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**Doctoral School of Pharmaceutical Sciences**

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## Zsófia Hegedüs

# Molecular mimicry of conformationally diverse β-sheets by using α/β-peptide foldamers

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## A. INTRODUCTION AND AIMS

β-Sheet secondary structures are frequent among protein-protein interactions (PPIs) and also present among membrane interacting peptides. From a pharmaceutical point of view, these interfaces are usually undruggable by small molecule compounds because of solvent exposed surface and complex biding mechanism. The need for alternative solutions that target these interactions is reflected by the increasing number of biotherapeutics, but their application may be limited by poor pharmacokinetics, stability and immunogenicity. Non-natural β-sheet mimetic structures may overcome these disadvantages, but the construction of artificial β-sheets is an enduring challenge. The use of β-sheets as therapeutics is further hindered by their high aggregation propensity, which may result in toxic compounds.

With the help of non-natural self-organizing polymers (foldamers) both the β-sheet design and the advantageous properties may be achieved. β-Peptide foldamers have been successfully applied in molecular recognition processes targeting proteins and membranes, but these results have been achieved mainly by using helical secondary structures. The mimicry of β-sheets therefore requires further investigations; only a few sheet-like structures are known with biological activity.

Our goal was to establish design strategies for conformationally diverse β-sheet folding systems by using α/β-peptide foldamers based on a selected peptide participating in protein-protein and membrane interactions. The antiangiogenic and antimicrobial anginex was chosen as a model system, which exhibits diverse structural features. It has random coil conformation in solution, and the bioactive β-sheet is formed only during interaction with the target membrane. Anginex exerts biological activity through interaction with cell membrane and a β-sandwich protein, the tumor nursing galectin-1, which was identified as its main target protein.

Our design strategy for the α/β-peptide foldameric analogs relied on a top-down approach, systematic amino acid substitutions were made in the β-sheet region of anginex. After synthesis, the structural features were characterized by NMR spectroscopy and circular dichroism (CD) techniques, which allowed the description of the local conformational preferences and the overall folding tendencies of the analogs. We observed and analyzed the favorable substitutions and destructuring effects. In order to investigate bioactivity, the binding to galectin-1 was characterized by isothermal titration calorimetry (ITC) and *in vitro* bioactivity assays were performed by Ildikó Makra, Lymphocyte Signal Transduction Group, in the Biological Research Centre.

## B. METHODS

### NMR spectroscopy

Local conformational preferences of the β-amino acids and their secondary structure propensity were investigated by NMR spectroscopic methods. After optimization of measurement conditions, NMR spectra were recorded at 0.5 mM peptide concentration, pH 5.6 at 37°C. Resonance assignment was carried out with the help of 2D TOCSY, NOESY and $^{13}$C-HSQC experiments. Turn stability and conformational rigidity of the $\beta^3$-amino acids were investigated *via* the chemical shift difference of diastereotropic Hα protons of glycine residues or β-amino acids, respectively. The local conformational preferences of $\beta^3$-amino acids were investigated through scalar couplings and NOE intensity ratios. Secondary structure propensity at the amino acid level was investigated by the comparison of the detected chemical shifts to a random coil chemical shift set. This was combined into a secondary structure propensity (SSP) score, which indicated helical and β-sheet forming tendency.

