Constraining an Irregular Peptide Secondary Structure through Ring-Closing Alkyne Metathesis

Philipp M. Cromm[a, b], Kerstin Wallraven[c], Adrian Glas[b, d], David Bier[d, e, g], Alois Fürstner[b, f], Christian Ottmann[e, g] and Tom N. Grossmann[b, c, d].

Macrocyclization can be used to constrain peptides in their bioactive conformations, thereby supporting target affinity and bioactivity. In particular, for the targeting of challenging protein–protein interactions, macrocyclic peptides have proven to be very useful. Available approaches focus on the stabilization of α-helices, which limits their general applicability. Here we report for the first time on the use of ring-closing alkyne metathesis for the stabilization of an irregular peptide secondary structure. A small library of alkyne-crosslinked peptides provided a number of derivatives with improved target affinity relative to the linear parent peptide. In addition, we report the crystal structure of the highest-affinity derivative in a complex with its protein target 14-3-3.z. It can be expected that the alkyne-based macrocyclization of irregular binding epitopes should give rise to new scaffolds suitable for targeting of currently intractable proteins.

Peptide-based drugs have recently experienced a renaissance, with several approvals and many candidates in clinical trials.[1] Protein–protein interactions (PPIs), which are mainly mediated by shallow and extensive interaction surfaces, are hardly addressable by small-molecule entities, and so peptides and peptidomimetics are stepping up to fill the void.[2, 3] For the targeting of PPIs, peptides feature many advantages over classic small-molecule compounds because they can cover larger surfaces, provide straightforward access to structural diversity, and show low toxicity in humans.

However, unmodified short L-peptides often suffer from low target affinity and bioactivity, hampering their therapeutic potential. These drawbacks are mainly caused by the high flexibility of short peptides in their unbound state.[4] In this context, peptide macrocyclization has emerged as an important strategy for enforcing conformational constraint.[5] Preorganization of the peptide in solution prior to binding reduces the entropic penalty upon target engagement, thereby increasing the stability of the resulting complex.[6] Macrocyclization of natural peptide sequences yields class A peptidomimetics, which are defined as modified peptides that harbor only minor backbone and side chain alterations.[5]

Class A peptidomimetics have been widely used to mimic secondary structure elements for the inhibition of PPIs through the application of different cyclization strategies.[3, 4] Helix stabilization has often been pursued by use of the hydrocarbon peptide stapling approach[7, 8] and hydrogen bond surrogates (HBSS),[9] two methods that use ring-closing olefin metathesis (RCM) for the formation of a hydrophobic crosslink. Recently, we reported a new synthetic method utilizing ring-closing alkyne metathesis (RCM, Scheme 1) for the formation of constrained helices.[10] Importantly, the linear and rigid alkyne moiety within the macrocycle allows the installation of a hydrophobic crosslink, known to be beneficial for bioavailability,[7] and gives access to new molecular geometries. Especially in cases in which the crosslink mediates the interaction with the target protein, an extended set of synthetically accessible architectures can be crucial for the identification of high-affinity binders. Here we report the use of RCAM for the stabilization...
of an irregular peptide secondary structure, to yield macrocyclic peptides with improved target affinities relative to their linear parent peptide. In addition, a crystal structure of the highest-affinity alkynyl derivative in a complex with its protein target 14-3-3 is presented.

