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13. ABSTRACT (Maximum 200 Words) Overexpression of Her-2 (HER2/neu/erbB-2) is found in 25-30% of human breast cancers and correlates with more aggressive tumors and a poorer prognosis. Therapies targeted at Her-2 have a great therapeutic potential for the treatment of breast cancers. The specific monoclonal antibody against Her-2, Herceptin (Trastuzumab), is currently an effective therapy for the treatment of human breast cancer with Her-2 overexpression. But Herceptin is effective in only about one-third of breast cancers with Her-2 overexpression, indicating new strategies for targeting Her-2 are needed. In breast cancer cells with Her-2 overexpression, abnormal cell proliferation is caused by the extremely high level of signal transduction, mediated by homodimerization of Her-2 and/or heterodimerization of Her-2 with EGFR, Her-3 and Her-4. Thus, agents that directly block the Her-2 dimerization may have a great therapeutic potential for treating Her-2 overexpressing breast cancer. The recently determined X-ray structures of Her-2 in complex with Herceptin and Her-2 show that Her-2 contains a hairpin loop (an "arm"), protruding from the domain II. Importantly, a similar hairpin loop is also found in the X-ray structures of EGFR and Her-3. Based upon the critical structural information, we have designed, synthesized and evaluated several cyclized small-molecule peptides as a new strategy to target the Her-2 protein by blocking its dimerization with members of EGFR receptors (EGFR, Her-2, and Her-3).					
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Introduction:

Overexpression of Her-2 is found in 25-30% of human breast cancers and correlates with more aggressive tumors and a poorer prognosis. The specific monoclonal antibody against Her-2, Herceptin, is effective in only about one-third of breast cancers with Her-2 overexpression, indicating new strategies for targeting Her-2 are needed.

In breast cancer cells with Her-2 overexpression, abnormal cell proliferation is caused by the extremely high level of signal transduction, mediated by homodimerization of Her-2 and/or heterodimerization of Her-2 with EGFR, Her-3 and Her-4. Thus, agents that directly block the Her-2 dimerization may have a great therapeutic potential for treating Her-2 overexpressing breast cancer. The recently determined X-ray structures of Her-2, EGFR and Her-3 suggest that the hairpin loop protruding from the domain II in Her-2 may be crucial for its dimerization. Our basic hypothesis is that cyclized peptides designed to mimic this hairpin loop in Her-2 may bind to Her-2 and/or other EGFR receptors, directly block the dimerization of Her-2, and shutdown the signal transduction mediated by Her-2. In this proposal, we propose to design and synthesize cyclized peptides which mimic the hairpin loop in Her-2 and perform initial but critical experiments to test whether such cyclized peptides will block dimerization of Her-2, and Her-2 mediated signal transduction in model cells and breast cancer cells. If successful, our study will provide an exciting new approach for targeting Her-2, and Her-2 mediated signaling in breast cancer.

RESULTS

Design of cyclized small-molecule Her-2 peptides

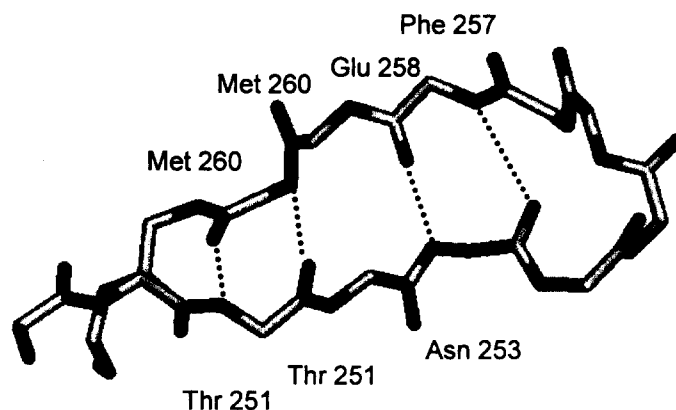
The recently determined X-ray structures of Her-2 suggest that the hairpin loop protruding from the domain II in Her-2 may be crucial for its dimerization. We hypothesized that that cyclized peptides designed to mimic this hairpin loop in Her-2 may bind to Her-2 and/or other EGFR receptors, directly block the dimerization of Her-2, and shutdown the signal transduction mediated by Her-2.

Based upon the x-ray structure of Her-2, the segment between residues Val250 and Pro261 forms a hairpin loop (**Figure 1**). To understand critical residues in this hairpin loop for dimerization, we have superimposed the three-dimensional (3D) structure of the hairpin loop in Her-2 on the 3D structure of the corresponding EGFR hairpin loop in domain II through molecular modeling. The hairpin loop in domain II of the EGFR has been shown to be responsible for the homodimerization of EGFR based upon the x-ray structure of the homodimer of EGFR in complex with EGF. This loop has also been implicated in hetero-dimerization of EGFR with other members of EGFR members (Her-2, Her-3). Our analysis showed that three residues in Her-2, namely Tyr252, Phe257 and Glu258 may be mainly responsible for the Her-2 dimerization with EGFR members (**Figure 2**). These three residues in Her-2 correspond to Tyr246, Tyr251 and Asn252 in the EGFR hairpin loop (**Figure 3**), which were shown to be responsible for homodimerization of the EGFR based upon the x-ray crystal structure.