### Circular dichroism

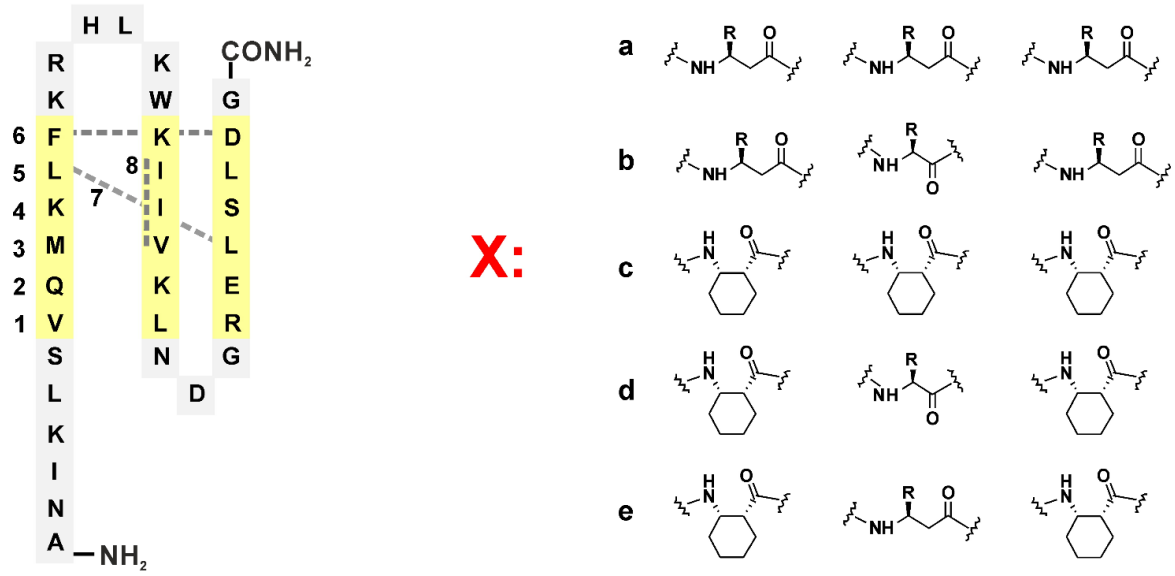
The overall conformation of the sequences in solution and upon interaction with the membrane mimicking phospholipid were investigated by using circular dichroism (CD) measurements. Spectra were recorded at a peptide concentration of 100 μM without and with 2.5 mM of the structure inducing dodecyl-phosphocholine (DPC). Spectra were analyzed with the convex constraint algorithm (CCA+), and quantitative estimation of the secondary structure content was carried out. Temperature-dependent CD measurements were carried out in the range 5 - 75°C in order to investigate the contribution of hydrophobic interactions to the β-sheet folding.

### Isothermal titration calorimetry

Isothermal titration calorimetric experiments were performed with anginex and its target protein galectin-1 using a Microcal VP ITC calorimeter. After the optimization of the measurement conditions, anginex and analogs were titrated to galectin-1 at pH 7.4, 35 °C using 15 μM protein concentration.

## C. Results and Discussion

1. Twenty different β-amino acid-containing α/β-peptides were designed in order to investigate the rules of β-sheet folding in an α/β-foldameric system derived from the antiangiogenic peptide, anginex (Figure 1). The design strategy involved substitutions with β-amino acids in matching positions, using $\beta^3$-amino acids without side-chain alterations and the conformationally constrained 1*R*,2*S*-aminocyclohexane carboxylic acid (*RS*-ACHC), which was selected on the basis of stereochemical considerations. The designed peptides were successfully synthesized after the optimization of the synthesis conditions.

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|---|---|
| **anginex-Gly** | ANIKLSVQMKLFKRHLKWKIIVKLNDGRELSLDG-$NH_2$ |
| **1a-e** | ANIKLS**X**QMKLFKRHLKWKIIVK**X**NDG**X**ELSLDG-$NH_2$ |
| **2a** | ANIKLSV**X**MKLFKRHLKWKIIV**X**LNDGR**X**LSLDG-$NH_2$ |
| **3a-e** | ANIKLSVQ**X**KLFKRHLKWKII**X**KLNDGRE**X**SLDG-$NH_2$ |
| **4a** | ANIKLSVQM**X**LFKRHLKWKI**X**VKLNDGREL**X**LDG-$NH_2$ |
| **5a-e** | ANIKLSVQMK**X**FKRHLKWK**X**IVKLNDGRELS**X**DG-$NH_2$ |
| **6a** | ANIKLSVQMKL**X**KRHLKW**X**IIVKLNDGRELSL**X**-$NH_2$ |
| **7a** | ANIKLSVQMK**X**FKRHLKWKI**X**VKLNDGRE**X**SLDG-$NH_2$ |
| **8a** | ANIKLSVQMKLFKRHLKWK**XXX**KLNDGRELSLDG-$NH_2$ |

**Figure 1.** The designed anginex analogs. Substitution position is indicated by arabic numbers, starting with the first triplet ($Val^7$-$Leu^{24}$-$Arg^{28}$) from the N terminal. Substitution pattern is indicated with dashed lines: **1**-**6** in registry substitutions, **7** – diagonal, **8** – sequential. The amino acid patterns used in the substitution positions (X, Y, Z) are designated by letters **a**-**e**. Compound numbering is generated by the number of the substitution position combined with the letter of the substitution pattern.

2. NMR spectroscopic studies revealed diverse behavior regarding turn initiation and local conformational preferences depending on the substitution position and amino acid type. β-Amino acid substitutions close to the turn segment increased its flexibility. Local conformational preferences of the $\beta^3$-amino acids revealed that amino acids with bulky side-chains were able to adopt the desired *gauche* conformation that fits to the hydrogen bonding pattern of the β-sheet but may result in impaired side-chain packing (Figure 2).

