Published in final edited form as:

J Biomol Struct Dyn. 2012 ; 30(5): 594–606. doi:10.1080/07391102.2012.687525.

Inhibition of Protein-Protein Interaction of HER2-EGFR and HER2–HER3 by a Rationally Designed Peptidomimetic

Sashikanth Banappagari¹, Miriam Corti², Seth Pincus², and Seetharama Satyanarayanajois^{*,1}

¹Department of Basic Pharmaceutical Sciences, College of Pharmacy, University of Louisiana at Monroe, Monroe, LA 71201

²Research Institute for Children, Children's Hospital, 200 Henry Clay Avenue, New Orleans, LA 70118

Abstract

Protein-protein interactions (PPI) play a crucial role in many biological processes, and modulation of PPI using small molecules to target hot spots has therapeutic value. As a model system we will use PPI of human epidermal growth factor receptors (EGFRs). Among the four EGFRs, EGFR-HER2 and HER2–HER3 are well known in cancer. We have designed a small molecule that is targeted to modulate HER2-mediated signaling. Our approach is novel because the small molecule designed disrupts dimerization not only of EGFR-HER2 but also of HER2–HER3. In the present study we have shown, using surface plasmon resonance (SPR) analysis, that a peptidomimetic, compound **5**, binds specifically to HER2 protein extracellular domain and disrupts the dimerization of EGFRs. To evaluate the effect of compound **5** on HER2 signaling *in vitro*, Western blot and PathHunter assays were used. Results indicated that compound **5** inhibits the phosphorylation of HER2 kinase domain and inhibits the heterodimerization in a dose-dependent manner. Molecular modeling methods were used to model the PPI of HER2–HER3 heterodimer.

Keywords

EGFR; HER2; Peptidomimetic; Homology modeling; Surface plasmon resonance; Protein-protein interactions; PathHunter assay

Introduction

Protein-protein interactions (PPI) play a crucial role in many biological processes, including transmembrane signal transduction, cell regulation, and the immune response (Wells & McClenden, 2007; Reichmann et al., 2007). It has been established that human diseases can be caused by aberrant protein-protein interactions (Ryan & Mathews, 2005), either by a loss of interactions or through the formation of complexes by overexpression of proteins on the cell surface. Understanding the structural details and modulation of protein-protein

*Address Correspondence to, Seetharama D. Satyanarayanajois, Assistant Professor, Department of Basic Pharmaceutical Sciences, University of Louisiana at Monroe, 1800 Bienville Drive, Monroe LA 71201 USA, Tel: (318)-342-1993; Fax: (318)-342-1737, jois@ulm.edu.

Supplementary Material

Supplementary material consisting of HPLC, MS, and purity data along with SPR immobilization data, and control compound data can be obtained free of charge from the authors.

Supporting Information Available: HPLC, MS, and purity data along with SPR immobilization data, control compound data. These can be obtained free of charge from the authors or can be purchased from Adenine Press for _____

interactions has significance in human health (Fuller, Burgoyne & Jackson, 2009; Sperandio, Reynes, Camproux, & Villoutriex, 2010). PPI have a wide range of affinities and possess a high degree of specificity for carrying out cellular processes. Analysis of 3D structures of protein complexes has suggested that the PPI surfaces are large, covering 700–3000 Å², and the binding surfaces are relatively flat (Bahadur, Chakrabarti, Rodier & Janin, 2004). Detailed studies of PPI interaction surfaces and the amino acid residues by several researchers have indicated that there are “hot spots” on the protein-protein interface area where major contributions to binding free energy are provided. These hot spots have core regions consisting mostly of aromatic amino acid residues such as Tyr, Trp, and Phe (Bahadur, Chakrabarti, Rodier & Janin, 2004; Moreira, Fernandes & Ramos, 2007; Chene, 2006). All of these studies indicate that PPI are concentrated in crucial hot spots, which are hydrophobic in nature and small enough to accommodate only a small molecule. Modulation of PPI using small molecules to target hot spots has therapeutic value (Wells & McClenden, 2007; Khan et al., 2011; Drozdov-Tikhomirov et al., 2001; Liang et al., 2000). Our interest is to design peptidomimetics to modulate the protein-protein interactions. As a model system, we will use PPI of human epidermal growth factor receptors (EGFRs).

The human epidermal growth factor receptor (EGFRs/HERs) system of receptor tyrosine kinases plays an important role in cell growth and differentiation (Ferguson, 2008). The receptor system consists of four members: HER1 or EGFR and HER2–4. These receptors also play a key role in tumor progression. These proteins have an extracellular domain (ECD) (Burgess et al., 2003), a transmembrane helix, a cytoplasmic kinase domain, and a regulatory region. Ligand binding to EGFR or HER3 leads to heterodimerization of these proteins, which leads to cell signaling. Different possible dimers such as EGFR-HER2, HER2-HER3, and HER2-HER4 have been proposed in the literature (Shankaran, Wiley & Resat, 2006; Tao & Maruyama, 2008). Among these, EGFR-HER2 and HER2-HER3 are well known in cancer. HER2 overexpression and its dimerization leads to an aggressive form of breast cancer (Landgraf, 2007; Nahta & Esteva, 2003; Hynes & Lane, 2005). It has been reported that, apart from the EGFR-HER2 dimer, HER2-HER3 dimers are the major contributors to HER2-overexpressing breast cancer (Baselga & Swain, 2009; Lee-Hoeflich et al., 2008). Hence, the protein-protein interactions between HERs are a good model system for our study. The structure and functional properties of EGFR have been studied in depth, including the structural details of the homodimer of the ECD domain of EGFR (Ogiso et al., 2002). However, heterodimers of HERs have not been studied in atomic detail. Modeling the PPI of HER2, HER3 and EGFR and understanding the hydrophobic hot spots in these interactions and PPI modulation by small molecules have potential clinical significance. The involvement of extracellular domains of these proteins in dimerization and signaling has been studied. However, at present, the crystal structure of heterodimers has not been reported. Domains II and IV of ECD of EGFR and HER2 are known to be important in PPI (Berezov et al., 2002; Saxon & Lee, 1999), and domain II interactions have been studied in EGFR. Small molecules and antibodies have been targeted to HER2 (Lombardi et al., 2011; Huang et al., 2010; Chen, 2009; Arcangeli et al., 2008). The domain IV C-terminal is near the transmembrane domain, and the details of its interaction in dimerization have not been elucidated. In the crystal structures of HER2, the electron density of the domain IV C-terminal is faded and not well defined. However, binding of the antibody trastuzumab to domain IV of HER2 is known to be clinically significant (Chang, 2007).