As a basis for the design of alkynyl-crosslinked peptides, we used the so-called Esp peptide (14-3-3 binding motif of exoenzyme S). Exoenzyme S is a virulence factor of the bacterium Pseudomonas aeruginosa, and it inactivates various small GTPases through ADP-ribosylation upon interaction with its cofactor 14-3-3 to cause severe cellular damage. Esp resembles an irregularly structured binding motif that recognizes 14-3-3 mainly through hydrophobic contacts. Inhibition of the 14-3-3–exoenzyme S interaction would be expected to reduce the pathogenicity of P. aeruginosa. Previously, we had developed two crosslinked peptides—β₁₁₂ and β₁₀₈—that stabilize the bioactive irregular peptide structure of Esp and bind 14-3-3 with sub-micromolar affinities. In each case a fully saturated hydrocarbon crosslink was applied for conformational restriction. The recently reported alkynyl-based macrocyclization of peptides through on-resin RCAM enables the introduction of a rigid alkynyl into the crosslink to provide additional constraints. To investigate the consequences of crosslink rigidification on 14-3-3 binding and the applicability of RCAM for the stabilization of irregular secondary structures, we designed a series of alkynyl-macrocyclized peptides based on the two peptides β₁₁₂ and β₁₀₈ (Scheme 1). The two peptides each contain two unnatural amino acids, at positions i and i+3, for crosslinking [Scheme 2A, amino acids 422 (X) and 425 (Y) based on the exoenzyme S sequence]. Peptide β₁₀₈ features an eight-carbon linker (absolute configurations: X : R, Y : S) whereas β₁₁₂ has a 12-carbon linker (absolute configurations: X : S, Y : S). A total of eight alkynyl-macrocyclized Esp derivatives based on these two architectures were synthesized. They can be divided into two families (Scheme 2B): one is based on β₁₀₈ and contains an R- and an S-configured building block (X and Y, respectively) with carbon linkers varying between eight and ten atoms (Scheme 2B, peptides A–D). The other family, based on β₁₁₂, incorporates two S-configured building blocks (X and Y) and crosslinks with between ten and twelve carbon atoms (Scheme 2B, peptides E–H). Linker lengths and configurations were inspired by previously reported results.

In the design process of the alkynyl-crosslinked derivatives, we aimed to position the alkynyl moiety in the center of the hydrocarbon crosslink. Peptides with even numbers of carbon atoms have the triple bond located in the middle of the crosslink (A, D, E, and H, Scheme 2B), whereas peptides with odd numbers of linker atoms exist as two regioisomers (B/C and F/G, Scheme 2B). All peptides were synthesized by Fmoc-based solid-phase peptide synthesis (SPPS) with incorporation of unnatural α-methyl, α-alkynyl amino acids 1–5 (Scheme 1, Figure S1 in the Supporting Information) at positions X and Y (Scheme 2B). RCAM was performed according to established protocols with use of the latest versions of molybdenum-mediated alkynyl metathesis.

**Scheme 1.** Synthesis of alkynyl-macrocyclized peptides. α-Methyl, α-alkynyl amino acids Fmoc-1–5-OH are introduced into the peptide sequence by means of SPPS. Macrocyclization is performed on solid phase through molybdenum-mediated alkynyl metathesis.
Binding affinities towards 14-3-3\(\zeta\) were determined in a fluorescence polarization (FP) assay (Scheme 2B, Figures 52–54). Dissociation constants (\(K_d\) values) of alkyne-macrocyclized peptides A–H with 14-3-3\(\zeta\) show a broad range depending on linker length and absolute configuration of building blocks at positions X and Y. Of the \(\beta_{12}\)-derived peptides (A–D, R/S-configured), peptide A appears to be the best 14-3-3 binder (\(K_d[A]=0.54\ \mu\text{M}\)), showing a 1.6-fold improved affinity in relation to the unmodified ESp sequence (\(K_d[\text{ESp}]=0.84\ \mu\text{M}\), Scheme 2B). In this respect it is interesting to note that the crosslink in peptide A has the same number of carbon atoms (eight) as in alkyne-crosslinked peptide \(\beta_{10}\). Increases in linker length (peptides B–D) result in a tremendous loss in target affinity (\(K_d>10\ \mu\text{M}\)). The \(\beta_{12}\)-derived peptides (E–H, S/S-configured) show generally higher affinity for 14-3-3\(\zeta\) than their R/S-configured analogues. Peptides E and H reveal \(K_d\) values in the sub-micromolar range (Scheme 2B). Interestingly, whereas one of the regioisomers (peptide G) binds 14-3-3\(\zeta\) with a \(K_d\) value in the low micromolar range (\(K_d[G]=1.35\ \mu\text{M}\), the other one (peptide F) does not show any binding to 14-3-3\(\zeta\) in the FP assay (\(K_d[F]>10\ \mu\text{M}\)). Of all the alkyne-cyclized peptides, H (Figure 1A) is the highest-affinity binder (\(K_d[H]=0.31\ \mu\text{M}\)), exhibiting a dissociation constant in the same range as the alkyne-crosslinked peptide \(\beta_{12}\) (\(K_d[\beta_{12}]=0.10\ \mu\text{M}\)) and showing higher affinity for 14-3-3 than \(\beta_{10}\) and unmodified peptide ESp. Peptide H bears the S-configured building block 3 at positions X and Y (Figure 1A), forming a 12-carbon crosslink in analogy to the unsaturated parent peptide \(\beta_{12}\). The binding affinity of peptide H was verified in an orthogonal binding assay based on microscale thermophoresis (MST), providing affinities in good agreement with the FP results (\(K_d=0.44\ \mu\text{M}\), Figures S5 and S6 and Table S2).

