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A SPR strategy for high-throughput ligand screenings based on synthetic peptides mimicking a selected subdomain of the target protein: A proof of concept on HER2 receptor

Luca Monfregola [a], Rosa Maria Vitale [b], Pietro Amodeo [b], Stefania De Luca [a,*]

[a] Istituto di Biostrutture e Bioimmagini IBB-CNR, 80134 Naples, Italy
[b] Istituto di Chimica Biomolecolare ICB-CNR, 80078 Pozzuoli (NA), Italy

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ABSTRACT

The discovery of pharmaceutical agents is a complex, lengthy and costly process, critically depending on the availability of rapid and efficient screening methods. In particular, when targets are large, multidomain proteins, their complexity may affect unfavorably technical feasibility, costs and unambiguity of binding test interpretation.

A possible strategy to overcome these problems relies on molecular design of receptor fragments that are: sensible targets for ligand screenings, conformationally stable also as standalone domains, easily synthesized and immobilized on chip for Biacore experiments.

An additional desirable feature for new ligands is the ability of selectively targeting alternative conformational states typical of many proteins.

To test the feasibility of such approach on a case with potential applicative interest, we developed a surface plasmon resonance (SPR)-based screening method for drug candidates toward HER2, a Tyr-kinase receptor targeted in anticancer therapies. HER2 was mimicked by HER2-DIVMP, a modified fragment of it immobilized onto the sensor surface specifically modeling HER2 domain IV in its bounded form, designed by structural comparison of HER2 alone and in complex with Herceptin, a monoclonal therapeutic anti-HER2 antibody.

This design and its implementation in SPR devices was validated by investigating Herceptin- HER2-DIVMP affinity, measuring its dissociation constant (K_D = 19.2 nM). An efficient synthetic procedure to prepare the HER2-DIVMP peptide was also developed. The HER2-DIVMP conformational stability suggested by experimental and computational results, makes it also a valuable candidate as a mold to design new molecules selectively targeting domain IV of HER2.

1. Introduction

Membrane receptors represent very common targets for drugs and diagnostic molecules. However, they present several potential problems when used for screenings of new ligands. In fact: (a) they are often difficult to purify/express and manipulate; (b) their binding and activity tests may easily be slow, complex, expensive and poorly reproducible; (c) their structural characterization, alone or in complex with ligands, is from difficult to impossible, and, even in most favorable cases, it is a slow process and implies severe simplification of the true system; (d) their complexity makes difficult to ascertain, in principle, if the binding site of a ligand corresponds to that targeted in its design; (e) receptors may exhibit multiple conformational states associated to 'bounded' or 'unbounded' forms to their effectors (i.e., 'active' vs 'inactive', or 'open' vs 'closed' forms); (f) when tests are performed on cells or cellular extracts, it is difficult to account for possible interferences from other highly-homologous receptor subtypes. These limitations are particularly severe in the case of high-throughput screenings, such as those performed on combinatorial libraries of ligands.

In this view, the availability of simplified synthetic models that are both fully representative of the receptor binding properties relatively to preselected sites and conformational states, and easy to obtain and implement in reproducible high-throughput screenings of ligands, would play a strategical role in the development of new therapeutic or diagnostic molecules.

These molecules should, in principle, fulfill several requirements for maximum usability: (1) inclusion of highly structurally-conserved receptor regions, able to bind ligands with high-affinity and, possibly, selectivity for the full receptor, and, in the case of therapeutic agents, to interfere with the receptor activity; (2) optionally, in the case of multi-state proteins, stabilization of a preselected conformational state; (3) ease of synthesis, purification and

* Corresponding author. Tel.: +39 081 2536643; fax: +39 081 2536642.
E-mail address: stefania.deluca@cnr.it (S.De Luca).

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manipulation; (4) easy integration in fast, reliable and inexpensive assays for ligand affinity, able to catch the overall binding properties of the full receptor.