The 3D structure of the hairpin loop (**Figures 1 and 2**) in Her-2 shows that Val250 and Pro261 are in close proximity (approximately 5 Å), which makes it feasible to cyclize these two residues to form a cyclic peptide without distorting the conformation of this hairpin loop. One

additional glycine residue was added to each end of the peptide sequence for additional flexibility so that the final cyclized peptide would not have a significantly distorted conformation as the hairpin loop conformation in the Her-2 x-ray structure. The linear peptide with the same sequence was also designed and synthesized as the control peptide. The chemical structures of these two peptides are shown in Figure 4.

Figure 1. The Hairpin structure in Her-2 that we hypothesized to mediate Her-2 dimerization.



Her-2 sequence (residue 250): VTYN^TDTFESMP

Figure 2. Three residues (Tyr252, Phe257 and Glu258) predicted to be the most critical residues for Her-2 dimerization with EGFR members

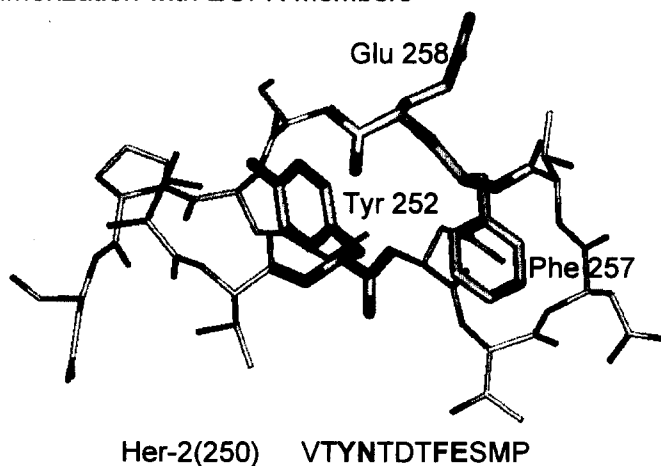


Figure 3. Three residues (Tyr246, Tyr251 and Asn252) that are shown to be the most critical residues for EGFR dimerization in the x-ray structure.

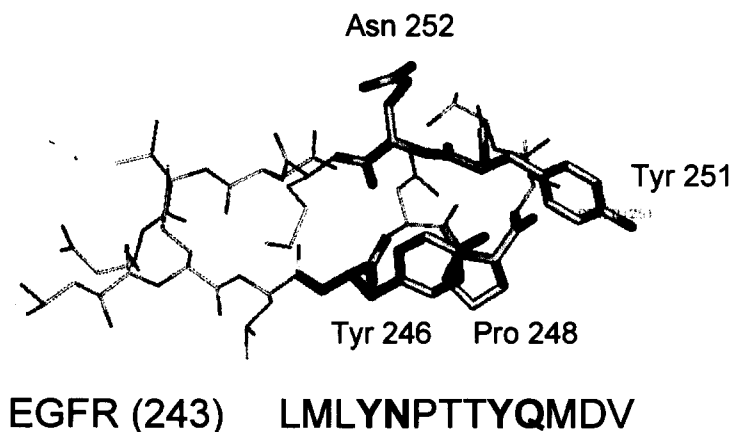


Figure 4. Two peptides designed and synthesized based upon the sequence of the hairpin loop in Her-2.
(a). Linear control peptide, denoted as **PHR-1**; (b). Cyclized peptide in which the cyclization was formed between the two glycine residues, denoted as **PHR-2**.

NH₂-Gly-Val-Thr-Tyr-Asn-Thr-Asp-Thr-Phe-Glu-Ser-Met-Pro-Gly-COOH

(a). Linear Her-2 peptide **PHR-1**

NH-Gly-Val-Thr-Tyr-Asn-Thr-Asp-Thr-Phe-Glu-Ser-Met-Pro-Gly-CO

(b). Cyclized Her-2 peptide **PHR-2**

Synthesis of the Her-2 peptides

The linear peptide GVTYNTDTFESMPG was synthesized by an automated SPPS (ABI 433A Peptide Synthesizer), using preloaded Fmoc-Gly-HMPB-BHA resin and a standard Fmoc chemistry (coupling with HBTU/HOBt/DIPEA). After synthesis peptide-resin was washed 5 times with DCM. The fully protected peptide (with an exception of N-end amino group) was

cleaved from the resin using following procedure: the peptide-resin was mixed with 5 ml cleavage solution (1% TFA, 1% EDT in DCM), and then the solution was filtered into 2 ml 10% pyridine solution in MeOH. This procedure was repeated 10 times, then the resin was washed (3xDCM, 3xMeOH, 3xDCM). The combined solutions were evaporated to the final volume ~10ml and 20ml of cold water was added to precipitate the peptide. Peptide was separated by centrifugation, washed twice with water and dried under a reduced pressure.

