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Phage selection of bicyclic peptides binding Her2

Philippe Diderich, Christian Heinis *

Institute of Chemical Sciences and Engineering, Ecole Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland

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ABSTRACT

Aberrant expression of the epidermal growth factor receptor Her2 has been implicated in various malignancies including breast cancer. Monoclonal antibodies and an antibody–drug conjugate targeting Her2 have found wide clinical application. Herein, we aimed at developing Her2-specifc ligands based on peptides that have a 100-fold smaller molecular weight than antibodies. Such peptides could potentially offer advantages in the development of ligand–drug conjugates, such as ease of synthesis and conjugation, higher molecule-per-mass ratios, and better tumor penetration. Panning of large bicyclic peptide phage display libraries against Her2 yielded a range of Her2-specific ligands having different formats and binding motifs. Strong sequence similarities among several of the isolated peptides indicated that they interact with Her2 in a specific manner. The best bicyclic peptide obtained after affinity maturation bound Her2 with a K_D of 304 nM. The diverse peptide ligands may offer valuable starting points for the development of high-affinity Her2 binders with potential application for tumor imaging and therapy.

1. Introduction

Human epidermal growth factor receptor 2 (Her2/neu, ErbB-2; here abbreviated Her2) is a plasma membrane-bound receptor tyrosine kinase that is over-expressed in 15–30% of breast cancer patients. It is strongly associated with increased disease recurrence and poor prognosis.[1] Her2 can heterodimerize with the homologous proteins ErbB-1 (EGFR), ErbB-3, and ErbB-4 and is considered to be the preferred dimerization partner.[2] Dimerization leads to autophosphorylation of tyrosine residues within the cytoplasmic domain of the receptors, resulting in cell growth and division.

Monoclonal antibodies targeting Her2 have found wide application in therapy. Two monoclonal antibodies (trastuzumab and pertuzumab) and one antibody–drug conjugate (trastuzumab emtansine) are in clinical use.[3] While antibodies have dramatically improved the treatment of Her2-positive cancers, they suffer from a range of limitations that are associated with their large size of 150 kDa.[4] Antibodies do not homogenously distribute in solid tumors. As a consequence a fraction of the protein target may not be reached. Furthermore, site-selective conjugation of drugs to specific sites in antibodies is challenging. Many antibody–drug conjugates are heterogeneous mixtures of structures having varying antibody-to-drug ratios. In imaging applications, whole antibodies are slowly cleared and exhibit low tissue contrast.

Toward the generation of smaller Her2-specific ligands, various protein scaffolds were exploited including protein A (Affibodies),[5] designed ankyrin repeat proteins (DARPins),[6,7] and Fyn kinase SH3 domain (Fynomers).[8] Libraries of these proteins were generated and screened by in vitro display techniques, such as phage display or ribosome display. Affibodies were reported to penetrate more efficiently into tissue compared to an IgG antibody.[9] DARPins with very high affinity for Her2 showed good accumulation in Her2 over-expressing SK-OV-3 xenografts in mice.[10] Many different conjugates of Affibodies and DARPins have been reported.[11,12] Chemical conjugation of small molecules to these proteins is technically less complex and more efficient than conjugation reactions with antibodies. Affibodies can be synthesized chemically.[13]

Even smaller Her2 ligands were developed based on disulfide-cyclized peptides. Murali and Greene generated a peptidomimetic named AHNP, based on the shared structural properties of the heavy chain CDR3 in two Her2-specific antibodies.[14] AHNP bound with sub-micromolar affinity to Her2 and inhibited proliferation of Her2-expressing cancer cells.[14,15] Quinn and co-workers isolated the Her2-specific peptide KCCYSL from a hexapeptide phage display library.[16] The peptide containing two adjacent cysteines bound Her2 with a K_D of 295 nM and was evaluated for tumor imaging and targeting in mice.[17] The successful isolation of Her2-specific ligands from hexapeptide libraries suggested that better and/or more diverse peptide ligands might be isolated from libraries of longer peptides. Longer peptides can potentially form more contacts with the target and thus should be able to bind more tightly.

Our laboratory is specialized in the phage selection of bicyclic peptide ligands. Bicyclic peptides have two peptide rings that can both engage in binding and they typically bind with higher affinity

* Corresponding author. Tel.: +41 21 693 9350; fax: +41 21 693 9895; e-mail address: christian.heinis@epfl.ch (C. Heinis).

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than linear or monocyclic analogues. The bicyclic configuration reduces the conformational flexibility and renders them more resistant to proteases. Bicyclic peptide ligands can be evolved in vitro by phage display as follows. Linear, random peptides containing three cysteine residues are displayed on the surface of filamentous phage and cyclized with a tri-functional thiol-reactive reagent. Such combinatorial libraries of bicyclic peptides are then subjected to affinity selection toward a desired target. Bicyclic peptide inhibitors have been successfully developed for a range of protease targets, including urokinase-type plasminogen activator,[18] plasma kallikrein,[19] cathepsin G,[19] and coagulation factor XIIa.[20] Herein, we describe the development of bicyclic peptide ligands against the extracellular domain (ECD) of Her2. The ECD of Her2 is composed of four domains. Unlike proteases, which had previously served as targets of bicyclic peptides, Her2 has no narrow substrate binding pocket or cleft and we expected the development of ligands to be more challenging. By applying different phage selection strategies, we succeeded in developing bicyclic peptides binding Her2. Several different consensus motifs and peptide formats were identified. The best ligands bound Her2 with nanomolar affinity.

