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Foldameric probes for membrane interactions by induced β-sheet folding

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Design strategies were devised for α/β-peptide foldameric analogues of the antiangiogenic anginex with the goal of mimicking the diverse structural features from the unordered conformation to a folded β-sheet in response to membrane interactions. Structure-activity relationships were investigated in the light of different β-sheet folding levels.

β- Sheet interfaces are rarely targets of pharmacological intervention despite their frequent occurrence in protein-protein interactions or among membrane-interacting peptides, which is probably due to the challenges associated with the β-sheet design. A special subgroup of membrane-interacting β-sheet peptides demonstrate unique internal conformational diversity: the conformation is unstructured in solution and the bioactive β-sheet forms only during interactions with membranes. We set out to mimic this diverse behaviour in a model involving the use of α/β-peptide foldamers in order to devise design strategies for foldameric β-sheets and to acquire a deeper understanding of the underlying mechanism of membrane–foldamer interactions.

The driving forces of β-sheet folding have been studied in a number of natural peptide sequences of designed β-hairpin, β-sheet and higher-ordered β-sandwich models. Mimicking secondary structures with peptidic foldamers has become successful among helices, whereas only a few bioactive strand or β-sheet-like structures are known. In small hairpin models, the matching α to β-amino acid substitution resulted in an altered hydrogen-bond pattern and side-chain orientation. The promising strategy of using αα to β or ββ substitution did not prove suitable in a more complex tertiary structure since it tolerated only minor amino acid alterations.

To investigate the impact of the non-natural amino acid substitutions on the β-sheet folding and bioactivity of a three-stranded antiparallel β-sheet, anginex (anx) with antiangiogenic and antitumour activity was used as a model system. Anginex may be a particularly suitable target since it has been shown to exert its activity in part through interactions with cell membranes. The prevailing random coil conformation can be induced to adopt a β-sheet fold by membrane mimetics, and the resulting β-sheet structure being essential for its biological activity. We describe here that cyclically constrained β-amino acid substitution at particular positions in the β-sheet is advantageous, as the analogues can retain biomimetic behaviour. Moreover, the activity can be tuned by the inducibility of the β-sheet folding.

In β1-amino acid-containing short turns, the N(1)-Ca-C(=O) torsion angle (δ) exchanges between anti and gauche conformations. For the proper peptide bond orientation that fits the natural hydrogen-bonding pattern, gauche conformation is required. Our earlier results revealed that β-amino acids with bulky side-chains have this conformational preference, but the side-chains are misaligned leading to lowered hydrophobic interactions and folding. The desired gauche conformation can be fixed by using constrained β-amino acids, while better overlapping and a higher contribution to the hydrophobic interactions are expected from a cyclic side-chain (Fig. S1). 1R,2S-cis-Aminocyclohexanecarboxylic acid (1R,2S-ACHC) was chosen for substitution on the basis of the following stereochemical considerations: (i) the Cβ configuration should match that of the natural amino acids, (ii) a six-membered saturated cyclic amino acid with a cis-relative configuration gives a higher β-sheet content as compared with other cyclic amino acid types.

Three matching positions were chosen for substitution on the hydrophobic face of the peptide (Fig. 1) so as to maximize the geometrical fit between the hydrogen-bonding network.
around the β-amino acids. The derivatives were prepared in four different patterns with substitution in all three (a and b) or only in the peripheral strands (c and d). The synthesis was carried out by using microwave-assisted peptide coupling and also with a novel continuous flow method17, which needs an amino acid excess of only 1.5 equivalents for the β-residues.