In our previous studies, we have reported the design of a small molecule, H-Arg-[3-amino-3(naphthyl)-propionic acid]-Phe-OH (compound **5**, Table I) (Satyanarayanajois, Villalba, Jianchao, & Lin, 2009; Banappagari, Satyanarayanajois & Ronald, 2010; Banappagari, Satyanarayanajois & Ronald, 2011) based on the protein-protein interaction in the crystal structure of the HER2-antibody trastuzumab complex (Cho et al., 2003). Trastuzumab (anti-HER2) binds to HER2 on the C-terminal portion of domain IV. This

interaction is known to block proteolytic cleavage in HER2 and indirectly affect dimerization with other HERs that induce signaling pathways (Molina et al., 2001). Thus, blocking any interaction in domain IV on HER2 can disrupt signaling pathway(s) that promote cell proliferation and cancer (Franklin et al., 2004). The small molecule, compound **5**, showed antiproliferative activity against HER2-overexpressing breast cancer cell lines with IC₅₀ in the lower micromolar range (Satyanarayanajois, Villalba, Jianchao, & Lin, 2009). Using fluorescence assay, competitive binding, and circular dichroism spectroscopy, we have shown that compound **5** binds to domain IV of the HER2 protein (Banappagari, Satyanarayanajois & Ronald, 2010). The interaction of domain IV of HER2 with EGFR or HER3 is not clear. Blocking domain IV of HER2 with antibodies is known to suppress the growth of cancer cells by indirectly inhibiting the dimerization mechanism of HER2 with other receptors. It is also known that the C-terminal portions of the domains interact with one another in EGFR and HER2, and that the absence of domain IV of HER2 changes the heteromeric signaling of EGFR (Kumagai et al., 2003). The aim of the present study is to show that the designed peptidomimetic, compound **5**, binds specifically to HER2 protein and, in particular, to domain IV and disrupts the dimerization of EGFRs. We have used SPR analysis, Western blot, and PathHunter assay to show that the compound **5** binds specifically to HER2 domain IV and disrupts the protein-protein interactions of EGFR-HER2 as well as HER2-HER3. Molecular modeling methods were used to model the PPI of HER2-HER3 heterodimer and propose the possible binding region of compound **5** to disrupt the dimerization process.

Methods

Extracellular domains of pure recombinant proteins HER2, EGFR and HER3 were obtained from Bender MedSystems, eBioscience, San Diego, CA; Leinco Technologies, St. Louis, MO. Ligands for proteins EGFR and HER3, EGF and neuregulin 1 (NRG1) were obtained from Abcam, Inc., (Cambridge, MA). Peptides were custom synthesized by New England Peptides (Gardner, MA) or PolyPeptide Laboratories (San Diego, CA). The pure products were analyzed by HPLC, electrospray ionization mass spectrometry (ESI-MS), and high-resolution mass spectrometry (HR-MS). HPLC chromatograms showed that the peptides were > 95 % pure.

Surface Plasmon Resonance Experiments

Surface plasmon resonance experiments were performed using Biacore 3000 (GE Health Sciences) at 25 °C (Komolov et al., 2010; Berezov, Zhang, Greene & Murali, 2001; Myszk, 2000). Immobilization of compound **5**, EGFR, HER2, HER3- extracellular domains and domain IV-HER2 was done with the standard amine coupling procedure on a CM5 chip. Carboxyl groups on the sensor chip were activated by a solution containing 0.2 M N-ethyl-N-(dimethylaminopropyl) carbodiimide (EDC) and 0.05 M N-hydroxysuccinimide (NHS) (35 μ L solution, flow rate 5 μ L/min). Running buffer consisted of 0.01M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% Tween at pH 7.5. Regeneration buffers were 50% acid cocktail and glycine 100 mM at pH 1.7. For immobilization, HER2 was injected onto the chip surface at a rate of 10 μ L/min until approximately 7000 response units (RU) was reached. Unreacted activated groups were blocked with 1 M ethanolamine for 7 min. A reference surface was generated under the same conditions without compound **5** or HER2 protein injection. The sensogram obtained was double referenced (reference surface was used as blank to correct for instrument and buffer artifacts and zero nM analyte buffer to avoid bulk shift). For binding, dimerization, and inhibition of dimerization studies, compound **5** or HER2, EGFR, HER3 were injected at various concentrations at a flow rate of 20 μ L/min; for kinetics experiments (HER2-ECD was injected onto immobilized compound **5**) HER2 protein was injected at 30 μ L/min for 2.5 min (association) and dissociated for 10 min. For

competitive studies, two analytes were injected sequentially using the coinject mode at a flow rate of 20 $\mu\text{L}/\text{min}$. A control peptidomimetic (an analog of compound **5**, Table 1) that did not show antiproliferative activity in breast cancer cell lines was used to establish specific binding of compound **5** (negative control).

Circular Dichroism Experiments

Circular dichroism (CD) studies were carried out on a Jasco-J810 spectropolarimeter instrument equipped with temperature control. The concentration of the proteins used was in the range of 5 to 10 μM in deionized water. Spectra were collected from 360–190 nm using a 1 mm path length rectangular quartz cell. Each spectrum was the average of three scans taken at a scan rate of 50 nm/min with a spectral bandwidth of 0.1 nm. Calculation of the concentration of protein was based on the fact that the human epidermal growth factor receptors-ECD protein consists of 630 amino acid residues.

Western blot and phosphorylation

BT474 and MCF7 cells were maintained in RPMI-1640 supplemented with penstrep 1 mL/100 mL, insulin 1 mg/100 mL, and 10% FBS. SKBR3 cells were maintained in McCoy's medium supplemented with penstrep 1 mL/100 mL, insulin 1 mg/100 mL, and 10% FBS. After confluency, cells were trypsinized and triplicates were plated in T-75 flasks. After 24 hrs the cells were treated with either AG-825 0.35 μM or compound **5** 0.4 μM , or left untreated. Cells with compounds were incubated at 37 °C and 5% CO₂. After 24 hrs, cells were trypsinized, collected in 15 mL tubes, and centrifuged at 200 g. To the pellet, 200 μL of cell lysis buffer with 20 μL protease inhibitor and 20 μL phosphatase inhibitor were added. Tubes were vortexed for 1 min and then kept on ice for 10 min. This process is repeated twice. Cells were subjected to centrifugation at high speed (450 g) and the supernatant was collected into separate tubes and stored at –80 °C until further use. Protein concentration in the tubes was analyzed using Bradford's assay. 40 μg of protein from each sample was loaded on 10% SDS-polyacrylamide minigels. The gels were run at 30 mA, 125 V and then transferred at 30 V for 12–16 h at 4 °C onto a nitrocellulose membrane. After transfer, the membranes were blocked with 5% milk for 1 hr. The nitrocellulose membranes were then probed with specific primary antibodies against p-HER2 diluted 1:3000 in 5% milk for 15 h at 4 °C. After incubation with primary antibody, the membranes were washed five times with PBS and then incubated with secondary antibody to p-HER2 (anti-rabbit secondary antibody) diluted 1:2000 in 5% milk with 0.001% Tween 20 for 1 h. The membranes were then washed five times with PBS, and proteins were visualized using the Super Signal enhanced chemiluminescence kit (Pierce, Rockford, IL). The Kodak Gel Logic-1500 imaging system (Carestream Molecular Imaging, New Haven, CT) was used to visualize the luminescent proteins. All experiments were repeated at least three times. A representative Western blot image was used for final representation. The visualization of β -actin was used to ensure equal sample loading in each lane. Total HER2 protein was detected using an antibody to HER2 extracellular domain (Abcam).