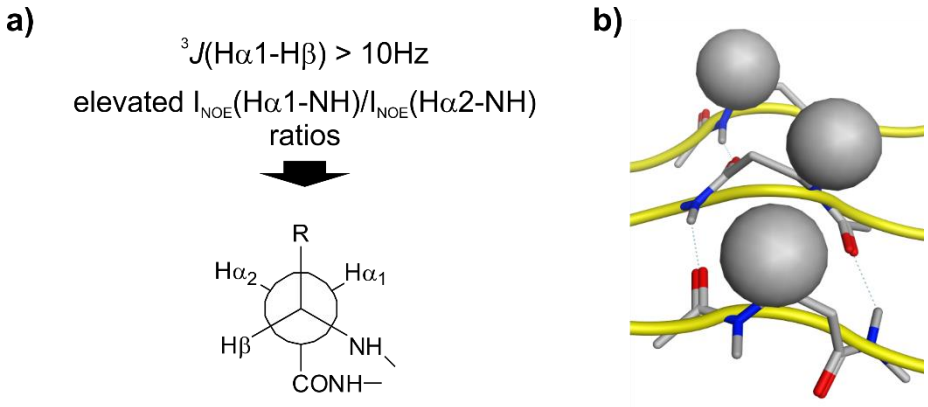


**Figure 2. a)** Observed coupling constant values and NOE intensity ratios for $\beta^3$-*h*Ile and $\beta^3$-*h*Val indicated conformational bias toward *gauche* conformation. **b)** Matching $\beta^3$-amino acid substitutions in *gauche* conformation.

3. Secondary chemical shift analysis and SSP score calculation were used to evaluate the secondary structure forming propensity at the amino acid level (Figure 3). $\beta^3$-Amino acid substitutions induced an undesired helix formation in the most flexible, first strand of the peptide while the use of constrained residues completely inhibited this tendency, which highlighted the advantage of the constrained amino acid to preorganize an extended conformation. Substitutions were the most tolerable close to the turn segment or in the peripheral chains. After addition of the membrane-mimicking phospholipid, the $^1$H NMR spectra of the compounds exhibited β-sheet folding, however signal assignment was prevented by signal intensity loss and line-broadening.

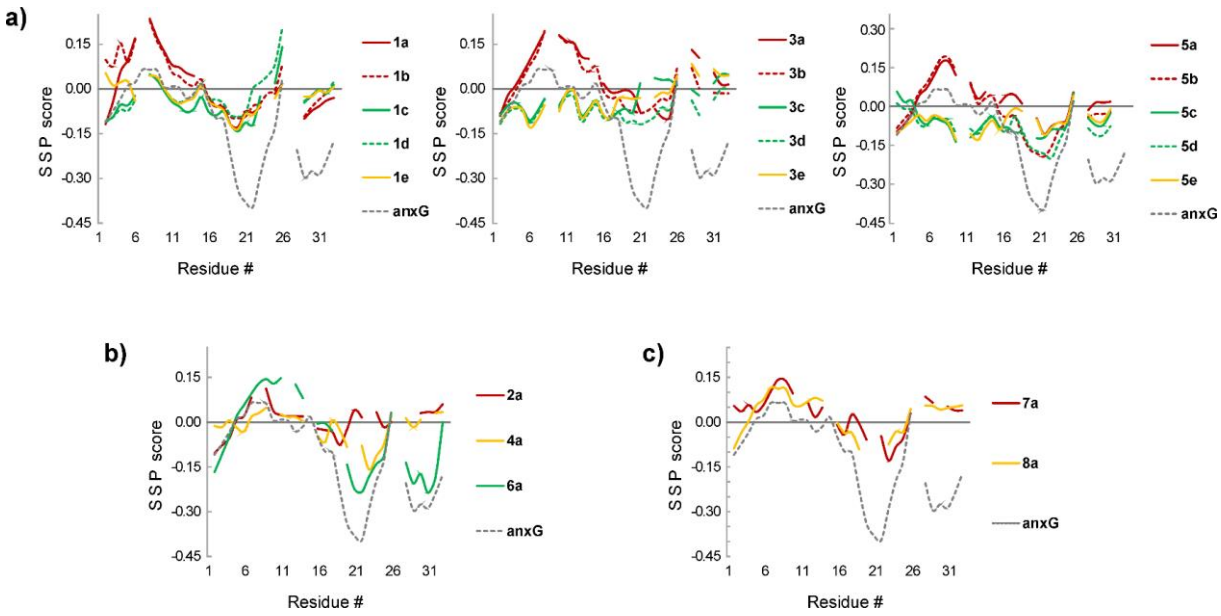


**Figure 3.** Envelopes of SSP scores indicating folding propensities for substitutions in **a)** the hydrophobic face, **b)** the hydrophilic face and **c)** non-matching positions. Positive and negative values correspond to helix and a β-sheet propensity, respectively.

