Structured Peptides

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Peptide Ligands Stabilized by Small Molecules**

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Abstract: *Bicyclic peptides generated through directed evolution by using phage display offer an attractive ligand format for the development of therapeutics. Being nearly 100-fold smaller than antibodies, they promise advantages such as access to chemical synthesis, efficient diffusion into tissues, and needle-free application. However, unlike antibodies, they do not have a folded structure in solution and thus bind less well. We developed bicyclic peptides with hydrophilic chemical structures at their center to promote noncovalent intramolecular interactions, thereby stabilizing the peptide conformation. The sequences of the peptides isolated by phage display from large combinatorial libraries were strongly influenced by the type of small molecule used in the screen, thus suggesting that the peptides fold around the small molecules. X-ray structure analysis revealed that the small molecules indeed formed hydrogen bonds with the peptides. These noncovalent interactions stabilize the peptide–protein complexes and contribute to the high binding affinity.*

Mimicking the binding site of antibodies by using small synthetic molecules has been a longstanding challenge.[1] The binding contacts of antibodies are mediated by small regions at the tip of the Y-shaped structures, the complementarity-determining regions (CDRs). In early attempts to generate synthetic antibody mimics, CDR-based peptide sequences were cyclized to stabilize the native conformation.[2] Later, multiple CDRs were anchored onto synthetic scaffolds.[1b,c,3] The various constrained synthetic antibody mimics displayed improved binding affinities compared to individual linear CDR-based peptides. However, the affinities were in all cases significantly lower than those of the parental antibodies.

Good binders that structurally resemble rationally designed antibody mimics were obtained by screening billions of bicyclic peptides by using phage display.[4] Bicyclic-peptide libraries were generated by cyclizing cysteine-rich peptides displayed on phage with the thiol-reactive compound 1,3,5-tris(bromomethyl)benzene (TBMB).[4,5] Isolated bicyclic peptides bound with affinities in the nanomolar or even picomolar range and showed high target selectivity.[4,6] A limitation of the bicyclic peptides is their rather flexible structure. 2D-NMR analysis of several phage-selected bicyclic peptides showed that they do not have a preferred three-dimensional folding in solution.[4,7] This conformational flexibility prevents the development of binders for challenging targets such as peptides or small molecules, and it may limit the binding affinity of bicyclic peptides developed against more tractable targets.

In this work, we raised the question of whether chemical linkers in bicyclic peptides could direct the structure of the peptide moiety by forming, in addition to the covalent bonds, noncovalent interactions with amino acids in the peptide (Figure 1 a). More specifically, we wondered whether the peptides anchored to small molecules and selected in vitro for