PathHunter assay

The PathHunter assay kit was obtained from DiscoverX Corporation (Fremont, CA). U2OS cells expressing HER2–HER3 protein with C-terminal probe peptide were suspended in cell plating (CP) reagent and placed into wells of a 96-well plate at a density of 1×10^4 cells per well. After coating, the cells were incubated at 37 °C, 5% CO₂ for 24 hours. Threefold dilutions of the agonist neuregulin 1 (NRG1) were prepared from the stock of agonist in CP reagent. Cells were incubated with the agonist for 3 h. Then, 55 μL of detection reagent per well was added and incubated at room temperature in the dark. After 60 min, luminescence from the cells was measured (Yin et al., 2009). EC₈₀ concentration of the agonist was

measured from the dose-response curve of the agonist on PathHunter-expressed cells. Compound **5** (antagonist) and control (Table 1) dilutions were then prepared in CP reagent at threefold dilution and added to the remaining wells of the plate. The plate was incubated for 60 min at 37 °C. After 60 min, the EC₈₀ dose of agonist (0.3 μM) was added to all the cells with compound **5** and control peptide. The plate was incubated for 3 hours at 37 °C. Then 55 μL of detection reagent per well was added and incubated for 60 min at room temperature. Luminescence from the plate was read using a BioTek microplate reader (BioTek, Winooski, VT). A plot of luminescence vs. concentration of agonist (NRG1) or antagonist (compound **5** and control peptide) was obtained. From the dose response curves, IC₅₀ value of inhibition was obtained using GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA).

Modeling

The crystal structure of the HER3 extracellular domain in closed conformation was obtained from the Protein Data Bank (Cho & Leahy, 2002). Modeling of the HER3 extracellular domain in open conformation was carried out using templates of HER2 crystal structure (Fisher et al., 2010) and EGFR extracellular domains (Ogiso et al., 2002; Lu et al., 2010) and domain IV of HER3 in closed conformation. SWISSMODEL was used to model the starting structure (Arnold, Bordoli, Kopp & Schwede, 2010). The model was energy minimized using Insight II (Accelrys Inc. San Diego, CA) and evaluated for bond length, bond angle, and torsion angle using MolProbity (Chen et al., 2010). To generate the ligand-bound HER3 extracellular domain, EGFR extracellular domain in open conformation was used (Ogiso et al., 2002). HER3 and EGFR extracellular domain structures were overlapped. The overlapped structures had EGF ligand positioned in the pocket between domains I and II of HER3. The EGF structure was replaced by heregulin structure, and the structure of HER3 with heregulin-bound structure was minimized. The crystal structure of HER2 extracellular domain in complex with trastuzumab was used for the heterodimer model of the HER2–HER3 complex (Cho et al., 2003). The crystal structure of HER2 in complex with trastuzumab was missing residues 581–590, and 607–620 in domain IV. The crystal structure of HER2 in complex with tryptophan-rich antibody has the structure of a loop region covering residues 581–590 and 607–620 (Fisher et al., 2010). These missing residues were added to the HER2 crystal structure from the trastuzumab complex to generate the full-length extracellular domain of HER2 protein. The generated model was evaluated for quality by using Molprobity. The quality of the model was also evaluated by calculating solvent-accessible and solvent-excluded surface areas using autodock tools; the presence of hydrophobic residues on the protein surface was determined by hydrophobic and hydrophilic surface area calculations and representation using Maestro (Schrodinger LLC., Inc. Portland, OR).

To model the HER2–HER3 complex, the homodimer crystal structure of EGFR was used as a template (Ogiso et al., 2002). Structures of HER2 and HER3 over overlapped with EGFR dimer structure. Domains I to III of HER2 and HER3 were overlapped on the symmetric dimer of EGFR domains. In the overlapping position with the EGFR homodimer, HER2 and HER3 made contact with dimerization arm II and the C-terminal portion of domain IV. The models were compared with the recently reported crystal structure of the extracellular domain of the homodimer of EGFR (Lu et al., 2010) and orientation of domain IV of two molecules in the heterodimer was brought to close proximity using by overlapping with the homodimer crystal structure.

Results

Surface plasmon resonance analysis

Immobilization—In SPR experiments, one of the molecules (for example, HER2 protein, 200 nM) is immobilized on the sensor surface and the analyte (EGFR or HER3, 200 nM) continuously flows over the surface of a microflow cell (Komolov et al., 2010; Berezov, Zhang, Greene & Murali, 2001; Myszk, 2000). The real-time interaction between the two proteins is shown in a sensogram. For immobilization, HER2, EGFR, HER3, and domain IV of HER2 were injected onto the chip surface at a rate of 10 $\mu\text{L}/\text{min}$ until response units of approximately 7000 RU were reached (immobilization of HER2, EGFR, HER3, and HER2 domain IV are provided in Supporting Information). A stable surface of the immobilized proteins 7500 RU for HER2-ECD, 9000 RU for HER3-ECD, 7000 RU for EGFR-ECD, and 11,000 RU for domain IV-HER2 were obtained after injection.

Compound 5 binds to HER2-ECD—The binding of compound **5** to HER2 ECD was investigated by two methods: a) by immobilizing the HER2 ECD and using compound **5** as analyte and b) by immobilizing compound **5** and using HER2 ECD as analyte. Curves were double referenced by subtracting the effects of control flow cell and 0.0 nM of analyte buffer control to avoid bulk shifts. When compound **5** was injected at different concentrations (0, 5, 10, 25, 50, 75, and 100 μM) into the flow-cell, concentration-dependent binding to immobilized HER2-ECD was observed (Figure 1A).

When compound **5** was immobilized on the CM5 chip surface and HER2 ECD was injected at different concentrations (0, 5, 10, 20, 40, 80, and 160 μM) at a rate of 30 $\mu\text{L}/\text{min}$, binding of HER2 ECD to compound **5** was observed (Figure 1B) exhibiting kinetics of association and dissociation. The kinetic constant was estimated by global fitting analysis of the titration curve to the 1:1 Langmuir interaction model and the K_d value calculated was around 1.1 μM . The association rate constant was $2.1 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$ and the dissociation rate constant was $2.31 \times 10^{-4} \text{ s}^{-1}$. As a reference, a control peptide was immobilized onto a CM5 chip, and a series of concentrations of HER2-ECD was injected. No non-specific binding of the control compound to HER2 ECD was observed. Also, a peptidomimetic analog of compound **5** (Table I) was also injected at a concentration of 100 μM onto immobilized HER2-ECD on flow cell 2. No binding was observed for control compound to HER2 ECD compared to compound **5**, indicating the specific binding of compound **5** to HER2 ECD (Figure 1C).

The next important experiment was to determine the specificity of compound **5** for HER2 protein but not for the homologues receptors EGFR and HER3. To monitor the specificity, compound **5** was injected at a concentration of 100 μM onto immobilized HER2, EGFR, and HER3 on a CM5 sensor chip. Figure 2A shows the sensograms of flow cells 2, 3, and 4. Binding of compound **5** onto HER2-ECD was observed as shown by a 40 RU increase in response. However, no binding to EGFR and HER3 was observed, indicating that compound **5** interacts specifically with HER2-ECD.