2. Results and discussion

2.1. Phage selection of bicyclic peptides

Bicyclic peptides binding to the ECD of Her2 were isolated by phage display as follows. Linear peptides of the format CX_6CX_6C (6×6 library; C=cysteine, X=random amino acid) were displayed on the tip of filamentous phage and chemically cyclized with the tri-functional thiol-reactive compound, 1,3,5-tris(bromomethyl) benzene (TBMB, **1**).[19] The resulting bicyclic peptide library comprising >4 billion different peptides was subjected to three rounds of selection against the ECD of Her2. Her2 ECD was expressed as fusion with hGH (hGH–Her2 ECD) and immobilized on streptavidin- or neutravidin-coated magnetic beads after biotinylation. Sequencing of peptides isolated after two rounds of selection showed strong enrichment of one peptide (clone H1) (Fig. 1A).

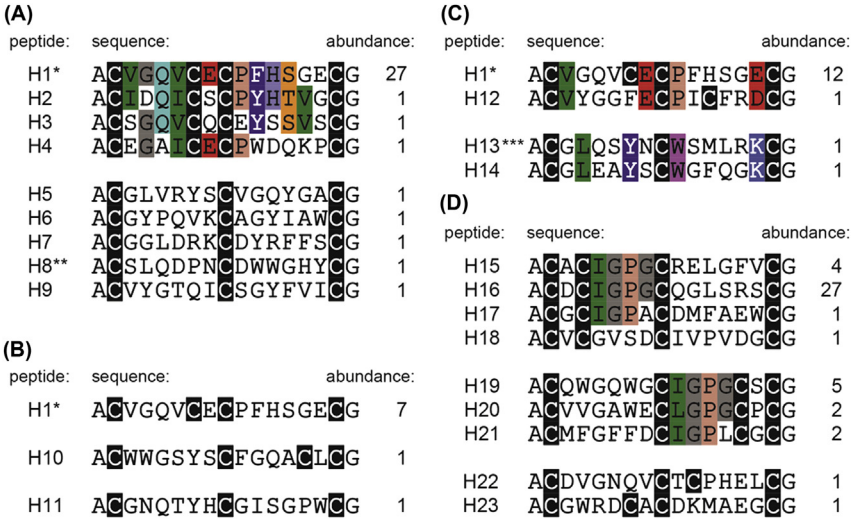

Fig. 1. Peptides isolated in phage selections. A phage peptide library of the form ACX_6CX_6CG-phage (X=random amino acid) was cyclized with **1** prior to affinity selection. Peptides isolated after two rounds of selection in three independent experiments are shown in panels (A)–(C). Similar amino acids are highlighted in color (Rasmol color code). Peptides marked with asterisks were found in two or more independent selection experiments (see also Fig. 3A). (D) Peptides isolated from the 6×6 phage library with air-oxidized peptides.

After the third round of selection, this dominant clone was found exclusively. Comparison of clone H1 with the other isolated peptides revealed a strong sequence homology between clones H1, H2, H3, and H4 (highlighted in color in Fig. 1A). The four peptides contained a fourth cysteine at position 7 and sequence homologies spread over the entire peptide. In previous work, we found that peptides with four cysteines are occasionally strongly enriched in phage selections.[21] A reason for selecting such peptides was found to be incomplete alkylation of the three cysteines with **1** and subsequent isolation of peptides that formed disulfide bridges during phage purification and affinity selection. To exclude that peptides with four cysteines were enriched in this work due to inefficient cyclization with **1**, the selections were repeated two additional times. In both of these phage selection experiments, clone H1 with a fourth cysteine in position 7 was again strongly enriched (Fig. 1B and C).

2.2. Characterization of bicyclic peptides with four cysteines

Peptide H1 was synthesized with an N-terminal fluorescein moiety to enable binding studies by fluorescence polarization. In a first experiment, the peptide was incubated under oxidizing conditions to promote formation of two disulfide bonds (Fig. 2A, upper sequences). All the three possible disulfide bond isomers were formed (H1a, b, and c) and could be separated chromatographically (Fig. 2B). None of the three peptides bound hGH–Her2 ECD at target concentrations as high as 2.5 μM. In a second experiment, we wanted to assess whether peptide H1 was binding to hGH–Her2 ECD when cyclized with TBMB. Cyclization of peptides containing four cysteines by **1** yields complex product mixtures that cannot be separated chromatographically. Each of the four cysteines in H1 was therefore substituted separately by alanine to obtain bicyclic peptides with the defined configuration (Fig. 2A, lower sequences). The peptides were synthesized with fluorescein linked to the N-terminus. None of the four peptides bound hGH–Her2 ECD in the fluorescence polarization assay, which led us to the speculation that the peptide was isolated in a different format. One possibility is that three cysteines reacted with TBMB and the fourth one remained free. Such peptides may be synthesized chemically with the help of orthogonal thiol-protecting groups. Yet another explanation might be that the peptide is forming key interactions through its N-terminus and that conjugation of fluorescein to the N-terminus in the synthetic peptides prevented binding. None of these hypotheses was further assessed.