4. Inherent and inducible folding of the foldamers were analyzed by CD. Without induction the foldamers displayed high random coil content; after provision of the membrane-mimicking DPC, β-sheet folding was apparent for most of the analogs. Quantitative estimation of the secondary structure content revealed different extent of β-sheet content and inducibility, that is, the difference between the uninduced and DPC-induced structure. The β-sheet formation showed preference for substitutions close to the turn and peripheral regions, which was in line with the NMR results.

   Temperature dependent CD measurements revealed that the stabilizing hydrophobic forces were scaled down in the foldameric analogs, which mostly explained the destructuring effect of β-amino acids. The presence and stabilizing factor of the hydrophobic forces were more pronounced at positions where the turn stability decreased. Substitutions did not result in increased aggregation or self-association, the determined hydrodynamic radii based on DOSY NMR measurements were close to the parent sequence.

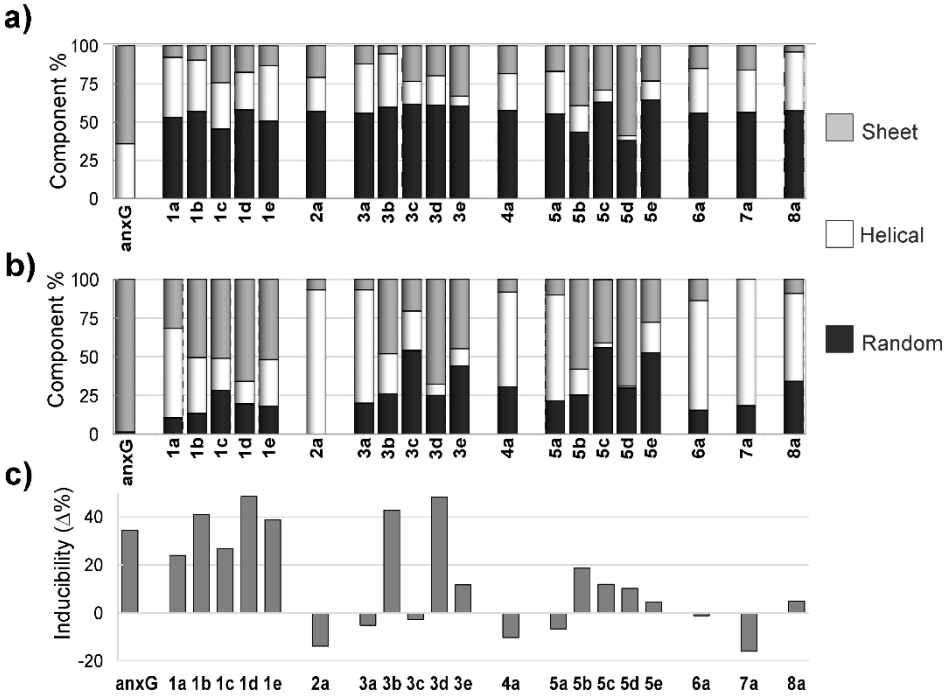


**Figure 4. a)** Secondary structure content in percentages of the analogs without induction and **b)** after induction with 2.5 mM DPC. Grey bars indicate β-sheet, white bars indicate helical and black bars correspond to random coil secondary structure content. **c)** β-Sheet inducibility for all analogs that is calculated by the difference in initial and induced β-sheet content and expressed in Δ%.

5. Investigation of the biological activity was carried out by ITC and *in vitro* experiments. Gal-1 anginex interaction was successfully characterized by ITC, which revealed 1:4 stoichiometry and high affinity. (Figure 5a) Most of the substitutions were detrimental to gal-1 binding, which may originate from the changed position of pharmacophore points or decreased folding. A number of analogs displayed *in vitro* activity close to the parent sequence, which were in correlation with the β-sheet inducibility, which pointed to the fact that the structure formation upon interaction with the target membrane is essential for bioactivity (Figure 5b).