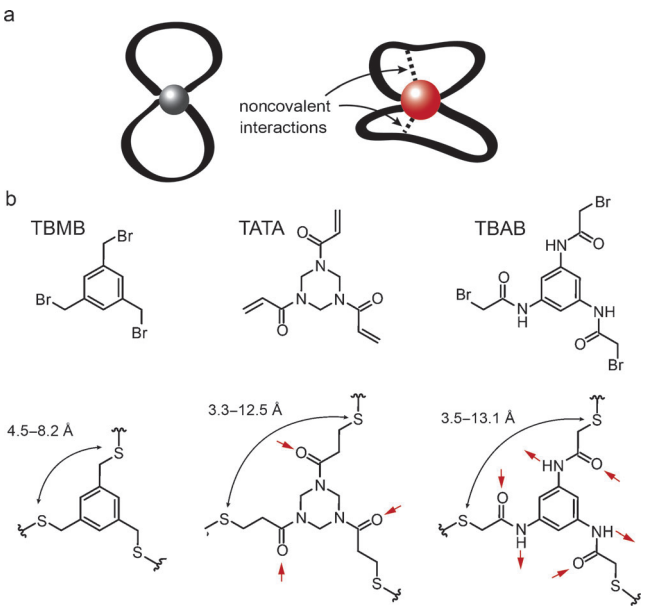

Figure 1. Structurally diverse small molecules for the phage selection of bicyclic peptides. a) The structures of previously developed and structurally characterized bicyclic peptides were not directed by the central chemical linker (illustrated by a gray ball; left). Small molecules with polar groups (illustrated as a red ball) are envisioned to form H-bonding interactions (black dashed lines) with the peptide loops and to serve as nucleating scaffolds (right). b) Structures of the small molecules, each containing three thiol-reactive groups, before and after reaction. Potential H-bond donors and acceptors are indicated by red arrows. Computed distance ranges between the sulfur atoms of the cyclized peptides are indicated.

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binding to protein targets would fold around the small molecules and employ them as scaffolds. We addressed this question by subjecting random peptides, covalently linked to various small molecules, to affinity selections against a protein target and analyzing the sequences and structures of the isolated peptide ligands. We envisioned that noncovalent interactions would preorganize the peptide loops to reduce the entropic penalty upon binding to the target. Furthermore, we hoped that noncovalent interactions between the peptide and a nucleating core would stabilize the peptide–target complex.

A recently solved first X-ray structure of a target-bound bicyclic peptide UK18, which inhibits the serine protease urokinase-type plasminogen activator (uPA), showed that the central hydrophobic linker does not form noncovalent interactions with the peptide loops and thus does not serve as a nucleating scaffold.[6a] To promote the formation of hydrogen bonds between the small molecules and amino acids in the peptides, we applied the hydrophilic small molecules 1,3,5-triacryloyl-1,3,5-triazinane (TATA) and *N*,*N*′,*N*″-(benzene-1,3,5-triyl)-tris(2-bromoacetamide) (TBAB), each of which has multiple polar groups and three thiol-reactive groups (Figure 1b).[7] As a control, we applied the small molecule TBMB,[4,5] a reagent that has a hydrophobic benzene ring at its center and has previously been shown not to serve as a nucleating scaffold for bicyclic peptides.[6a] The three small molecules have similar but not identical dimensions, with the cysteine residues in the cyclized peptides held at different distances relative to each other (Figure 1b). The optimal concentration of small-molecule reagent needed for the cyclization of peptides fused to the phage coat protein pIII were found to be 40 μM and 20 μM for TATA and TBAB, respectively (Figure S1a in the Supporting Information). Treating the phage with the same or slightly higher concentrations of the compounds did not affect their infectivity (Figure S1b).

A peptide phage library of the format XCX_4CX_4CX–phage (4×4 library; X = any amino acid; library size 7.3×10^8) was allowed to react with the three thiol-reactive small molecules. The libraries were subjected to two or three iterative rounds of phage selection against human uPA, a protease involved in tumor growth and invasion.[8] The isolated peptides showed consensus sequences that were strongly biased by the small-molecule structures linked to them (Figure 2). Most of the consensus sequences were found exclusively in selections with one of the three small molecules. Selection with TBMB yielded one unique consensus sequence (group A, Figure 2a), that with TATA yielded two unique consensus sequences (groups D and E, Figure 2b), and that with TBAB yielded one unique consensus sequence (group H, Figure 2c). The consensus motifs "TAR" and "SAR" were found in selections with each of the three small molecules (groups B, F, and I in Figure 2). The same type of experiment was performed with a different peptide library of the format XCX_3CX_3CX–phage (3×3 library; library size 5.6×10^8). In this experiment too, the consensus sequences of the isolated peptides were strongly

