the neuroprotective pentapeptide LPFFD and the fluorescent dye ThT was studied in the presence of Aβ1-42 fibrils. The experiments yielded highly intriguing results:
   3.1. ThT could not fully and immediately replace LPFFD from Aβ and could not prevent the weak binding of the pentapeptide, and LPFFD did not decrease the extent of the NMR spectroscopy-detected ThT binding, but rather increased it.
   3.2. No solution NMR spectroscopically visible monomeric or oligomeric Aβ1-42 signal accompanied the resonances of the studied ligands in the spectra.
   3.3. The disaggregation mechanism of neuroprotective peptides is unlikely and ligand-induced flocculation and sedimentation processes could be proposed (Figure 4), which were confirmed by DLS and TEM experiments.

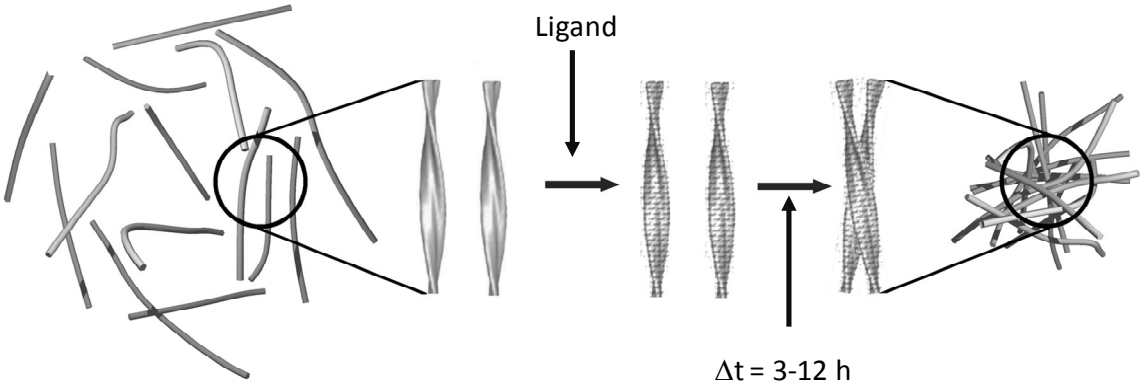

Figure 4. Schematic representation of the ligand-induced flocculation of fibrillar Aβ1-42 by non-covalent cross-linking.

4. A foldamer library was screened with STD and trNOE techniques testing their binding to soluble Aβ1-42 oligomers.
   4.1. A hexapeptide foldamer was found which exhibited weak binding to Aβ. The weak interaction could be enhanced by following the principles of multivalent ligands (Figure 5): the tetravalent generation-zero poly(amidoamine) conjugate of the peptide exhibited nanomolar binding to oligomers. The binding of the tetravalent compound showed a two-step-binding with a low nanomolar and a submicromolar apparent dissociation constants.
   4.2. Initial structure-activity relationship studies revealed that compounds with different recognition elements or with divalent construction exhibited only weak interaction, which suggests that the pharmacophore is specific.