The strategical importance of these molecules would be further increased in the absence of known natural ligands for the receptor/domain under investigation, when development of strategies requiring high-throughput screening techniques, such as those based on the synthesis of combinatorial libraries of ligands, are widely employed.

Among different possible candidates endowed with most of these prerequisites, human epidermal growth factor receptor-2 (HER2) was selected to test this strategy to develop a potentially effective and selective screening method.

Human epidermal growth factor receptor-2 (HER2) is a member of the epidermal growth factor[1] (EGFR) or HER family of tyrosine kinase receptors that also includes HER1 (Erb1), HER3 (Erb3), and HER4 (Erb4). Erb receptors are crucial for mediating cell proliferation, differentiation, and survival. The overexpression of HER2 protein has been observed in a variety of tumors, in particular, breast and ovarian cancers,[2] as well as tumors of the lung, salivary gland, kidney,[3] bladder, and has been associated with poor clinical outcomes.[4] Anti-cancer therapies targeting these receptors are currently being explored. Trastuzumab (Herceptin), a recombinant humanized monoclonal anti-HER2 antibody which has been approved for clinical use, is reported to bind HER2 with nanomolar affinity[5,6] and inhibits the growth of HER2-expressing breast cancer cells.[7,8] ErbB receptors consist of an extracellular regions organized in four domains (I–IV) followed by a single membrane-spanning region and a cytoplasmic tyrosine kinase domain. Activation of these receptors requires their homo- or hetero-dimerization induced by ligand binding to the extracellular region. HER2 is an universal ErbB co-receptor but singularly devoid of a high-affinity ligand. The crystal structure[9] of the extracellular regions of HER2 alone (rat) and in complex (human) with the Herceptin antigen-binding fragment (Fab) has shown that HER2 adopts an open conformation unlike the other receptor family members, whose unliganded forms arrange in an autoinhibited or closed conformation, with domains II and IV strictly interacting. The absence of an autoinhibited conformation for HER2 could partially account for its cancer-inducing potential when over-expressed. A potential role of domain IV in mediating receptor–receptor interactions was also evidenced by a work of Berezov et al. where a series of peptides derived from domain IV of erbB receptors were able to inhibit ligand-induced receptor self-associations.[10] In its constitutionally-open conformation, HER2 domain IV is fully exposed and its C-terminal portion encompasses the binding pocket that in HER3 and HER1 inactive states hosts a large loop from domain II. As Herceptin has been demonstrated[9] to bind specifically domain IV of HER2, this region represents a possible target for other new anticancer drugs and diagnostic agents.

2. Experimental

2.1. Materials and methods

Solid-phase peptide synthesis was performed on a fully automated Multisyntech Syro I synthesizer. Analytical RP-HPLC runs were carried out on a HP Agilent Series 1100 apparatus using a Phenomenex (Torrance, CA) C18 column, 4.6 250 mm with a flow rate of 1.0 mL min^{-1}. Preparative RP-HPLC was carried out on a Shimadzu 8A apparatus equipped with an UV Shimadzu detector using a Phenomenex (Torrance, CA) C18 column, 22 × 250 mm with a flow rate of 20 mL min^{-1}. For all the RP-HPLC procedures the system solvent used was H_2O 0.1% TFA (A) and CH_3CN 0.1% TFA (B), with a linear gradient from 20% to 80% B in 20 min. Mass spectral analyses were carried out on Finnigan Surveyor MSQ single quadrupole electrospray ionization mass spectrometer coupled with a Finnigan Surveyor HPLC (Finnigan/Thermo Electron Corporation San Jose, CA, USA).

PyBOP, HOBt, Rink Amide MBHA resin all Fmoc-amino acid derivatives were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland). All other chemicals were obtained from Aldrich (St. Louis, MI), Fluka (Milwaukee, WI) or LabScan (Stillorgan, Dublin, Ireland) and were used without further purification, unless otherwise stated.