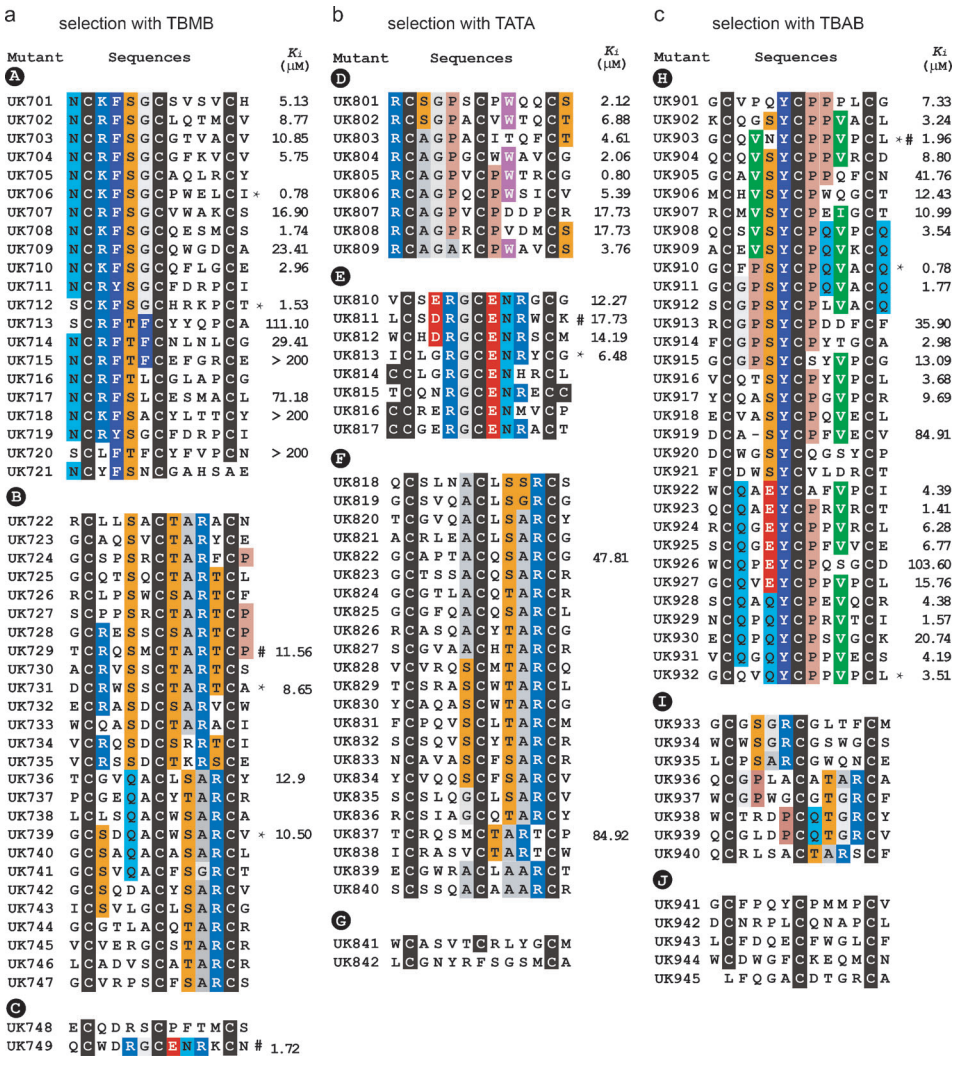

Figure 2. Peptides isolated in phage selections against uPA from the 4×4 library after cyclization with the chemical compounds TBMB (a), TATA (b), and TBAB (c). Peptides with similar sequences are arranged in groups and the similarities are highlighted in color. The indicated inhibition constants (K_i) are averages of at least two measurements. Standard deviations were smaller than 20% in all cases and are not indicated. * Indicates peptides that were also cyclized with the other two chemical compounds (see Figure 3c). # Indicates peptides that were cocrystallized with uPA (see Figure 4).

