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Engineered Two-Helix Small Proteins for Molecular Recognition

Jack M. Webster,[a] Rong Zhang,[a] Sanjiv S. Gambhir,[b] Zhen Cheng,*[b] and Faisal A. Syud*[a]

Because of the rapid advancement of molecular biology, numerous biomarkers that are aberrantly expressed in tumor tissues have been identified and studied to correlate their presence and expression levels with the extent of tumor physiological traits such as malignancy, invasiveness, metastasis, and resistance to therapy.[1] Biological molecules that can specifically recognize these tumor targets and biomarkers are thus highly important and are under intensive investigation. These biomolecules might provide a unique opportunity for the development of drugs for targeted cancer therapy or imaging probes for cancer molecular imaging.[2]

Recently, many in vitro display technologies (phage, bacterial, yeast, ribosome, and mRNA displayed) in conjunction with rational protein design and engineering have been used to construct nonimmunoglobulin protein libraries with well-defined scaffolds. The rapid progress of these protein display technologies has led to the discovery of many novel small proteins and peptides with high affinity and specificity for a variety of molecular targets.[3] Among these small proteins, affibody molecules have received significant attention. Affibody molecules are engineered small protein scaffolds with 58 amino acid residues, ~7 kDa molecular weight (M_W), and a three-helix bundle structure, and are derived from one of the IgG-binding domains of staphylococcal protein A (SPA; Figure 1 A).[3a] Affibody molecules against desired targets (e.g., human epidermal growth factor receptor type 2 (HER2), epidermal growth factor receptor (EGFR), HIV-1 gp120, etc.) have been quickly identified and selected by using phage-display library technology.[3a,4]

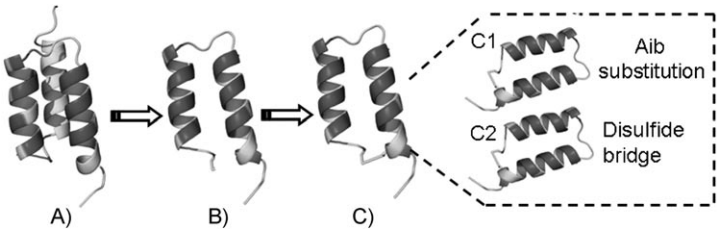

Figure 1. Strategies for developing 2-helix small proteins for molecular recognition. A) Based on the 3-helix affibody molecule, one helix was removed to generate B) a free 2-helix protein. C) The truncated 2-helix protein was further constrained and stabilized by using different methods for affinity maturation, such as inclusion of unnatural amino acid aminoisobutyric acid (Aib) and cyclization of protein through disulfide bridge.

[a] *Dr. J. M. Webster, R. Zhang, Dr. F. A. Syud*
Biosciences, Global Research, General Electric Company
1 Research Circle, Niskayuna, NY 12309 (USA)
Fax: (+1) 518-387-7765
E-mail: syud@crd.ge.com

[b] *Prof. S. S. Gambhir, Prof. Z. Cheng*
Molecular Imaging Program at Stanford
Department of Radiology and Bio-X Program, Stanford University
1201 Welch Road, Lucas Expansion, P020A Stanford, CA 94305 (USA)
Fax: (+1) 650-736-7925
E-mail: zcheng@stanford.edu

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HER2, a transmembrane protein and a member of erbB family of receptor tyrosine kinase proteins, is a well-established tumor biomarker that is overexpressed in a wide variety of cancers including breast, ovarian, lung, gastric, and oral cancers.[5] HER2 amplification and activation promote tumor cell proliferation, growth, migration, adhesion, and invasiveness. It has been found that HER2 over-expression is usually associated with high-grade aggressive tumors, and a poor prognosis.[5] Therefore, HER2 has great value as a molecular target for therapeutic intervention, as a prognostic indicator of patient survival, and as a predictive marker of response to anti-neoplastic therapy. HER2-binding affibody molecules (both the 7 kDa monomeric and 14 kDa dimeric affibody constructs) have been successfully developed and extensively studied in conjunction with a variety of radiolabels for therapeutic and diagnostic imaging applications.[6] These research efforts have clearly demonstrated that 3-helix protein scaffold affibody molecules are a promising new class of cancer-targeting ligands.