For SPR experiments was used a four channel BIAcore 3000 optical sensor instrument (Uppsala, Sweden). All experiments were performed at 25 °C on SA sensor chip (sensor chip with immobilized streptavidin), also obtained from BIAcore, and by using Hepes-buffered saline (HBS) as running buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4).

Far uv CD spectra were collected at room temperature on a Jasco Model J-715 spectropolarimeter using a solution 4.2 μM of HER2-DIVMP in 10 mM phosphate buffer, pH 7.2, in 1-cm quartz cells

2.2. Synthesis

The two peptide sequences (557–580) and (591–607) were synthesized by solid-phase method, with standard Fmoc procedure on a fully automated synthesizer. Appropriate Fmoc-amino acid derivatives were employed (Fmoc-Asn(Trt)-OH, Fmoc-Ile-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Val-OH, Fmoc-Cys(Acm)-OH, Fmoc-Tyr(OtBu)-OH, Fmoc-His(Trt)-OH); Rink Amide MBHA resin (0.28 mmol g^{-1} substitution; 42 μmol scale) was used as solid support, as it releases peptides amidated at C-terminus upon acid treatment. All couplings were performed twice for 20 min by using an excess (4 equiv) of each amino acid derivative. The amino acids were activated in situ by the standard HOBt/PyBOP/ DIPEA protocol. Fmoc deprotection was performed with 20% piperidine in DMF. The last coupling for peptide (557–508) was performed using Biotin, while for peptide (591–607) the synthesis was ended with the acetylation (4.7% acetic anhydride and 4% pyridine in DMF) of the N-terminal amino acid residue. The peptide cleavage from the solid support and the simultaneous removal of all protecting groups from the amino acid residues was carried out by suspending the fully protected compound-resins in TFA/H_2O/TIS (97:2:1) for 3 h followed by filtration. The solution was then concentrated and the crude product isolated by precipitation into cold diethyl ether.

RP-HPLC and mass spectrometry analysis confirmed the presence of the desired compounds. Biot-HER2(557–580)[Cys^{565}(Acm), Cys^{574}-(Acm)]: t_R = 11.46 min; $[M+2H]^{++}$ = 1480.0 *m*/*z* (calcd = 1480.7); HER2-(591–607): t_R = 10.12 min; $[M+H]^{+}$ = 1973.0 *m*/*z* (calcd = 1972.9).

The procedure to activate the thiol group of Cys601 is described as follows. DTNP (3–5 equiv) was dissolved in the minimum amount of acetic acid/water (3:1) and the peptide HER2(591–607) (1 equiv) was added in one portion previously dissolved in the same system solvent. The reaction was monitored by RP-HPLC and after it was complete (4–6 h), water was added to reach a proportion of 9 to 1 and the solvent was eliminated by lyophilization. The solid obtained was washed with 0.1% TFA in water and it allowed the removal (in the form of a yellow powder) of the excess of DTNP which resulted insoluble in the aqueous solution.

The desired compound, obtained in excellent yield, was analyzed and identified by RP-HPLC and mass spectrometry, respectively. HER2(591–607)[Cys^{601}(*p*-Npys)]: t_R = 14.99 min; $[M+2H]^{++}$ = 1063.9 *m*/*z*(calcd = 1064.0).

In order to bind the two peptide chains synthesized, to an ammonium acetate buffer solution (pH 3.5–6.5) of HER2(591–607)[Cys^{601}(*p*-Npys)] (1 equiv) was added Biot-HER2(557-580)[Cys^{565}(Acm), Cys^{574}(Acm)] (1.2 equiv), previously dissolved in the minimum volume of the same buffer, in several portion. The reaction was easily monitored by the intense yellow coloration due to the release of 5-nitro-2-pyridinethiol. The RP-HPLC and the mass spectrometry analysis revealed that the two peptides were quantitatively bound via disulfide bridge.

HER2-DIVMP[Cys^{565}(Acm), Cys^{574}(Acm)]: t_R = 13.49 min; $[M+2H]^{++}$ = 2464.8 *m*/*z* (calcd = 2465.6).