Compound 5 binds specifically to domain IV of HER2—HER2 extracellular domain IV is an important site for stabilizing the HER2-mediated dimers with the EGFR family of receptors such as EGFR and HER3. Also, domain IV is the binding site of the antibody trastuzumab, which is used in treating breast cancer. Compound **5**, a peptidomimetic, is designed to interact specifically with domain IV of HER2 protein. To demonstrate that the compound **5** binds to domain IV of HER2, we immobilized domain IV-HER2 on a CM5 sensor chip via an amine coupling process. A stable surface of approximately 11000 RU was obtained in flow cell 2 of a CM5 sensor chip. Compound **5** was then injected onto domain IV-HER2 on a CM5 sensor chip at the rate of 10 $\mu\text{L}/\text{min}$ to monitor the interaction of

compound **5**. For comparison, trastuzumab, which is known to specifically interact with domain IV of HER2, was also injected onto the CM5 sensor chip. Figures 2B and C represent the sensograms of compound **5** and trastuzumab binding to domain IV of HER2. The next important experiment was to evaluate whether compound **5** and trastuzumab compete for the same binding site on domain IV-HER2. To monitor the competitive binding, trastuzumab and compound **5** were coinjected onto the immobilized domain IV-HER2. In the presence of trastuzumab, there was no interaction of compound **5** with HER2 domain IV (Supporting Information). This indicates that compound **5** may bind near the trastuzumab binding site on domain IV-HER2.

Inhibition of HER2 mediated dimerization by compound 5—Dimerization of EGFRs, in particular, EGFR-HER2 and HER2-HER3, plays an important role in activating downstream signaling (Ferguson, 2008; Burgess et al., 2003). The ultimate goal in designing the compound **5** was to block HER2-mediated dimerization with the EGFR family of receptors EGFR and HER3 with HER2. To investigate the effect of compound **5** in blocking HER2-mediated dimerization process, HER2 was immobilized onto a CM5 chip and EGFR or HER3 was coinjected at 200 nM concentration with and without compound **5**. EGFR and HER3 were pre-activated with EGF and NRG1 ligands by incubating at a concentration of 20 nM for 15 min. Figures 3A and B represent the sensograms for coinjection experiments of EGFR and HER3 with or without compound **5**, respectively. Activated EGFR and HER3 showed binding to HER2-ECD in the absence of compound **5**, exhibiting heterodimerization of the extracellular regions of the EGFR proteins. In the presence of compound **5**, heterodimerization interaction was blocked. To validate the dimerization ability of commercially obtained protein extracellular domains (Bender MedSystems, eBioscience, San Diego, CA; Leinco Technologies, St. Louis, MO), proteins EGFR and HER3 were studied using SPR in the absence of their ligands EGF and neuregulin (Abcam), respectively. EGFR and HER3 did not show any binding (no change in sensogram) to immobilized HER2 in the absence of their ligands, suggesting an absence of heterodimerization without the ligands. In the presence of ligands, EGFR and HER3 exhibited changes in the sensograms (open conformation is needed for EGFR and HER3 to dimerize with HER2) with kinetics of association and dissociation.

Circular Dichroism

CD experiments were performed to evaluate the secondary structure of the extracellular domains of proteins HER2, EGFR, and HER3. Protein samples that were used in biacore studies were commercially obtained of HER2, HER3, and EGFR. To verify that these proteins have intact secondary structure, CD spectra of all the proteins were obtained in water. CD spectra of proteins showed a negative band around 217 nm, suggesting β -sheet secondary structure of the proteins (Venyaminov & Yang 1996) (Supporting Information).

Western blot analysis of phosphorylation

Transphosphorylation of HER2-mediated dimers activate the downstream signaling cascade that results in cell growth and proliferation (Ferguson, 2008; Burgess et al., 2003; Gijssen et al., 2010). Thus, inhibiting transphosphorylation of HER-mediated dimers plays a major role in inhibiting cell growth and proliferation. SPR experiments provide information about dimerization of pure proteins. Furthermore, the proteins used had only ECD. To evaluate the effect of compound **5** on the dimerization of full-length EGFRs in the native form in HER2-overexpressing breast cancer cell lines, the effect of compound **5** on the phosphorylation of HER2 protein kinase domain in BT-474 and MCF-7 cells was investigated using Western blot. If compound **5** inhibits the receptor heterodimerization/association in cells, then one should see a decrease in the phosphorylation of HER2 protein. Phosphorylation was detected using p-HER2, an antibody that specifically binds to phosphorylated HER2. Figures 4A&B

represents the Western blot analysis of the phosphorylation level in treated and untreated BT-474 and MCF-7 cells respectively. Cells treated with compound **5** at 0.4 μM concentration and AG-825 (a kinase inhibitor) (Gijssen et al., 2010; Levitzki & Gazit, 1995) showed a decreased level of p-HER2 compared to the level of p-HER2 in untreated cells. Comparison of the inhibition of phosphorylation of AG-825, a standard kinase inhibitor, and control suggested that compound **5** showed significant inhibition of phosphorylation of HER2. These results clearly suggest that compound **5** inhibits the full-length receptor association in the native form and, hence, phosphorylation of the kinase domain.

PathHunter assay

The PathHunter tyrosine kinase functional assay kit monitors dimerization and kinase activation of receptor tyrosine kinases (Yin et al., 2009). In the PathHunter assay (PathHunter technology is a registered trademark of DiscoveRx, Fremont, CA), The HER2–HER3 pair is expressed in U2OS cells by transfecting with cDNA for the EGFR protein pairs. A small peptide epitope is expressed recombinantly on the intracellular C-terminal part of HER2/HER3. An SH2 domain is co-expressed with a larger sequence, termed enzyme acceptor (EA), forming SH2-EA fusion protein. Activation of receptors results in dimerization of receptor tyrosine kinase (HER2–HER3); the kinase domain is phosphorylated, and phosphorylated tyrosine acts as a recruitment site for downstream signaling molecules containing SH2 domains. The interaction of the C-terminal peptide and SH2-EA fusion protein forces complementation of the two enzyme fragments. This action results in the formation of fully complemented β -galactosidase, the activity of which is measured using a chemiluminescence detection reagent in the cellular environment (Kumagai et al., 2003). Addition of NRG1, a ligand for HER3, at different concentrations induces heterodimerization of HER2–HER3 and phosphorylation, which results in binding of the SH2 domain protein and β -galactosidase activity. The activity of agonist NRG1 at different concentration is shown in Figure 5. When compound **5** was incubated with cells and NRG1 at a EC_{80} concentration (0.3 μM from the dose-response curve), the β -galactosidase activity was decreased, suggesting inhibition of dimerization of HER2–HER3 in U2OS cells. The IC_{50} value of inhibition was $0.260 \pm 0.002 \mu\text{M}$ for compound **5**. A control peptidomimetic that has structure similar to that of compound **5** was also evaluated for heterodimer inhibition activity. The control peptide did not exhibit any decrease in β -galactosidase activity, indicating the formation of heterodimers of HER2–HER3.