Cyclization: The protected peptide (0.02 mmol) was dissolved in the solution of 0.3 mmol DIEA in 500 ml DCM and 2ml DMF, then PyAOP (0.1 mmol) and HOAt (0.1 mmol) were added. The solution was mixed 24h, and then evaporated. A residue was dissolved in DMF (2 ml). The peptide was precipitated with 30 ml of water, separated by centrifugation, washed 3 times with water, and dried under a reduced pressure.

Deprotection: The fully protected cyclic peptide was dissolved and mixed 1h with 4 ml of a deprotection mixture (2.5% EDT, 2.5% water, 1% TIS in TFA), the solution was filtered into 20 ml of cold Et₂O, after 10 min. at -10°C, the precipitated peptide was separated by centrifugation, washed 4 times with cold Et₂O, and dried 1h under a reduced pressure.

The crude cyclic peptide was dissolved in 50% of acetonitrile in water, lyophilized, and purified by HPLC (Vydac C₁₈ RP preparative column, 2 x 25 cm). The cyclic peptide was checked by analytical HPLC (Vydac C₈ RP column) and MALDI-TOF MS (Kratos Axima CFR; an observed monoisotopic mass: 1522.62 Da, a theoretical mass 1522.61 [M+Na⁺]).

Testing the activity and specificity of the cyclized and linear control peptides

As our first screening, we have evaluated these two peptides using the MDA-MB-453 human breast cancer cells overexpressing Her-2 for their activity in inhibition of cell growth using the standard MTT-based assay. As expected, the linear peptide PHR-1 did not show any significant activity up to 100 μ M. Very surprisingly, the cyclized peptide PHR-2 did not show any appreciable activity up to 100 μ M.

Design of the second generation of the cyclized Her-2 peptides

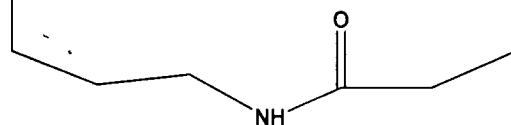
The unexpected lack of the activity of PHR-2 suggested that the cyclization strategy used in our first generation of the cyclized Her-2 peptide via direct cyclization of the backbone of the peptide may have caused the distortion of the desired hairpin loop conformation as shown in the x-ray structure of Her-2. To overcome this problem, we have designed the second generation of the cyclized peptide through cyclization of the side chains of two residues. The side chains of Thr251 and Met260 are in close proximity in the x-ray structure of Her-2 but are not directly involved in the interaction with the other monomer protein in the modeled dimer structures of Her-2:Her-2 and Her-2:EGFR. Therefore, we have replaced these two residues with Lysine and Glutamate, respectively. Cyclization of the side chains of the Lysine and Glutamate residues leads to the design of PHR-3. The synthesis of this peptide is ongoing and is expected to be complete shortly. Our modeling studies show that this cyclized peptide mimics the hairpin loop conformation in Her-2 perfectly, raising the hope that this cyclized peptide may be effective in blocking the homo-dimerization of Her-2 and hetero-dimerization of Her-2 with other EGFR members.

Figure 5. Design of the second generation of cyclized Her-2 peptide (PHR-3) through cyclization of the two side chain residues.

NH₂-Val-Thr (251)-Tyr-Asn-Thr-Asp-Thr-Phe-Glu-Ser-Met (260)-Pro-COOH



NH-Val-Lys-Tyr-Asn-Thr-Asp-Thr-Phe-Glu-Ser-Glu-Pro-COOH



Cyclized Her-2 peptide **PHR-3**

Key Research Accomplishments:

Cyclized Her-2 peptides were designed and synthesized with the goal to target the dimerization of Her-2 with EGFR members (e.g. Her-2, EGFR, Her-3). The first generation of designed peptides was not successful. Possible reasons were identified and new generation of conformationally constrained peptide was designed to further test our hypothesis and design strategy.

Reportable Outcomes:

None

Conclusions:

Based upon the experimentally determined crystal structure of Her-2, as well as EGFR and Her-3, we have hypothesized that conformationally constrained cyclized peptides that were designed to mimic the hairpin loop in domain II of Her-2 may be able to bind to Her-2 and block the dimerization of Her-2 with members of EGFR. The first generation of designed peptides was not successful. Possible reasons for the lack of the activity for the designed cyclized peptide were identified and the second generation of conformationally constrained peptide was designed to further test our hypothesis and design strategy. If the newly designed cyclized peptide is proved to be active in our cell-based and cell-free assay, this new idea could lead to the development of an entirely new class of anti-cancer agents for the treatment of human breast cancer by targeting the activity of Her-2 and other EGFR members.