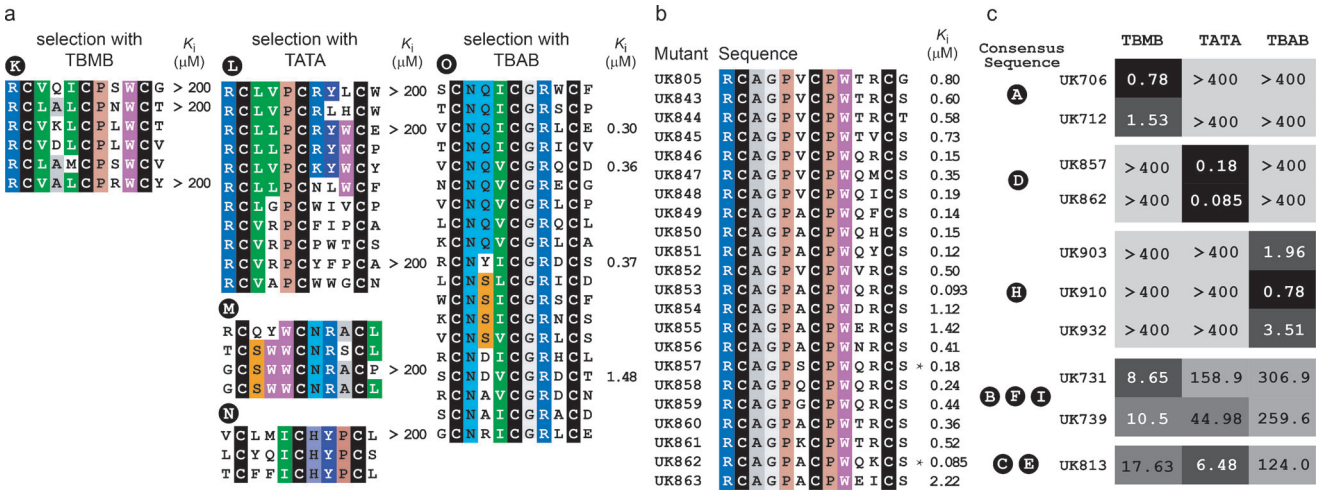

Figure 3. Phage selected peptides and their activities. All indicated K_i values are averages of at least two measurements. Standard deviations were smaller than 20% in all cases and are not indicated. a) Peptides isolated in phage selections against uPA from the 3×3 library cyclized with each of the three chemical compounds. b) Affinity maturation of the peptide UK805, which was cyclized with TATA. c) The peptides marked with an asterisk in Figures 2 and 3b were cyclized with each of the three small molecules and the K_i value for uPA measured.

influenced by the small molecule used in the selections (Figure 3a), thus suggesting that the peptides fold closely around these structures or even form noncovalent interactions with them.

75 of the identified bicyclic peptides were synthesized and their inhibition of uPA measured (Figures 2a–c and 3a). Most of the inhibitors showed K_i values in the micromolar range and several showed K_i values in the high nanomolar range. In the selection with the 4×4 library, all three molecules yielded good binders (K_i = 0.87–0.8 μM). In the selection with the 3×3 library, by far the best binders were isolated from pools of peptides cyclized with the new small molecule TBAB (best K_i = 0.3 μM). The generation and testing of 20 mutants of one bicyclic peptide, UK805, yielded a ninefold improved inhibitor (UK862; K_i = 85 nM, Figure 3b). This affinity is slightly weaker than that of the best bicyclic peptide inhibitor of uPA (UK202, which contains the unnatural amino acid D-Ser; K_i = 28 nM[9]) but it is remarkably good considering the short length of the peptide (13 amino acids versus 17 amino acids in UK202).

To verify that the different consensus sequences indeed evolved as a result of the chemical and/or structural differences between the three small molecules, we swapped the small molecules in the best inhibitory peptides (Figure 3c; these peptides used are indicated with an asterisk in Figure 2a–c and Figure 3b). Most of the peptides were found to be more than 400-fold less active when cyclized with the two small molecules not used for their selection. The peptides with the consensus motifs TAR and SAR, which were found after selection with each of the three small molecules (for example UK731 and UK739; groups B, F, and I), inhibited uPA when cyclized with any one of the three compounds. However, for these peptides too, the inhibitory activities were highest when the peptides were cyclized with the small molecule applied in their selection (Figure 3c). The swapping experiment confirmed that the peptides had adapted to the structural and chemical environment of the small molecules. Despite their comparable sizes, the three small molecules offer different environments to the peptides.