In our previous report, we have shown that between the 7 kDa monomeric and 14 kDa dimeric affibody constructs, despite higher in vitro HER2-binding affinity of the dimeric construct, the monomer has significantly improved in vivo properties: better tumor uptake and faster clearance from background organs such as blood and liver; this highlights the great potential of using monomeric affibody for molecular imaging.[6a] These results have also motivated us to continue to explore protein scaffolds that are even smaller than the 3-helix, 7 kDa affibody. It is expected that further size reduction of the affibody construct could alleviate unspecific organ uptake, provide higher target tumor-to-organ uptake ratios, and provide both better therapeutic and imaging applications. Moreover, such size reduction also opens opportunities to more facile and economically viable synthetic generation strategies. Additional advantages that are expected for smaller protein constructs include the potentially lower immunogenic potential, fast clearance rate, quick tumor accumulation and relatively short in vivo biological half life. In this communication, we describe strategies that have been successfully used to develop 2-helix small proteins, a smaller version of affibody constructs for HER2 recognition (Figure 1).

It has been widely reported that for affibody proteins the binding segment comprises the surface-exposed thirteen amino acid residues (9, 10, 11, 13, 14, 17, 18, 24, 25, 27, 28, 32, and 35) localized in the N-terminal helices 1 and 2 (Figure 1, amino acids highlighted in red). The third helix of the affibody is expected to only contribute to the structural rigidity of the molecule, thereby reducing the entropic cost upon bind-

ing.[3a,g,f] Therefore, to make an even smaller version of affibody constructs, our first trial was to truncate the C-terminal helix from the affibody (Figure 1). By using a high-affinity HER2 affibody binder $Z_{HER2:342}$ with a K_D of 22 pM as a template protein,[6f] a C-terminal helix-truncated small protein **1** was produced. Unfortunately, surface plasmon resonance (SPR) analysis by using BIAcore analysis showed that this peptide had nondetectable binding to HER2-Fc that was immobilized on a chip (Figure 2). This finding is consistent with previously reported ing disulfide bond.[9] Peptide **3** with the disulfide bond showed an affinity with a K_D of 259 nM (Figure 3). CD spectroscopy analysis revealed the helix content to be similar to that obtained from the sequence mutations alone, with a helix content also at about 10%. It was also found that although peptide **3** bound to HER2, it did not bind to the control proteins mesenchymal epithelial transition factor (c-Met) and human IgG (data shown in the Supporting Information); this suggests reasonable specificity of this peptide even though it lacks helicity. To probe whether placement of the disulfide bond was indeed optimal we toggled the position of the cysteine residues around positions 5 and 39, and also moved the bond along the axis of the two helices forming pairs between residues 9 and 34, 9 and 35, 13 and 30, 13 and 31, 16 and 27, and 17 and 27. For disulfides at the axis location simi-

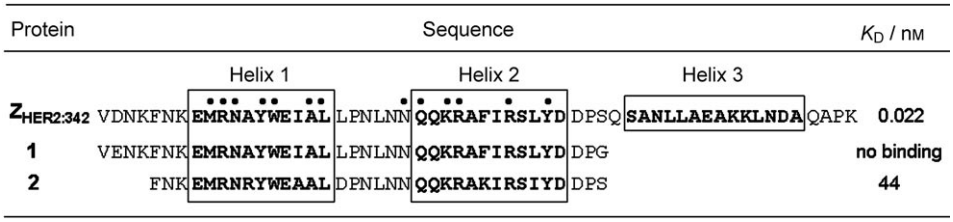


Figure 2. Amino acid sequence of a 3-helix affibody $Z_{HER2:342}$ and its mutants, 2-helix small protein **1** and **2**. The HER2-binding site is localized in helices 1 and 2 and is highlighted with black dots (●).