Modeling of heterodimers

The crystal structure of the homodimer of the ECD domain of EGFR has been reported. However, heterodimers of EGFRs have not been elucidated. It should be noted that models of homo- and –heterodimers of EGFRs proposed by different research groups is available in the literature (Ferguson, 2008; Burgess et al., 2003; Shankaran, Wiley & Resat, 2006; Tao & Maruyama, 2008; Landgraf, 2007; Baselga & Swain, 2009; Ogiso et al., 2002). Our aim is to model the heterodimer of HER2–HER3 in the light of reported crystal structures and available data from the literature to explain the experimental results we have obtained. Based on SPR and Western blot analysis results, we proposed a model for EGFR-HER2, HER2–HER3 interactions and explored the importance of domain IV of HER2 in the heterodimerization and modulation of heterodimerization by compound **5**. In an earlier report we proposed a model for HER2-EGFR interaction (Banappagari, Satyanarayanajois & Ronald, 2010) that is consistent with experimentally observed results from the literature (Ferguson, 2008; Burgess et al., 2003). In the model of EGFR-HER2, the dimerization arms of domain II in both proteins interact with one another, stabilizing the dimer. In this report, we propose a model for the extracellular domain of HER2–HER3 interaction based on homology modeling and using a template of EGFR homodimer structure. The structure of open conformation HER3 ECD is not available. Modeling of HER3 ECD was based on the

closed conformation of HER3 and the crystal structure of the open conformation of EGFR and HER2. EGFR, HER2 and HER3 share the sequence similarity (Burgess et al., 2003). The proposed model of ECD of HER3 in open conformation is shown in Figure 6. The overall conformation of HER3 ECD is similar to that of EGFR with domains I and III forming a cleft that accommodates the ligand (neuregulin/heregulin). Domain IV was stretched away from domains I–III. The quality of the model was evaluated by Molprobtity (Chen et al., 2010), which indicated that 87.5% of residues were in the favored region and 97.8% of the residues were in the allowed region of the Ramachandran map. The quality of the model was also evaluated by calculating solvent-accessible and solvent-excluded surface areas; these areas were compared with those of the reported crystal structure of EGFR (Lu et al., 2010). The solvent-accessible surface area for HER3 ECD was 32,532 Å², which was comparable to that of the crystal structure EGFR ECD (32,094 Å²). The solvent-excluded surface area for HER3 ECD was 27,188 (for the EGFR crystal structure 27,240 Å). Representation of the presence of hydrophobic residues on the surface of protein using hydrophobic and hydrophilic surface area calculations (Maestro) suggested that the majority of the surface area of the extracellular domain of HER3 is hydrophilic (Supporting Information). Comparison of the crystal structure of EGFR ECD and the proposed model of HER3 indicated that the packing of the core of the HER3 protein was similar to that of EGFR (Lu et al., 2010).

To model the heterodimer of HER2–HER3, the HER2 crystal structure from the trastuzumab antibody complex was used (Cho et al., 2003). However, the crystal structure did not have the C-terminal part of domain IV well defined; hence, domain IV was modeled based on the HER2 crystal structure with a tryptophan-rich antibody (Fisher et al., 2010) and domain IV of EGFR in the homodimer of EGFR. Domain IV was added to the HER2 crystal structure. EGFR homodimers were used as templates to model HER2–HER3 heterodimers (Burgess et al., 2003). The resulting heterodimer model indicated that the C-terminal parts of domain IV of HER2 and HER3 interact with one another. The proposed heterodimer model for HER2–HER3 is shown in Figure 7A. Protein-protein interaction was observed in domain II and domain IV of HER2 and HER3. The interaction of the dimerization arm of domain II was similar to that observed in the crystal structure of EGFR homodimer. Phe257 of HER2 interacts with Tyr282 and Phe294 of HER3 on one side of the dimerization arm (Figure 7B). Phe268 and Leu291 of HER2 interact with Phe270 and Tyr265 of HER3 on the other side of the dimerization arm. There is also a hydrophobic interaction of Leu249 and Tyr267 of HER2 with Gly318 of HER3, which stabilizes domain II of the heterodimer. In domain IV, Leu586 and Tyr588 of HER2 form hydrophobic interactions with Tyr601 and Pro599 of HER3. There was a cation- π interaction between Lys583 of HER2 with Tyr601 of HER3. A hydrogen-bonding interaction was observed between Asp585 of HER2 with Glu615, stabilizing the C-terminal portion of domain IV. When we compared the 3D structure of the EGFR homodimer with that of the HER2–HER3 heterodimer, the overall structures were similar (Lu et al., 2010). The difference was in the orientation of domains I and III, as expected. HER2 does not have any known ligand and, hence, the ligand binding domains I and II are close to each other. There was a difference in the structure of loops in the C-terminal part of domain IV.

Discussion

HER2-mediated heterodimerization plays a major role in cell growth and proliferation. Small molecules have been targeted to block the signaling by kinase domain inhibitor. There are few reports of small molecules or peptides targeting the extracellular domains of EGFRs (Berezov et al., 2002; Berezov, Zhang, Greene & Murali, 2001; Dakappagari et al., 2005; Allen et al., 2007; Yang et al., 2010). Among the extracellular domains, domain II is well-known in the dimerization process and is targeted for inhibition of dimerization in the case

of EGFR. The importance of domain IV of EGFRs is not very clear, although the antibody trastuzumab is known to target domain IV of HER2 and has shown efficacy in treatment of HER2-positive breast cancer patients (Huang et al., 2010; Arpino et al., 2007; Ahn & Vogel, 2011). The mechanism of trastuzumab in blocking the signal for cell growth via domain IV is not clear, and trastuzumab does not block HER2–HER3 interaction. Our results clearly suggest that a designed peptidomimetic binds to domain IV of HER2 protein and blocks the heterodimerization of EGFR-HER2 as well as HER2–HER3. SPR analysis clearly suggests that compound **5** binds specifically to domain IV and inhibits the heterodimerization process of EGFRs. Although SPR studies were conducted with pure proteins of extracellular domains of EGFRs, our Western blot studies suggest that compound **5** inhibits heterodimerization and phosphorylation *in vitro* in native cellular environments. Furthermore, PathHunter assay results suggested that compound **5** inhibits the heterodimerization of HER2–HER3 in a dose-dependent manner with an IC_{50} value of 0.260 μ M. This inhibition results in the inhibition of phosphorylation of the kinase domain. To understand the molecular mechanism of blocking of signaling by binding of compound **5** to domain IV, molecular modeling studies were carried out. The heterodimer of HER2–HER3 suggests that C-terminal parts of domain IV interact with one another apart from domain II. The C-terminal portion of domain IV is known to form weak interactions to stabilize the heterodimer of EGFRs. Modeling suggests that amino acid residues Phe573 to Trp592 of domain IV of HER2 may be involved in the interaction and stabilization of heterodimerization. Binding of compound **5** to this region may destabilize the heterodimer and block the phosphorylation of kinase domain and, hence, the signal for cell growth. This can be clearly seen the inhibition of phosphorylation of HER2 in the presence of compound **5** in BT-474 cell lines that overexpress HER2 protein. Compound **5** was designed based on the HER2-trastuzumab complex and interaction of trastuzumab with the C-terminal part of domain IV. Compound **5** inhibits the heterodimerization of EGFR-HER2, HER2-HER. However, trastuzumab does not directly inhibit the dimerization of EGFR-HER2. To explain this, we compared the proposed model of EGFR-HER2, HER2–HER3 with a recently available crystal structure of a homodimer of EGFR with C-terminal domain IV (Lu et al., 2010). If we overlap the HER2 structure with the EGFR homodimer, it is clear that trastuzumab binds to a site on domain IV that is slightly above the dimerization site of domain IV. Based on our modeling and SPR and Western blot studies, we propose that compound **5** binds near the dimerization surface of domain IV of HER2 as shown in Figure 8. It should be noted that determination of exact binding site of compound **5** on HER2 protein domain IV requires isotopic labeling of protein and NMR chemical shift perturbation studies. In an earlier report we have carried out docking studies to indicate the possible binding site (Satyanarayanajois, Villalba, Jianchao & Lin, 2009; Banappagari, Satyanarayanajois & Ronald, 2010). Domain IV interaction in the EGFR monomer is dominated by hydrophobic residues, which clearly suggests that PPI in domain IV are hydrophobic in nature. The importance of domain IV in HER2 in the dimerization process and subsequent signaling is not well understood. Trastuzumab, which binds to domain IV of HER2, does not disrupt the dimerization process. Studies related to domain IV of EGFR which is homologous to HER2 protein have been reported (Burgess et al., 2003; Ogiso et al., 2002; Cho et al., 2003; Lu et al., 2010). It is known that mutation of domain IV of EGFR ECD can impair the ability of ligands to bind to it and induce phosphorylation (Saxon & Lee, 1999). It is also reported that domain IV contributes to the stabilization of ligand-induced dimer (Ferguson, 2008). Peptides modeled on disulfide-bonded modules of domain IV of EGFR and HER2 can disrupt EGFR dimerization (Berezov et al., 2002; Berezov, Zhang, Greene & Murali, 2001). However, there are controversial reports in the literature about the involvement of the C-terminal part of domain IV in the dimerization process. It is reported that mutation of domain IV in EGFR did not yield any conclusive results about the involvement of dimerization and its disruption (Yuzawa et al., 2007; Dawson et al., 2005).