To verify the binding site of peptide H on 14-3-3\(\zeta\), FP competition experiments were performed. Treating a complex of 14-3-3\(\zeta\) and fluorescein-labeled ESp with increasing concentrations of unlabeled peptide H resulted in full displacement of ESp (half maximal inhibitory concentration (IC\(_{50}\)=1.19\ \mu\text{M}, Figure 1B), thus confirming an overlapping binding site of ESp and H. Using unlabeled ESp as competitor, we observed less efficient displacement (IC\(_{50}=4.65\ \mu\text{M}\)), which is in agreement with its lower affinity for 14-3-3\(\zeta\).

To elucidate the details of molecular recognition between peptide H and 14-3-3\(\zeta\), we attempted to acquire a crystal structure of peptide H in complex with 14-3-3\(\zeta\). For this reason, H was co-crystallized with 14-3-3\(\zeta\)Ac (aa 1–230), providing crystals that diffract in space group P2\(_1\)2\(_1\)2\(_1\). The resulting crystal structure (PDB ID: 5J31) provides the 

\[
\begin{align*}
\text{Figure 1. A) Chemical structure of alkyne-crosslinked 14-3-3\(\zeta\) binding peptide H (R = FITC-PEG or acetyl), B) FP-based displacement assay of N-terminally acetylated peptides [ESp (gray) and H (red)] competing with FITC-PEG-labeled ESp (10 nM) from 14-3-3\(\zeta\) (full length, 2 \(\mu\text{M}\)). Measurements were performed in triplicate (error = 1\(\sigma\)).}
\end{align*}
\]
We appreciate the help of the beamline staff at Swiss Light Source PXII-X10SA.

Keywords: macrocyclization \cdot peptide secondary structures \cdot peptidomimetics \cdot protein–protein interactions \cdot ring-closing alkyne metathesis

Acknowledgements

P.M.C. is grateful to the Studienstiftung des Deutschen Volkes for a Fellowship. This work was supported by the German Research Foundation (DFG, Emmy Noether program GR3592/2-1 and SFB1093), the European Research Council (ERC starting grant, no. 678623), AstraZeneca, Bayer CropScience, Bayer HealthCare, Boehringer Ingelheim, Merck KGaA, and the Max Planck Society.

Figure 2. A) 14-3-3Δ dimer with 2Foh–Fcalc electron density of peptides H (red, PDB ID: 5J31). B, C) Left: Side view of superimposed structures of peptide H (red) and ESp (white) or β12 (blue) in complex with 14-3-3Δ (gray/orange). Interacting side chains of peptides are shown in stick presentation (crosslink, L423, L426, D427, Y, L428). Hydrophobic pocket of 14-3-3Δ is highlighted orange (N42, S45, V46, F117, P165, I166, L172, N173, D213, L216, I217, L220). Right: Front view of superimposed peptides in complex with 14-3-3Δ, showing exclusively the amino acid side chains at positions X and Y (ESp: L422, A425) used to incorporate crosslinks.

We appreciate the help of the beamline staff at Swiss Light Source PXII-X10SA.

Keywords: macrocyclization \cdot peptide secondary structures \cdot peptidomimetics \cdot protein–protein interactions \cdot ring-closing alkyne metathesis