results, in which the truncation of the corresponding 3-helix construct from the Z-domain of protein A reduced the binding affinity against IgG_1 by approximately 10^5-fold.[7]

To improve the binding affinity of the 2-helix small protein, the strategy of natural amino acid mutation was applied to the peptide **1**. Braisted et al. have identified a number of amino acid mutations that are beneficial to the affinity of their truncated 38-mer peptide against IgG_1, including A13R, I17A, L20D, F31K, L35I, amongst others.[7] It was found that the inclusion of the mutations increased the helicity of their peptide by up to 50% relative to 11% for the wild type. This was still significantly below the helicity of the parent protein, but was sufficient to raise the affinity up to a K_D of 43 nM.[7] We adapted the sequence mutations specifically listed to our truncated peptide to obtain peptide **2**, but we retained the residues expected to interface with HER2. The peptide **2** demonstrated a K_D of 44 nM against HER2 by quantitative, kinetics-based SPR analysis. Interestingly, circular dichroism (CD) analysis revealed that the helix content of peptide **2** remained fairly low: qualitatively the spectrum was dominated by a random-coil conformation, and quantitatively it showed helix conformation of about 10%. It is hence not surprising that the affinity of peptide **2** is still three log orders lower than $Z_{HER2:342}$. This also suggested that an increase in the population of folded peptide and that mimicking the parent 3-helix affibody was required to further increase the affinity.

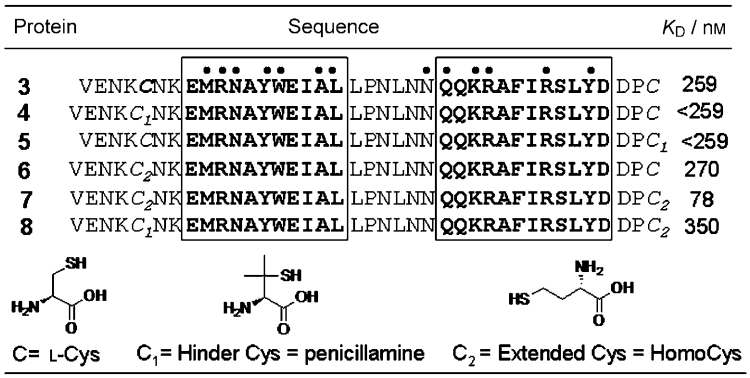


Figure 3. Structural stabilization of mutant small proteins by disulfide bridge formation.

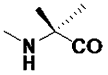
The use of disulfide bonds, specifically between two naturally occurring cysteine residues, is prevalent in the literature towards stabilizing the structure of truncated proteins and peptides.[8] Visual inspection of the known structure of the affibody Z domain suggested that the cysteine substitution of Phe5 and Ser39 might allow the formation of a favorable, constrainlar to that of peptide **3**, relative binding studies by SPR showed that the original placement of the bond was indeed optimal. Other constructs showed a decrease in binding by a factor of 2–3. To move the disulfide bond along the axis, we chose cysteine positions that balanced being distal from the binding site but potentially maintaining sufficient distance and torsion to form a disulfide bond. However, as the bond was moved along the axis towards the loop connecting the two helices, complete abrogation of the binding affinity was observed. Because the helical axis rotates slightly moving down along the axis, presumably it is harder to maintain the optimal balance of the correct distance and rotation between the helices to allow optimal binding to HER2.