Homo- and heteronuclear NMR spectra were measured to assign NH, Hx, Cx, and Cβ chemical shifts (Table S4) and the folding propensity at the amino acid level was characterized by chemical shift analysis.18 Moderate upfield shifts were observed for the NH and Hx protons of the (1R,2S)-ACHC, and downfield shifts for the Cx chemical shifts close to the β1-amino acid substitutions, which indicated a loss of β-sheet propensity; nevertheless, the structure-independent neighbouring effects of β-amino acids could not be entirely excluded. Secondary structure propensity (SSP) scores were calculated by use of the SSP program20; the resonances of β-amino acids and glycines were excluded because of the lack of reference values and Cβ chemical shifts, respectively. Without induction, anginex displayed moderate β-sheet propensity, with SSP scores of -0.2 to -0.4 for the second (K19-L24) and third (R28-D33) (Fig. S2), and around zero for the first (V7-F12) chain. The SSP scores of all the analogues were generally lower than that observed for the parent peptide (Fig. 2a-c and Fig. S2). 1c exhibited the highest β-sheet propensity in the range from -0.1 to -0.2, and small negative values for the first strand. Sequences with β1-amino acid replacements (1d, 2d and 3d) promoted a helix in the first strand, while the constrained residues completely suppressed this tendency. These results were in line with the helix-promoting potencies of the open-chain β1-amino residues, and suggested that the conformational preferences of the β-amino acid substitutions exerted strong effects on the secondary structure of the entire chain. In all three strand segments, substitutions were favourable only close to the second turn (3a and 3b). In the other tested positions, the peripheral chain substitutions were better tolerated.

The variability of the SSP values close to the tighter turn of anginex12 (N25–G27) may indicate the role of the turn stability in the folding. The conformational preference of this turn could be measured by the chemical shift difference (Δδ) observed for the diastereotopic Hx protons of G27. The closer the substitutions were situated with respect to the turn, the lower were the Δδ values (Fig. 2d), which indicated destabilization. This was generally seen for analogues 3a, 3b and 3c, and may be attributed to the loss of possible ionic interactions upon the mutation of R28.

The 1H-NMR spectra exhibited resonance broadening after the provision of a membrane-mimicking environment by the addition of dodexyclphosphocholine (DPC), which is known to enhance the β-sheet formation of anginex.12 Several analogues displayed downfield-shifted Hx and NH resonances (Figs. S7 and S8) indicating the presence of β-sheet segments.26 Moreover, Hx-Hx NOEs of β-sheet origin were observed for 3c (Fig. S9). Signal assignment was prevented by the resonance broadening, which may be due to the slow chemical exchange between folded and unfolded populations.26

In order to analyse the overall folding tendencies, β-sheet contents based on CD measurements were quantitatively estimated with and without the membrane-mimicking DPC (Fig. S3). All CD data were analysed by using the convex constant algorithm (CCA+) software21, and the data were deconvoluted to three component curves (Fig. S3) with average NRMSD of 1.9% of the fitted curves (Table S1). The spectrum of a particular component was assigned on the basis of similarity to the reference CD curve of the secondary structure of the given peptide22, including random coil, β-sheet and short helix segments. The resulting secondary structure contents were determined as percentages of the overall structure. The structure of anginex was β-sheet to extents of ~64% and...
in the absence or presence of, 2.5 mM DPC, respectively (Fig. 3). Great diversity in β-sheet content was observed for the analogues: in the intervals 5–64% and 20–98% before and after the addition of DPC, respectively. Inducibility was defined here as the increase in β-sheet content, which varied in the range from -3 to 49%. Peptides 1c and 1d exhibited the highest inherent β-sheet contents, but their inducibilities in the membrane-mimicking environment were low. Higher inducibilities were observed for substitutions with substitutions

Fig. 3 Secondary structure contents obtained by deconvolution by using the CCA+ software (a) without induction and (b) after induction with 2.5 mM DPC. Components observed: random coil (black bars), turn or short helical segment (white bars) and β-sheet (grey bars). Average NRMSDs of the fitting were 3.4% for (a) and 4.1% for (b).

It has previously been shown that appropriate orientation of the side-chains facilitates the hydrophobic interactions that play crucial roles in β-sheet folding, and they are scaled up by increasing temperature.23 Temperature-dependent CD measurements carried out to probe these interactions revealed increasing β-sheet content as the temperature was elevated (Fig. S5). The maximum β-sheet content of anginex was reached at 60 °C, and structural melting occurred at higher temperatures (Fig. 4). 1c and 1d exhibited similar biomimetic behaviour with rapidly increasing β-sheet content up to 55–60 °C; this could be explained by the appropriate fit of the side-chains into the tightly packed core regions. The hydrophobic cluster provided by the cyclic side-chains made a larger contribution to the hydrophobic interactions near the turn segment (3a, 3b and 3c) as compared with the core substitutions (2a, 2b and 2c). This was in line with the observation that the degree of hydrophobically driven stabilization was more pronounced in β-hairpins in which the hydrophobic cluster was close to the turn segment.24 Further, a decrease in the conformational stability of the turns (indicated by NMR) may result in an increased weight of hydrophobic interactions as a compensation for the substitution-induced turn destabilization.