The results presented here clearly suggest that disruption of domain IV interaction between EGFRs leads to disruption of heterodimerization and, hence, blocks the phosphorylation of the kinase domain. Compound **5** with hydrophobic groups in the center part of the molecule may bind to the C-terminal hydrophobic part of HER2 and disrupt the dimerization process. The inhibition of the dimerization process of EGFR-HER2, HER2-HER3 has clinical significance. It is known that, apart from EGFR-HER2, HER2-HER3 is important in breast cancer (Lee-Hoeflich et al., 2008). It has been reported that in HER2-overexpressing breast cancer HER2-HER3 is the important partner. Thus, **5** and analogs of **5** that target domain IV of HER2 and inhibit PPI of HER2-HER3, EGFR-HER3 are therapeutically useful.

Conclusions

Peptidomimetic **5** designed based on the HER2: trastuzumab crystal structure was shown to be a dual inhibitor of EGFRs heterodimerization. Compound **5** binds specifically to ECD of HER2 and, in particular, to domain IV, as shown by SPR analysis. Western blot analysis indicated that compound **5** inhibits the phosphorylation of HER2 protein, suggesting that inhibition of dimerization by compound **5** results in blocking of signaling for cell growth. Modeling studies suggested that the C-terminal part of domain IV of EGFR, HER2, and HER3 is important in stabilizing the dimer and that compound **5** may bind to the hydrophobic pocket near the C-terminal of domain IV of HER2. Compound **5** acts as a dual inhibitor of heterodimerization of EGFRs. Further investigation is needed to evaluate the effect of compound **5** on blocking of heterodimerization and its effect on tumor growth using *in vivo* studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The project described was supported by the National Center for Research Resources (5P20RR016456-11) and the National Institute of General Medical Sciences (8 P20GM103424-11) from the National Institutes of Health. Banappagari was supported by LBRN summer research program to conduct SPR analysis presented in this manuscript. Surface plasmon resonance was performed in the Molecular Interaction Core of the Louisiana Vaccine Center, supported by the LA Board of Regents Post-Katrina Support Fund. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources or the National Institutes of Health. Herceptin was purchased at the pharmacy of Children's Hospital, New Orleans.

References

- Ahn ER, Vogel CL. Dual HER2-targeted approaches in HER2-positive breast cancer. *Breast Cancer Research and Treatment*. 2011; 131:371–383. [PubMed: 21956210]
- Allen S, Garrett JT, Rawale SV, Jones AL, Philips G, Forni G, Morris JC, Oshima RG, Kaumaya TP. Peptide vaccines of the HER-2/neu dimerization loop are effective in inhibiting mammary tumor growth in vivo. *Journal of Immunology*. 2007; 179:472–482.
- Arcangeli C, Cantale C, Galeffi P, Gianese G, Paparcone R, Rosato V. Understanding structural/functional properties of immunoconjugates for cancer therapy by computational approaches. *Journal of Biomolecular Structure and Dynamics*. 2008; 26:35–48. [PubMed: 18533724]
- Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL Workspace: A web-based environment for protein structure homology modeling. *Bioinformatics*. 2010; 22:195–201. [PubMed: 16301204]
- Arpino G, Gutierrez C, Weiss H, Rimawi M, Massarweh S, Bharwani L, Placido SD, Osborne KC, Schiff R. Treatment of human epidermal growth factor receptor 2-overexpressing breast cancer xenografts with multiagent HER-targeted therapy. *Journal of the National Cancer Institute*. 2007; 99:694–705. [PubMed: 17470737]