To explore how rigidity of the disulfide bridge could affect affinity we used a number of unnatural amino acid analogues of cysteine (Figure 3). Such mutants allowed the utilization of the thiol chemistry, which was developed to cyclize the truncated two-helix. Homocysteine is an unnatural amino acid derivative analogous to the naturally occurring cysteine but with an extra methylene group in the side chain; it is expected to

bestow more flexibility relative to the side chain of naturally occurring cysteine. The cysteine derivative penicillamine (Pen) has two methyl groups attached to the β-carbon of the side chain and is expected to be more sterically hindered. Various combinations of L-cysteine, and the extended and hindered side chain containing cysteine variants were studied (Figure 3, proteins **4**–**8**). By using SPR, relative binding studies identified the variant containing two extended cysteine to have the highest affinity. Kinetic analysis revealed the dissociation constant, K_D, of the construct **7** against HER2 to be 78 nM.

Helix-promoting amino acids are well documented in the literature, and we hypothesized that such amino acids could rigidify our 2-helix construct further. Aminoisobutyric acid (Aib) is particularly well-documented in the literature to promote α-helix formation.[10] We identified positions 8, 12, and 16 on the N-terminal helix and positions 26, 30, and 34 on the C-terminal helix as possible sites for mutation to Aib in our construct (Figure 4, **9**–**12**). We further postulated that such mutations

Protein	Sequence	K_D / nM
9	VENK*C*NK**XMRNXYWEXAL**LPNLNN**QQKRAFIRSLYD**DP*C*	n.b.
10	VENK*C*NK**EMRNAYWEIAL**LPNLNN**QXKRAXIRSXYD**DP*C*	n.b.
11	VENK*C*NK**XMRNXYWEXAL**LPNLNN**QXKRAXIRSXYD**DP*C*	n.b.
12	VENK*C*NK**XMRNXYWEXAL**LPNLNN**QXKRAXIRSXYD**DP*C*	182



X = α-aminoisobutyric acid (Aib)

Figure 4. Inclusion of unnatural amino acid (Aib) mutations so as to improve binding affinity; n.b.: no binding; ●: HER2 binding sites of the small proteins.

might by themselves be insufficient to completely recover the binding affinity, and its impact could be more apparent on a peptide with some binding affinity. Hence, all mutations were combined with a disulfide bridge stabilization strategy. A peptide (**9**) was generated with all the mutations on the N-terminal helix, one (**10**) with all the mutations on the C-terminal helix, and one (**11**) with all six mutations. All peptides contained a disulfide bridge between C5 and C39. It was interesting to find that the inclusion of the mutations completely abolished all binding to HER2. A possible hypothesis for this might be that the amino acids that were substituted take part in, directly or indirectly, the binding interaction with HER2, and a mere structural stabilization of the helices is insufficient. Comparison of the Z-domain of protein A, from which the affibody scaffold is derived, and the B-domain reveals that the G29A substitution significantly stabilizes the second helix and increases the rate of folding.[11] We therefore hypothesized that at least Aib substitution into this corresponding position in our 2-helix should increase the binding affinity by increasing the helix stability. Indeed, the Ala29Aib substitution (peptide **12**) generated the expected modest but noticeable increase in affinity by a factor of about 1.5 relative to peptide **3**.

Finally, we combined the most impactful strategies for further improvement of the affinity of the 2-helix for HER2 (Figure 5). A variant of peptide **3** was generated containing

Protein	Sequence	K_D / nM
13	*C*NK**EMRNRYWEAAL**DPNLNN**QQKRAKIRSIYD**DP*C*	15
14	C_2NK**EMRNRYWEAAL**DPNLNN**QQKRAKIRSIYD**DPC_2	5
15	C_2NK**EMRNRYWEXAL**DPNLNN**QQKRXKIRSIYD**DPC_2	5

Figure 5. Affinity improvement strategies; C_2: HomoCys; X: Aib.

natural and unnatural amino acid mutations (Aib) and the flexible cysteine analogues (peptide **5**). The N-terminal segment VENK was further removed to allow more facile synthesis because previous NMR spectroscopy studies have shown this segment to be unstructured and it represents a superfluous region.[7] SPR analysis showed that peptide **14** and **15** have a binding affinity of 5 nM. Interestingly, CD spectroscopy revealed peptide **15** to have the highest population of helix conformers, which is still low at approximately 15%.