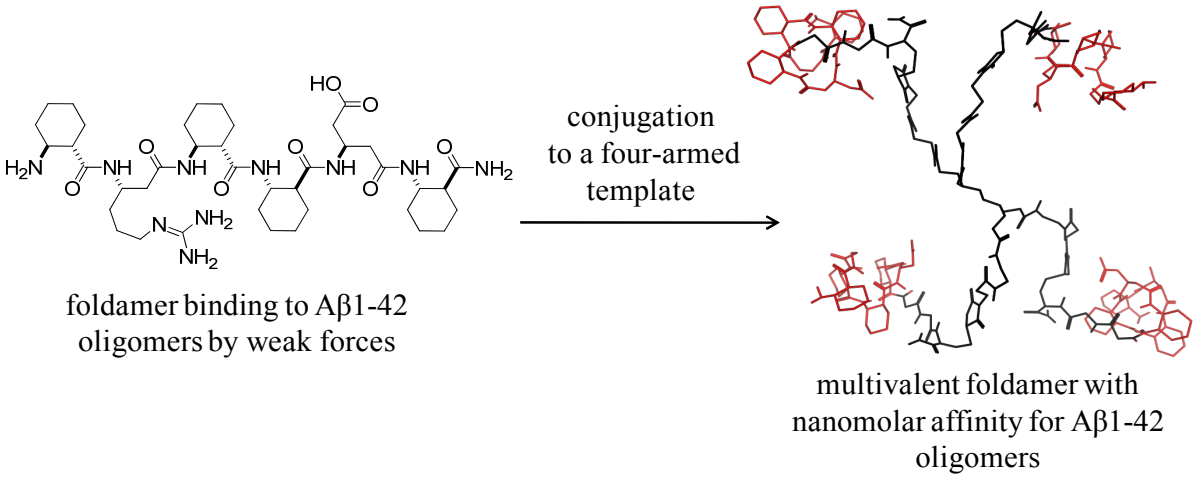

Figure 5. Applying the principle of multivalency to foldamers: a compound with nanomolar affinity for Aβ1-42 oligomers was achieved.

D. List of publications and lectures

List of publications and lectures

Full papers related to the thesis

I. A. Hetényi, L. Fülöp, T. A. Martinek, **E. Wéber**, K. Soós, B. Penke
Ligand-induced flocculation of neurotoxic fibrillar Aβ(1–42) by noncovalent crosslinking.
ChemBioChem **2008**, *9*, 748–757. IF: 3.945*

II. **E. Wéber**, A. Hetényi, B. Váczi, É. Szolnoki, R. Fajka-Boja, V. Tubak, É. Monostori, T. A. Martinek
Galectin-1–Asialofetuin interaction is inhibited by peptides containing the Tyr-Xxx-Tyr motif acting on the glycoprotein.
ChemBioChem **2010**, *11*, 228–234. IF: 3.945

III. K. E. Kövér, **E. Wéber**, T. A. Martinek, É. Monostori, G. Batta
^{15}N and ^{13}C group-selective techniques extend the scope of STD NMR detection of weak host–guest interactions and ligand screening.
ChemBioChem **2010**, *11*, 2182–2187. IF: 3.945

IV. L. Fülöp, I. M. Mándity, G. Juhász, V. Szegedi, A. Hetényi, **E. Wéber**, Z. Bozsó, D. Simon, M. Benkő, Z. Király, T. A. Martinek
A foldamer-dendrimer conjugate neutralizes synaptotoxic β-amyloid oligomers.
PLoS ONE **2012** submitted

Other full papers

1. E. Háznagy-Radnai, B. Réthy, S. Czigle, I. Zupkó, **E. Wéber**, T. Martinek, G. Falkay, I. Máthé
Cytotoxic activities of Stachys species.
Fitoterapia **2008**, *79*, 595-597. IF: 1.899

2. G. Benedek, M. Palkó, **E. Wéber**, T. A. Martinek, E. Forró, F. Fülöp
Efficient synthesis of hydroxy-substituted cispentacin derivatives.
Eur. J. Org. Chem. **2008**, *20*, 3724–3730. IF: 3.206

3. I. M. Mándity, **E. Wéber**, T. A. Martinek, G. Olajos, G. Tóth, E. Vass, F. Fülöp
Design of peptidic foldamer helices: A stereochemical patterning approach.
Angew. Chem. Int. Ed. **2009**, *48*, 2171-2175. IF: 12.730

4. G. Benedek, M. Palkó, **E. Wéber**, T. A. Martinek, E. Forró, F. Fülöp
Efficient synthesis of 3,4- and 4,5-dihydroxy-2-amino-cyclohexanecarboxylic acid

enantiomers.
Tetrahedron: Asymmetry **2009**, *20*, 2220-2225. IF: 2.484

5. M. Palkó, G. Benedek, E. Forró, **E. Wéber**, M. Hänninen, R. Sillanpää, F. Fülöp
Synthesis of mono- and dihydroxy-substituted 2-aminocyclooctanecarboxylic acid enantiomers.
Tetrahedron: Asymmetry **2010**, *21*, 957-961. IF: 2.484

6. Z. Bozsó, B. Penke, D. Simon, I. Laczkó, G. Juhász, V. Szegedi, Á. Kasza, K. Soós, A. Hetényi, **E. Wéber**, H. Tóháti, M. Csete, M. Zarándi, L. Fülöp
Controlled in situ preparation of Aβ1-42 oligomers from the isopeptide 'iso-Aβ1-42', physicochemical and biological characterization.
Peptides **2010**, *31*, 248-256. IF: 2.654