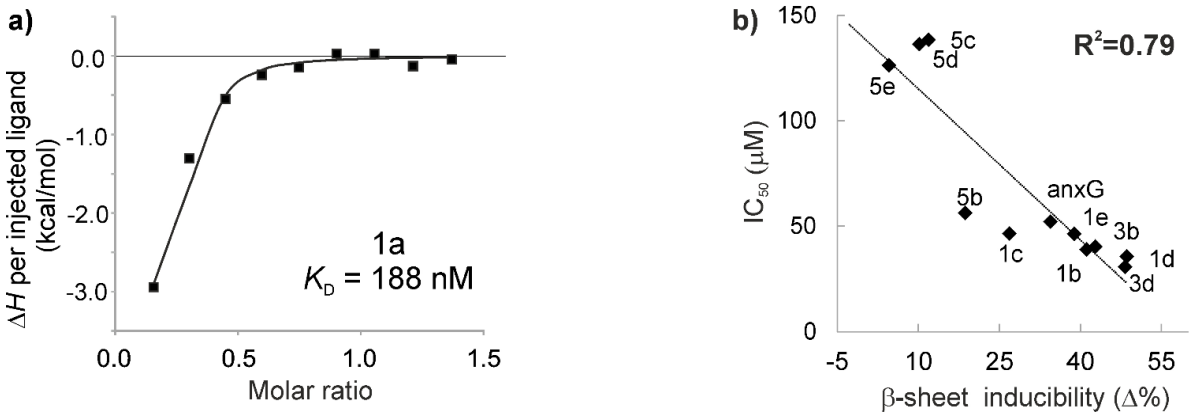


**Figure 5. a)** ITC enthalpogram of **1a** titrated to galectin-1, showing 1:4 stoichiometry and nanomolar $K_D$ **b)** Correlation between β-sheet inducibility and $IC_{50}$ of the analogs having substitutions in the hydrophobic face.

## Full papers related to the thesis

I. Hegedüs, Z., Wéber, E., Kriston-Pál, É., Makra, I., Czibula, Á., Monostori, É., & Martinek, T. A. (2013). Foldameric α/β-peptide analogs of the β-sheet-forming antiangiogenic anginex: structure and bioactivity. *Journal of the American Chemical Society*, **135** (44), 16578-16584.
IF: 11.444

II. Hegedüs, Z., Wéber, E., Végh, L., Váczi, B., Tubak, V., Kriston-Pál, É., & Martinek, T. A. (2015). Two-stage interaction of the tumor nursing galectin-1 with the antiangiogenic peptide anginex. *Journal of Thermal Analysis and Calorimetry*, **120** (1), 449-456.
IF: 2.042

III. Hegedüs, Z., Makra, I., Imre, N., Hetényi, A., Mándity, I. M., Monostori, É., & Martinek, T. A. (2016). Foldameric probes for membrane interactions by induced β-sheet folding. *Chemical Communications*, **52** (9), 1891-1894.
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## Other full papers

I. Németh, L. J., Hegedüs, Z., & Martinek, T. A. (2014). Predicting Order and Disorder for β-peptide Foldamers in Water. *Journal of chemical information and modeling*, **54** (10), 2776-2783.
IF: 3.738

*The impact factors for the year 2015 are given.

## Scientific lectures related to the thesis

1. Hegedüs Zs., Wéber E., Kriston-Pál É., Makra I., Czibula Á., Monostori É., Martinek T. A.
   Antiangiogén foldamer β-szendvics analógok, szerkezet és bioaktivitás
   XXXVI. Kémiai Előadói Napok
   Szeged, 2013. Október 28 - 30

2. Zs. Hegedüs, E. Wéber, É. Kriston-Pál, I. Makra, É. Monostori, T. A. Martinek
   β-sandwich forming propensity and biological activity of foldameric anginex analogs
   Poster presentation
   2013 Symposium on Foldamers, Paris, 12. April 2013

3. Hegedüs Zs., Wéber E., Makra I., Czibula Á., Monostori É., Martinek T. A.
   β-redős foldamerek tervezése az angiogenezis gátló anginex analógiájára, szerkezetvizsgálat
   MTA Peptidkémiai Munkabizottság Tudományos Ülése
   Balatonszemes, 2014. május 28-30.

4. Zs. Hegedüs, E. Wéber, I. Makra, É. Monostori, T. A. Martinek
   Redesigning an antiangiogenic β-sheet peptide anginex to α/β peptide foldamers
   Poster presentation
   7th Central Europe Conference, Chemistry Towards Biology
   Katowice, 12. September 2014

5. Zs. Hegedüs, E. Wéber, I. Makra, É. Monostori, T. A. Martinek
   Membrán kölcsönhatások vizsgálata foldamerekkel, a béta redő képző hajlamuk függvényében
   MTA Peptidkémiai Munkabizottság Tudományos Ülése
   Balatonszemes, 2015. május 20-22.