Four out of around 50 tested bicyclic peptides cocrystallized with uPA and the structures of the peptide–uPA complexes could be determined at resolutions between 1.49 and 1.85 Å (Figure 4 and Figure S2; these peptides are indicated with hash symbols in Figure 2). Two of these peptides were cyclized by TBMB (UK729, PDB entry 4MNV; UK749, PDB entry 4MNW), one by TATA (UK811, PDB entry 4MNX), and one by TBAB (UK903, PDB entry 4MNY). As shown in Figure 4, the peptides bind to the substrate-binding region of uPA in different orientations.

In all of the structures, the electron density of the small molecule was well resolved (Figure S2), thus allowing assignment of all of the atoms and identification of noncovalent interactions between the small molecule and the peptide (Figures S3–S5). In UK749, the mesitylene ring of TBMB does not form noncovalent interactions with amino acids in the peptide. In UK729, the mesitylene group of TBMB forms a hydrophobic contact with a small region of Gln4 but most of the mesitylene surface is entirely solvent-exposed. As found previously in the structure of the TBMB-cyclized bicyclic peptide UK18, the hydrophobic mesitylene core seems not to be an ideal molecule to serve as a structural scaffold. In the bicyclic peptide UK811, which was cyclized with TATA, the triazinane structure did not form any noncovalent interactions with the peptide despite its three potential H-bond donor groups. In contrast, in the bicyclic peptide UK903, which was cyclized with TBAB (the small molecule with the largest number of polar groups), we found multiple noncovalent interactions between the small molecule and the peptide. Specifically, the planar amide groups of TBAB form three H bonds with amino acids in the peptide, namely the side chain of Asn5 (H-bond length: 3.08 Å), the carbonyl oxygen atom of Cys7 (H-bond length: 2.93 Å), and the carbonyl oxygen atom of Val10 (H-bond length: 3.12 Å; Figure 5a–d). Peptide UK903 and TBAB form a dense

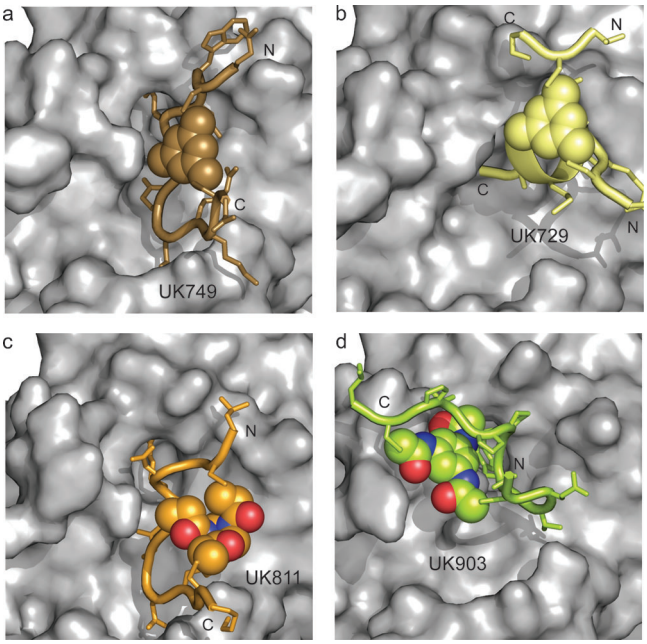

Figure 4. Cocrystal structures of bicyclic peptides and uPA. All four peptides bind to the active site of uPA (the same surface region of the protease is shown in all four cases). The chemical compound is shown as a space-filling model, the peptide backbone as a tube, and the side chains as stick representations. Detailed structures are shown in Figures S2–S5. H-bonding interactions are summarized in Tables S3–S10. a) UK749, which was cyclized with TBMB. b) UK729, which was cyclized with TBMB. The peptide is cleaved by uPA, whereby the active-site serine remains bound to the C terminus of the longer chain as an acyl-enzyme intermediate. c) UK811, which was cyclized with TATA. d) UK903, which was cyclized with TBAB.