Fig. 4 Change in β-sheet content in response to increasing temperature. β-Sh contents were calculated by using CCA+ deconvolution, with an average NRMSD of 1.2.

The effects of anginex and the α/β-analogues on the viability of murine bEnd.3 endothelial cells were analysed by flow cytometry. The cells underwent fragmentation on the addition of anginex and the α/β-analogues, indicating internal disruption of the cell membrane.11 The most effective analogue was 2c, the IC50 value of which was estimated to be 30.8 μM (Table 1). As it has been proposed that β-sheet formation is essential for the bioactivity of anginex,13 we carried out a structure–activity relationship analysis on the analogues. A correlation was not observed between the cell viability and the uninduced structure (Fig. 5a). Although expected, correspondence was not found either between the activity and the induced β-sheet content (Fig 5b). Another aspect of the structure–activity relation was therefore investigated, i.e. how the degree of inducibility correlated with the function. The analysis resulted in the novel finding that the determining factor in the biological activity was the inducibility rather than the pre-existing β-sheet content. Accordingly, linear regression between the inducibility and the bioactivity was successfully resulting in a regression coefficient of 0.88 (Fig. 5c and S11). This suggested that the conversion from the random coil to the β-sheet during the interaction with the membrane was the key element for the biological activity.

Fig. 5 Correlation between cell viability and β-sheet structure. Cell viability, measured at 50 μM peptide concentration, was plotted against β-sheet content % calculated by using the CCA+ software. Correlations are illustrated with (a) the uninduced β-sheet content; (b) the β-sheet content induced by DPC; and (c) the β-sheet inducibility (the difference between the induced and uninduced β-sheet contents, expressed in Δ%).

In order to gain a biophysical picture of the foldamer–membrane interaction, membrane leakage assays were performed by using carboxyfluorescein (CF) dequenching.
Selected analogues were added to large unilamellar vesicles of dioleyl-glycerophosphocholine (DOPC) alone, and mixed with dioleyl-glycerophosphoglycer (DOPG) in a molar ratio of 8:2. These mimic neutral and negatively charged membrane surfaces, respectively. All the analogues displayed an increased CF release upon interaction with the negatively charged lipids (Fig. S12), which was in accordance with the β-sheet folding propensity. 3c displayed decreased selectivity towards the negatively charged lipid vesicles, probably as a consequence of the loss of a positively charged side-chain upon the substitution of R28. On the other hand, 1c exhibited a leakage profile similar to that of the parent peptide, although it was less effective in the cell viability assay. The inactive and low β-sheet folding analogue 2a exhibited membrane leakage only at high concentrations. Close correlation between the results of the two experiments possibly cannot be expected, because of the different lipid composition and peptide/lipid ratio in the two experiments. We concluded, however, that foldamer–membrane interactions can contribute to the biological effects.

### Table 1. Estimated IC₅₀ values

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<th>IC₅₀ (µM)</th>
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<td>1a 138.6  1b 126.5  1c 136.5  1d 56.5</td>
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<td>2a n.a.  2b n.a.  2c 30.8  2d 40.4</td>
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In summary, the use of conformationally constrained β-amino acid substitutions successfully suppressed undesired helix formation in the presented β-sheet model sequence. (1R,2S)-ACHC mutations in the peripheral segments and at the near-turn positions were more favourable with respect to β-sheet formation. Some of the foldamer derivatives retained adaptive behaviour; their folding was highly inducible in a micellar environment. The structure–activity relationship study revealed that the inducibility of folding into a β-sheet was essential for biological activity. Tests of the direct effects of the foldamers on the membrane integrity indicated that the toxic effect of the foldamers on the epithelial cells depended on the foldamer–membrane interactions, although the responses of true biological membranes are more complex than those of synthetic membranes. These results contribute to a better understanding of the folding of α/β-peptide β-sheets, and especially their stimulus-response behaviour in the course of interactions with membranes.

### Notes and references

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