7. S. Patil, L. M. Saleena, K. Yong-Wah, **E. Wéber**, H. von Grafenstein
Expression and purification of isotopically enriched MHC binding immunogenic peptides for NMR studies.
Int. J. Pept. Res. Ther. **2011**, *17*, 137-145. IF: 1.034

8. A. Lakatos, B. Gyurcsik, N. V. Nagy, Z. Csendes, **E. Wéber**, L. Fülöp, T. Kiss
Histidine-rich branched peptides as Cu(II) and Zn(II) chelators with potential therapeutic application in Alzheimer's disease.
Dalton Trans **2012**, *41*, 1713-1726. IF: 3.647

9. Ł. Berlicki, L. Pilsl, **E. Wéber**, I. M. Mándity, C. Cabrele, T. A. Martinek, F. Fülöp, O. Reiser
Unique α,β - and α,α,β,β -Peptide Foldamers Based on cis-β - Aminocyclopentanecarboxylic Acid.
Angew. Chem. Int. Ed. 2012, doi: 10.1002/anie.201107702 IF: 12.730

*The impact factors for the year 2010 are given.

Scientific lectures related to the thesis

1. **Wéber E.**:
Amfifil β-peptid hélixek térszerkezete és asszociációs tulajdonságai
XXVIII. Országos Tudományos Diákköri Konferencia, Orvostudományi Szekció
Budapest, 2007. április 3–5.

2. **E. Wéber**, A. Hetényi, T. A. Martinek:
How not to lose hits in NMR binding tests: comprehensive optimization includes temperature
The 10th Central European NMR Symposium & Bruker NMR Users Meeting
September 29-30, 2008, Zagreb, Croatia, Abstr.: P11.

3. **Wéber E.**, Hetényi A.:
   Oldatfázisú szerkezeti biológiai adatok Galektin-1 tumourdajka fehérjéről
   IX. Clauder Ottó Emlékverseny
   Budapest, 2009. április 23-24.

4. **Wéber E.**, Hetényi A., Váczi B., Monostori É., Tóth G., Martinek A. T.:
   Galektin-1 tumourdajka fehérje, ahogy az NMR látja: funkció, dinamika, gátlás:
   MTA Peptidkémiai Munkabizottság Ülése
   Balatonszemes, 2009. május 26-28.

5. **Wéber E.**, Hetényi A., Váczi B., Monostori É., Tóth G., Martinek A. T.:
   Galektin-1 tumourdajka fehérje, ahogy az NMR látja: funkció, dinamika, gátlás
   XIV. Congressus Pharmaceuticus Hungaricus
   Budapest, 2009. november 13-15., Abstr.: P-13.

6. **Wéber E.**, Hetényi A., Fajka-Boja R., Szolnoki É., Batta Gy., Kövér E. K., Monostori É., Martinek A. T.:
   Galektin-1 kölcsönhatása laktózzal és YXY motívumot tartalmazó peptidekkel – NMR spektroszkópiás vizsgálatok
   MTA Peptidkémiai Munkabizottság ülése
   Balatonszemes, 2010. május 26-28.

7. K. E. Kövér, **E. Wéber**, T. A. Martinek, É. Monostori, G. Batta:
   ^{15}N- and ^{13}C group-selective STD NMR techniques for sensitive binding studies
   Joint EUROMAR 2010 and 17th ISMAR Conference
   July 4-9, 2010, Florence, Italy.

8. K. E. Kövér, **E. Wéber**, T. A. Martinek, É. Monostori, G. Batta:
   ^{15}N- and ^{13}C group-selective STD NMR techniques for the detection of weak host-guest interactions
   The 12th Central European NMR Symposium & Bruker NMR Users Meeting
   September 26-28, 2010, Graz, Austria, Abstr.: page 25.

9. **E. Wéber**, Z. Hegedűs, A. Hetényi, É. Szolnoki, T. A. Martinek:
   Towards foldamer inhibitors of the tumour nursing protein Galectin-1
   COST, Foldamers: design, synthesis and applications
   October 6-8, 2010, Bologna, Italy, Abstr.: PS-21.