- Bahadur RP, Chakrabarti P, Rodier F, Janin J. A dissection of specific and non-specific protein-protein interfaces. *Journal of Molecular Biology*. 2004; 336:943–955. [PubMed: 15095871]
- Burgess AW, Cho HS, Eigenbrot C, Ferguson KM, Garrett TP, Leahy DJ, Lemmon MA, Silwowski MX, Ward CW, Yokoyama S. An open-and-shut case? Recent insights into the activation of EGF/ ErbB receptors. *Molecular Cell*. 2003; 12:541–552. [PubMed: 14527402]
- Baselga J, Swain S. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. *Nature Reviews. Cancer*. 2009; 9:463–475.
- Berezov A, Zhang HT, Greene MI, Murali R. Disabling ErbB receptors with rationally designed exocyclic mimetics of antibodies: Structure-function analysis. *Journal of Medicinal Chemistry*. 2001; 44:2565–2574. [PubMed: 11472210]
- Berezov A, Chen J, Liu Q, Zhang HT, Greene MI, Murali R. Disabling receptor ensembles with rationally designed interface peptidomimetics. *Journal of Biological Chemistry*. 2002; 277:28330–28339. [PubMed: 12011054]
- Banappagari S, Satyanarayanajois SD, Ronald S. A conformationally constrained peptidomimetic binds to extracellular region of HER2 protein. *Journal of Biomolecular Structure and Dynamics*. 2010; 28:289–308. [PubMed: 20919746]
- Banappagari S, Ronald S, Satyanarayanajois SD. Structure-activity relationship of conformationally constrained peptidomimetics for antiproliferative activity in HER2-overexpressing breast cancer cell lines. *MedChemComm*. 2011; 2:752–759. [PubMed: 21887403]
- Chen VB, Arendall WB III, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW, Richardson JS, Richardson DC. MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallographica*. 2010; D66:12–21.
- Chen CYC. Weighted equation and rules—a novel concept for evaluating protein-ligand interaction. *Journal of Biomolecular Structure and Dynamics*. 2009; 3:271–282. [PubMed: 19795911]
- Chene P. Drugs targeting protein-protein interactions. *Chem Med Chem*. 2006; 1:400–411. [PubMed: 16892375]
- Cho HS, Leahy DJ. Structure of the extracellular region of HER3 reveals an interdomain tether. *Science*. 2002; 297:1330–1333. [PubMed: 12154198]
- Chang JC. HER2 inhibition: from discovery to clinical practice. *Clinical Cancer Research*. 2007; 13:1–3. [PubMed: 17200331]
- Cho HS, Mason K, Ramyar KX, Stanley AM, Gabell SB, Denney DW, Leahy DJ. Structure of the extracellular region of HER2 alone and in complex with herceptin Fab. *Nature*. 2003; 421:756–760. [PubMed: 12610629]
- Dawson JP, Berger MB, Lin CC, Schlessinger J, Lemmon MA, Ferguson KM. Epidermal growth factor receptor dimerization and activation require ligand-induced conformational changes in the dimer interface. *Molecular and Cellular Biology*. 2005; 25:7734–7742. [PubMed: 16107719]
- Drozdz-Tikhomirov LN, Linde DM, Poroikov VV, Alexandrov AA, Skurida GI. Molecular mechanisms of protein-protein recognition: whether the surface placed charged residues determine the recognition process? *Journal of Biomolecular Structure and Dynamics*. 2001; 19:279–284. [PubMed: 11697732]
- Dakappagari NK, Lunte KD, Rawale S, Steele JT, Allen SD, Phillips G, Reilly RT, Kaumaya TP. Conformational HER-2/neu B-cell epitope peptide vaccine designed to incorporate two native disulfide bonds enhances tumor cell binding and antitumor activities. *Journal of Biological Chemistry*. 2005; 280:54–63. [PubMed: 15507452]
- Ferguson KM. Structure-based view of epidermal growth factor receptor regulation. *Annual Review of Biophysics*. 2008; 37:353–373.
- Fuller JC, Burgoyne NJ, Jackson RM. Predicting druggable binding sites at the protein-protein interface. *Drug Discovery Today*. 2009; 14:155–161. [PubMed: 19041415]
- Franklin MC, Carey KD, Vajdos FF, Leahy DJ, De Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell*. 2004; 5:317–328. [PubMed: 15093539]
- Fisher RD, Ultsch M, Lingel A, Schaefer G, Shao L, Birtalan S, Sidhu SS, Eigenbrot C. Structure of the complex between HER2 and an antibody paratope formed by side chains from tryptophan and serine. *Journal of Molecular Biology*. 2010; 402:217–229. [PubMed: 20654626]

- Gijssen M, King P, Perera T, Parker PJ, Harris AL, Larijani B, Kong A. HER2 phosphorylation is maintained by a PKB negative feedback loop in response to anti-HER2 herceptin in breast cancer. *PLoS Biology*. 2010; 8:e1000563.
- Hynes EN, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nature Reviews, Cancer*. 2005; 5:341–354.
- Huang HJ, Lee KJ, Yu HW, Chen CY, Hsu CH, Chen HY, Tsai FJ, Chen CYC. Structure-Based and Ligand-Based Drug Design for HER 2 Receptor. *Journal of Biomolecular Structure and Dynamics*. 2010; 28:23–37. [PubMed: 20476793]
- Khan SH, Ahmad F, Ahmad N, Flynn DC, Kumar R. Protein-Protein Interactions: Principles, Techniques, and their Potential Role in New Drug Development. *Journal of Biomolecular Structure and Dynamics*. 2011; 28:929–938. [PubMed: 21469753]
- Komolov, KE.; Koch, KW. Application of surface plasmon resonance spectroscopy to study G-protein coupled receptor signaling in Surface Plasmon Resonance. In: De Moi, NJ.; Fischer, MJE., editors. *Methods in Molecular Biology*. Vol. 627. NY: Springer Science; 2010.
- Kumagai T, Katsumata M, Hasegawa A, Furuuchi K, Funakoshi T, Kawase I, Greene MI. Role of extracellular subdomains of p185c-neu and epidermal growth factor receptor in ligand-independent association and transactivation. *Proceedings of the National Academy of Sciences USA*. 2003; 100:9220–9225.
- Landgraf R. HER2(ERBB2):functional diversity from structurally conserved building blocks. *Breast Cancer Research*. 2007; 9:202–209. [PubMed: 17274834]
- Lee-Hoeflich ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP, Sliwkowski MX, Stern H. A central role of HER3 in HER2 amplified breast cancer: implications for targeting therapy. *Cancer Research*. 2008; 68:5878–5887. [PubMed: 18632642]
- Levitzi A, Gazit A. Tyrosine kinase inhibition: an approach to drug development. *Science*. 1995; 267:1782–1788. [PubMed: 7892601]
- Liang S, Li W, Xiao L, Wang J, Lai L. Grafting of protein-protein interaction epitope. *Journal of Biomolecular Structure and Dynamics*. 2000; 17:821–828. [PubMed: 10798527]
- Lombardi A, Gianese G, Arcangeli C, Galeffi P, Sperandei M. Bacterial cytoplasm production of an EGFP-labeled single-chain Fv antibody specific for the HER2 human receptor. *Journal of Biomolecular Structure and Dynamics*. 2011; 29:425–439. [PubMed: 22066531]
- Lu C, Mi LZ, Grey MJ, Zhu J, Graef E, Yokoyama S, Springer TA. Structural evidence for loose linkage between ligand binding and kinase activation in the epidermal growth factor receptor. *Molecular and Cellular Biology*. 2010; 30:5432–5443. [PubMed: 20837704]
- Molina MA, Codony-Servat J, Albancell J, Rojo F, Arribas J, Baselga J. Trastuzumab (herceptin), a humanized anti-HER2 receptor monoclonal antibody, inhibits basal and activated HER2 ectodomain cleavage in breast cancer cells. *Cancer Research*. 2001; 61:4744–4749. [PubMed: 11406546]
- Moreira IS, Fernandes PA, Ramos MJ. Hot spots—a review of the protein-protein interface determinant amino acid residues. *Proteins*. 2007; 68:803–812. [PubMed: 17546660]
- Myszka DG. Kinetic, equilibrium, and thermodynamic analysis of macromolecular interactions with BIACORE. *Methods in Enzymology*. 2000; 323:325–340. [PubMed: 10944758]
- Nahta R, Esteva FJ. HER2 targeted therapy: lessons learned and future directions. *Clinical Cancer Research*. 2003; 9:5078–5084. [PubMed: 14613984]
- Ogiso H, Ishitani R, Nureki O, Fukai S, Yamanaka M, Kim JH, Saito K, Sakamoto A, Inoue M, Shirouzu M, Yokoyama S. Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. *Cell*. 2002; 110:775–787. [PubMed: 12297050]
- Reichmann D, Rahat O, Cohen M, Neuvirth H, Schreiber G. The molecular architecture of protein-protein binding sites. *Current Opinion in Structural Biology*. 2007; 17:67–76. [PubMed: 17239579]
- Ryan DP, Mathews JM. Protein-protein interactions in human disease. *Current Opinion in Structural Biology*. 2005; 15:441–446. [PubMed: 15993577]
- Satyanarayanajois SD, Villalba S, Jianchao L, Lin GM. Design, synthesis, and docking studies of peptidomimetics based on HER2-herceptin binding site with potential antiproliferative activity