[1] A. A. Kaspar, J. M. Reichert, Drug Discovery Today 2013, 18, 807–817.
[2] a) D. J. Craik, D. P. Fairlie, S. Liras, D. Price, Chem. Biol. Drug Des. 2013, 81, 136–147; b) L. Nevola, E. Giralt, Chem. Commun. 2015, 51, 3302–3315; c) J. Spiegel, P. M. Cromm, G. Zimmermann, T. N. Grossmann, H. Waldmann, Nat. Chem. Biol. 2014, 10, 613–622; d) P. M. Cromm, J. Spiegel, T. N. Grossmann, H. Waldmann, Angew. Chem. Int. Ed. 2015, 54, 13516–13537; Angew. Chem. 2015, 127, 13718–13741.
[3] M. Pelay-Gimeno, A. Glas, O. Koch, T. N. Grossmann, Angew. Chem. Int. Ed. 2015, 54, 8896–8927; Angew. Chem. 2015, 127, 9022–9054.
[4] J. E. Bock, J. Gavenonis, J. A. Kritzer, ACS Chem. Biol. 2013, 8, 488–499.
[5] a) L. K. Henchey, A. L. Jochim, P. S. Arora, Carbohydr. Res. 2008, 12, 692–697; b) D. N. Houk, A. G. Leach, S. P. Kim, X. Zhang, Angew. Chem. Int. Ed. 2003, 42, 4872–4897; Angew. Chem. 2003, 115, 5020–5046.
[6] a) S. Wang, D. H.-C. Chou, Angew. Chem. Int. Ed. 2015, 54, 10931–10934; Angew. Chem. 2015, 127, 11081–11084; b) N. Assen, D. J. Ferreira, D. W. Wolan, P. E. Dawson, Angew. Chem. Int. Ed. 2015, 54, 8665–8668; Angew. Chem. 2015, 127, 8789–8792; c) L. Mendive-Tapia, S. Preciado, J. García, R. Ramón, N. Kielland, F. Albericio, R. Lavilla, Nat. Commun. 2015, 6, 7160; d) Y. H. Lau, Y. W, M. Rossmann, B. X. Tan, P. de Andrade, Y. S. Tan, C. Verma, G. J. McKenzie, A. R. Venkittaraman, M. Hyvönen et al., Angew. Chem. Int. Ed. 2015, 54, 15410–15413; Angew. Chem. 2015, 127, 15630–15633; e) Y. H. Lau, P. de Andrade, G. J. McKenzie, A. R. Venkitaraman, D. R. Spring, ChemBioChem 2014, 15, 2680–2683.
[7] P. M. Cromm, J. Spiegel, T. N. Grossmann, ACS Chem. Biol. 2015, 10, 1362–1375.
[8] a) J.-W. Kim, T. N. Grossmann, G. L. Verdine, Nat. Protoc. 2013, 6, 761–771; b) L. D. Walensky, G. H. Bird, J. Med. Chem. 2014, 57, 6275–6288.
[9] A. Patgiri, A. L. Jochim, P. S. Arora, Acc. Chem. Res. 2008, 41, 1289–1300.
[10] A. Fürstner, Angew. Chem. Int. Ed. 2013, 52, 2794–2819; Angew. Chem. 2013, 125, 2860–2887.
[11] P. M. Cromm, S. Schaubach, J. Spiegel, A. Fürstner, T. N. Grossmann, H. Waldmann, Nat. Commun. 2016, 7, 11300.
[12] C. Ottmann, L. Yasmin, M. Weyand, J. L. Veesenmeyer, M. H. Diaz, R. H. Palmer, M. S. Francis, A. R. Hauser, A. Wittinghofer, B. Hallberg, *EMBO J.* 2007, 26, 902 – 913.

[13] a) S. C. Masters, K. J. Pederson, L. Zhang, J. T. Barbieri, H. Fu, *Biochemistry* 1999, 38, 5216 – 5221; b) M. L. Henriksson, C. Sundin, A. L. Jansson, Å. Forsberg, R. H. Palmer, B. Hallberg, *Biochem. J.* 2002, 367, 617 – 628.

[14] A. Glas, D. Bier, G. Hahne, C. Rademacher, C. Ottmann, T. N. Grossmann, *Angew. Chem. Int. Ed.* 2014, 53, 2489 – 2493; *Angew. Chem.* 2014, 126, 2522 – 2526.

[15] A. Glas, T. Grossmann, *Synlett* 2015, 26, 1 – 5.

[16] a) J. Heppekausen, R. Stade, R. Goddard, A. Fürstner, *J. Am. Chem. Soc.* 2010, 132, 11045 – 11057; b) J. Heppekausen, R. Stade, A. Kondoh, G. Seidel, R. Goddard, A. Fürstner, *Chem. Eur. J.* 2012, 18, 10281 – 10299.

Manuscript received: June 24, 2016
Final article published: September 6, 2016