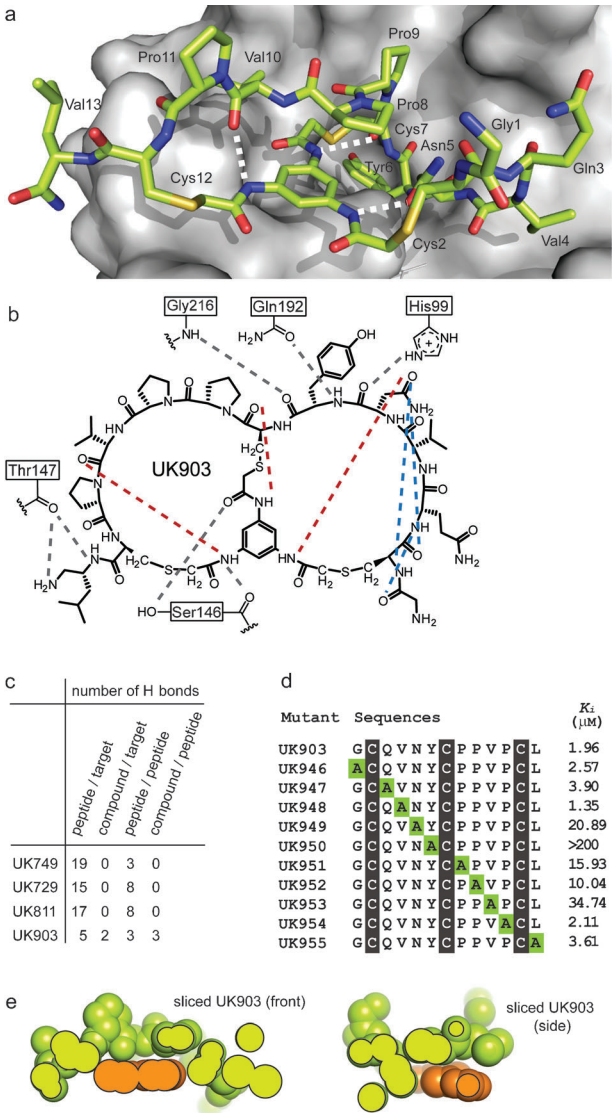

Figure 5. Structural properties of UK903. a) UK903 (shown as a stick representation) bound to uPA. H bonds between the benzenetriamide core and the peptide are shown as white dashed lines. b) Chemical structure of UK903 with H-bonding interactions shown as dashed lines. c) Comparison of the number of H bonds in each of the four bicyclic peptides cocrystallized with uPA. d) Alanine scan of UK903. e) UK903 represented as a space-filling model sliced in half along perpendicular planes. The peptide is shown in green and the ring of TBAB in orange.

structure with nearly no space in the interior that is accessible to water molecules (Figure 5e). The compactness and rigidity of the complex is underpinned by the exceptionally low B-factor values for the nine C-terminal residues and the benzenetriamide; these values were on average 16.5 $Å^2$ compared to 19.6 $Å^2$ for uPA in the cocrystal structure. The compact folding of UK903 resembles that of a protein.

The finding that hydrophilic small molecules can form noncovalent interactions with peptides that are wrapped around them is of great interest because these contacts could potentially help stabilize a three-dimensional structure in solution; this could in turn lower the entropic penalty upon binding to the target and thus result in higher binding affinities. The three observed H bonds between the peptide and the small molecule are unlikely to stabilize the peptide enough for it to adopt a defined structure in solution. However, these H bonds might allow transient preorganization of the peptide so that it more closely resembles its target-bound form and thus binds better than entirely flexible cyclic peptides. The H bonds undoubtedly stabilize the complex between the peptide and the target protein, as holds true for ligands with noncovalent intraligand interactions in general.