- against breast cancer cell lines. *Chemical Biology and Drug Design*. 2009; 74:246–257. [PubMed: 19703026]
- Saxon ML, Lee DC. Mutagenesis reveals a role for epidermal growth factor receptor extracellular subdomain IV in ligand binding. *Journal of Biological Chemistry*. 1999; 274:28356–28362. [PubMed: 10497195]
- Shankaran H, Wiley HS, Resat H. Modeling the effects of HER/ErbB1-3 coexpression on receptor dimerization and biological response. *Biophysical Journal*. 2006; 90:3993–4009. [PubMed: 16533841]
- Sperandio O, Reynes CH, Camproux AC, Villoutriex BO. Rationalizing the chemical space of protein-protein interaction inhibitors. *Drug Discovery Today*. 2010; 15:220–229. [PubMed: 19969101]
- Tao RH, Maruyama IN. All EGFR (ErbB) receptors have preformed homo- and heterodimeric structures in living cells. *Journal of Cell Science*. 2008; 121:3207–3217. [PubMed: 18782861]
- Venyaninov, SU.; Yang, JT. Determination of protein secondary structure in circular dichroism and the conformational analysis of biomolecules. In: Fasman, GD., editor. *Circular Dichroism and the Conformational Analysis of Biomolecules*. NY: Plenum Press; 1996. p. 69-108.
- Wells JA, McClendon CL. Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. *Nature*. 2007; 450:1001–1009. [PubMed: 18075579]
- Yang RYC, Yang KS, Pike LJ, Garland R, Marshall GR. Targeting the dimerization of epidermal growth factor receptors with small-molecule inhibitors. *Chemical Biology and Drug Design*. 2010; 76:1–9. [PubMed: 20456371]
- Yin H, Chu A, Li W, Wang B, Shelton F, Otero F, Nguyen DG, Caldwell JS, Chen YA. Lipid G protein-coupled receptor ligand identification using β -arrestin PathHunter™ assay. *Journal of Biological Chemistry*. 2009; 284:12328–12338. [PubMed: 19286662]
- Yuzawa S, Opatowsky Y, Zhang Z, Mandiyan V, Lax I, Schlessinger J. Structural basis for activation of the receptor tyrosine kinase KIT by stem cell factor. *Cell*. 2007; 130:323–334. [PubMed: 17662946]

Figure 1.

Sensograms of **A)** compound **5** interaction with immobilized HER2-ECD protein. The sensograms show dose-dependent binding of compound **5** injected at concentrations of a) 0.0, b) 5, c) 10, d) 25, e) 50, f) 75, and g) 100 μM . Injections were performed at the rate of 50 $\mu\text{L}/5\text{ min}$. A dose-dependent binding of compound **5** onto HER2-ECD can be observed. **B)** HER2 ECD interaction with immobilized compound **5**. The sensograms show dose-dependent binding of HER2 ECD injected at concentrations of a) 0.0, b) 5, c) 10, d) 20, e) 40, f) 80, and g) 160 μM . Injections were performed at the rate of 30 $\mu\text{L}/\text{min}$. The kinetic constant was estimated by global fitting analysis of the titration curve to the 1:1 Langmuir interaction model and the K_d value calculated was around 1.1 μM . **C)** the specific binding of compound **5** (100 μM) to HER2 ECD and control peptidomimetic (100 μM) that does not bind to HER2 ECD. Refer to Table I for the structure of the control peptidomimetic.

Figure 2.

Sensograms showing the **A**) interactions of compound **5** with HER2-ECD, EGFR-ECD, and HER3-ECD. Binding of compound **5** with HER2-ECD (40RU) can be seen whereas no binding can be seen with EGFR and HER3-ECD. Compound **5** specifically binds to HER2-ECD but not to the homologues receptors EGFR and HER3-ECD. **B**) interaction of compound **5** with domain IV of HER2 immobilized on a CM5 sensor chip (48 RU). **C**) interaction of trastuzumab with domain IV of HER2 immobilized on a CM5 sensor chip (850 RU).

Figure 3.

Sensogram showing the **A**) heterodimerization of EGFR-HER2 and its blockade by compound **5**. Compound **5** (100 μ M) binds to HER2 (38 RU). In the presence of compound **5** the interaction of EGFR-ECD with HER2-ECD was blocked whereas the activated EGFR-ECD (200 nM) binds to HER2-ECD in the absence of compound **5** (12 RU). **B**) the heterodimerization of HER2-HER3 and its blockade by compound **5**. Compound **5** (50 μ M) binds to HER2 ECD (14 RU). In the presence of compound **5** the interaction of HER3-ECD with HER2-ECD was blocked whereas the activated HER3 (200 nM) binds to HER2-ECD in the absence of compound **5** (23 RU).

Figure 4.

Western blot analysis of phosphorylated HER2. **A)** in BT-474 cells that overexpress HER2 protein upon treatment with compound **5** (0.4 μ M) and AG-825 (0.35 μ M). The visualization of β -actin was used to ensure equal sample loading in each lane. Phosphorylation was detected using p-HER2 antibody. Total HER2 protein is also shown. **B)** in MCF-7 cells that do not overexpress HER2 protein upon treatment with compound **5** (0.4 μ M) and AG-825 (0.35 μ M).

Figure 5.

Inhibition of heterodimerization of HER2–HER3 in HER2–HER3-transfected U2OS cells by compound **5** at different concentrations using PathHunter assay. Dose-response curve for agonist NRG1 and induction of dimerization is shown (filled circles). Dose-response curve for inhibition of heterodimerization by compound **5** (filled triangles) in the presence of 0.3 μM NRG1.

Figure 6.
Proposed model for HER3 extracellular domain showing domains I to IV in open conformation.

Figure 7.

A) Proposed model for PPI of extracellular domains of HER2 and HER3. Notice the absence of ligand for HER2. C-terminal portions of domain IV of HER2 and HER3 interact, stabilizing the heterodimer apart from domain II interactions. **B)** HER2 and HER3 domain II-interactions with residues in domain II that are important for dimerization are shown (magenta-HER2, blue-HER3).

Figure 8.
Proposed dimerization interface/binding site of compound 5 (shaded area) on domain IV of HER2 protein.

Table I

Structures of compound 5 and control compound along with antiproliferative activity against breast cancer cell lines.

Code number	Structure	IC ₅₀ μ M		
		SKBR-3	BT-474	MCF-7
5		0.396 $\pm$ 0.022	0.895 $\pm$ 0.029	16.9 $\pm$ 1.0
control	H-Lys-[3-amino-3(biphenyl)-propionic acid]-Phe-OH	>50	>50	>50