In summary, by starting with a high-affinity HER2-binding 3-helix affibody molecule ($Z_{HER2:342}$), we truncated a helix from the protein, adapted sequence mutations, and introduced a disulfide bridge to yield a 2-helix small protein **3** with modest HER2 affinity. We further explored several strategies (alternate placement and type of the disulfide bridge, inclusion of affinity-promoting, likely helix-promoting amino acid mutations) for affinity maturation. Finally, with a combination of these strategies, we successfully attained two 2-helix HER2 binders (**14** and **15**) with the high HER2-binding affinity of 5 nM. Currently, we are studying the in vivo performance (pharmacokinetics, tumor-targeting ability, etc.) of the truncated cyclic peptides by using different radiolabels. Our efforts clearly suggest that 2-helix small proteins against significant tumor targets such as HER2 can be obtained by rational design and protein engineering. A systematic set of tools that are described in this research have great translatability for developing many other 2-helix small proteins against desired targets. These novel 2-helix scaffold proteins (~4.6 kDa), two-thirds the size of the smallest affibody molecule, could become powerful tools in the fight against cancer.

Experimental Section

All the linear peptides were synthesized by using standard solid-phase peptide synthesis techniques (Fmoc chemistry with HBTU/HOBT activation). Cleavage of the peptides from the resin was accomplished by using a cocktail consisting of TFA/ethane-1,2-dithiol (EDT)/water/triisopropylsilane (TIPS) (94:2.5:2.5:1, *v*/*v*/*v*/*v*) for 4 h while being stirred at room temperature. Cyclization of the linear peptides was achieved by I_2 oxidation of the two acetamidomethyl (Acm)-protected thiols to form a disulfide bridge. Both crude linear and cyclized peptides were purified by a reverse-phase preparative HPLC with a Vydac protein and peptide C4 column. The mobile phase was solvent A (0.05% TFA in water, *v*/*v*) and solvent B (0.05% TFA in acetonitrile, *v*/*v*), and different gradients were used for purification of the peptides. The flow rate was typically 25 mL min^{-1}. The desired fractions were collected, frozen immediately, and lyophilized. The identity of the target peptides was confirmed by MALDI-TOF-MS or ESI-TOF-MS.

The HER2-binding affinity of proteins were measured in vitro by using surface plasmon resonance (SPR) detection with a Biacore 3000 instrument (GE Healthcare) according to the previously reported method.[6a] Briefly, the Fc-HER2 (R&D Systems, Minneapolis, MN, USA) was covalently attached to a CM-5 dextran-functionalized sensor chip (GE Healthcare) by using EDC and *N*-hydroxysuccinimide (NHS). A second flow cell on the same sensor chip without Fc-HER2 immobilization was used as a control. Prior to the kinetic study, binding of the target analyte was tested on both surfaces and a surface stability experiment was performed to ensure adequate removal of the bound analyte and regeneration of the sensor chip following treatment with NaCl (2.5 M) and NaOH (50 mM). SPR sensorgrams were analyzed by using the BIAevaluation software (GE Healthcare). SPR measurements were collected at eight analyte concentrations (0–100 nM protein) and the resulting sensorgrams were fitted to a 1:1 Langmuir binding model.

All CD spectra were obtained on an JASCO J-815 CD spectropolarimeter in the wavelength range of 250–185 nm by using 1 nm bandwidth, 0.5 nm resolution, 4 s averaging time, three scans per sample, 0 sdelay, and a path length of 1 mm. Spectra were recorded at 20 °C, unless otherwise noted, in a thermostated circular cuvette, with peptide concentrations of 15 μM K_2HPO_4 (13 mM) buffer at pH 8. Results were reported as mean residue ellipticity (MRW).

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