In summary, we demonstrate that small molecules at the center of bicyclic peptides can form noncovalent interactions with amino acids in the peptides and thus serve as structural scaffolds. We show that polar molecules are most suited as cores and are superior to the hydrophobic structure previously used in the directed evolution of bicyclic peptides. Our findings pave the way for the generation of synthetic antibody-mimicking structures, which has been a longstanding goal. Molecules combining a small size with excellent binding properties promise many advantages for clinical applications, including access to chemical synthesis, good tissue penetration, and a range of administration options.

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[1] a) G. Vlatakis, L. I. Andersson, R. Muller, K. Mosbach, *Nature* **1993**, *361*, 645 – 647; b) M. Mutter, P. Dumy, P. Garrouste, C. Lehmann, M. Mathieu, C. Peggion, S. Peluso, A. Razzaname, G. Tuchscherer, *Angew. Chem.* **1996**, *108*, 1588 – 1591; *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1482 – 1485; c) Y. Hamuro, M. C. Calama, H. S. Park, A. D. Hamilton, *Angew. Chem.* **1997**, *109*, 2797 – 2800; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 2680 – 2683.

[2] a) H. U. Saragovi, D. Fitzpatrick, A. Raktabutr, H. Nakanishi, M. Kahn, M. I. Greene, *Science* **1991**, *253*, 792 – 795; b) M. Levi, M. Sallberg, U. Ruden, D. Herlyn, H. Maruyama, H. Wigzell, J. Marks, B. Wahren, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 4374 – 4378; c) M. Favre, K. Moehle, L. Y. Jiang, B. Pfeiffer, J. A. Robinson, *J. Am. Chem. Soc.* **1999**, *121*, 2679 – 2685; d) B. W. Park, H. T. Zhang, C. Wu, A. Berezov, X. Zhang, R. Dua, Q. Wang, G. Kao, D. M. O'Rourke, M. I. Greene, R. Murali, *Nat. Biotechnol.* **2000**, *18*, 194 – 198.

[3] P. Timmerman, R. Barderas, J. Desmet, D. Altschuh, S. Shochat, M. J. Hollestelle, J. W. Hoppener, A. Monasterio, J. I. Casal, R. H. Meloen, *J. Biol. Chem.* **2009**, *284*, 34126 – 34134.

[4] C. Heinis, T. Rutherford, S. Freund, G. Winter, *Nat. Chem. Biol.* **2009**, *5*, 502 – 507.

[5] P. Timmerman, J. Beld, W. C. Puijk, R. H. Meloen, *ChemBioChem* **2005**, *6*, 821 – 824.

[6] a) A. Angelini, L. Cendron, S. Chen, J. Touati, G. Winter, G. Zanotti, C. Heinis, *ACS Chem. Biol.* **2012**, *7*, 817 – 821; b) V. Baeriswyl, S. Calzavarini, C. Gerschheimer, P. Diderich, A. Angelillo-Scherrer, C. Heinis, *J. Med. Chem.* **2013**, *56*, 3742 – 3746; c) I. R. Rebollo, A. Angelini, C. Heinis, *MedChemComm* **2013**, *4*, 145 – 150.

[7] S. Chen, J. Morales-Sanfrutos, A. Angelini, B. Cutting, C. Heinis, *ChemBioChem* **2012**, *13*, 1032 – 1038.

[8] P. A. Andreasen, R. Egelund, H. H. Petersen, *Cell. Mol. Life Sci.* **2000**, *57*, 25 – 40.

[9] S. Chen, D. Gfeller, S. A. Buth, O. Michielin, P. G. Leiman, C. Heinis, *ChemBioChem* **2013**, *14*, 1316 – 1322.