The removal of the Acm groups and the simultaneous oxidation reaction, in order to form the second disulfide bond between Cys565 and Cys574, were performed using iodine. The peptide Biot-HER2-DIVMP[Cys^{565}(Acm), Cys^{574}(Acm)] was dissolved in acetic acid/water (4:1) to a final concentration of 10^{-4} M. To this solution 10 equiv of iodine were added and the mixture reaction was stirred for 1 h. The final steps of HPLC purification and mass spectrometry characterization afforded the desired compound in high yield (30–40%). HER2-DIVMP: t_R = 13.85 min; $[M+3H]^{+++}$ = 1595.5 *m*/*z* (calcd = 1596.0).

2.3. SPR experiments

Before the immobilization procedure, the SA chip was first cleaned with three consecutive 1-min injection of 30 μL of a solution of 1 M NaCl in 50 mM NaOH. After the cleaning process, Biot-HER2-DIVMP, diluted in running buffer (HBS) to 10 mg/μL, was injected for 300 s using a flow rate of 10 μL/min. The maximum immobilization level of the biotinylated peptide on the SA chip surface (flow cell 2) was reached after 9 injections and the sensorgram baseline was increased of 1600 RU. The flow cell 1 of the SA chip was used as blank surface.

Six different concentrations of antibody Herceptin ranging from 200 nM to 2200 nM in running buffer were injected over each surface (flow cell 1 and flow cell 2) at flow rate of 30 μL/min for 100 s. The dissociation step was performed at the same flow rate in HBS buffer for 500 s. Finally, the chip surface was regenerated by 200 s injections of 50 mM NaOH and 1 M NaCl.

The signal of each binding experiment was corrected for non specific binding by subtracting the signal obtained for a blank surface.

The kinetic rate constants ($K_a = 3.33 \times 10^4\ M^{-1}\ s^{-1}$; $K_d = 6.38 \times 10^{-4}\ s^{-1}$) were calculated using the BIAevaluation 4.1 software. Global analysis was performed using the simple 1:1 Langmuir binding model. The equilibrium dissociation constant (K_D = 19.2 nM) was calculated from the rate constants. The correctness of the used fitting model was assessed by the residual values (a measure of the variance between the predicted model and the Experimental data) randomly distributed along the zero and the x^2 value obtained (0.306) less than 1.

The specificity of the interaction between Her2-DIVMP and Herceptin was checked by using as negative control an anti-human antibody against the peptide.

2.4. Computational methods

Starting models of HER2-DIVMP in its free form and complexed to Herceptin Fab were obtained by imposing the crystal structure of the extracellular domain of human HER2 complexed to Herceptin Fab (PDB entry: 1N8Z) to the corresponding HER2-DIVMP residues. The resulting models were completed by addition of all hydrogen atoms and underwent energy minimisation with NAMD[12] package using Charmm22 force field.[13] Molecular dynamics simulations were run in solvent by confining the minimized complexes in rectangular TIP3P water boxes, with a minimal distance from the solute to the box wall of 1.2 nm. Counterions (Na^+ for HER2-DIVMP alone, Cl^- for HER2-DIVMP-Herceptin complex) were added to neutralize the system. Particle mesh Ewald method was applied to calculate long-range electrostatics interactions, setting to 14 Å the nonbonded cutoff. The solvated molecules were energy minimized through 1000 steps with solute atoms restrained to their starting positions using a force constant K_d of 1 kcal mol^{-1} Å^{-1} prior to molecular dynamics (MD) simulations. The systems were then equilibrated by 50 ps MD with all solutes (protein, peptide and ions) kept fixed, followed by 25 ps solute-restrained MD (K_d = 100 kcal mol^{-1} Å^{-1}), 25 ps solute-restrained MD (K_d = 50 kcal mol^{-1} Å^{-1}) and 25 ps solute-restrained MD (K_d = 1 kcal mol^{-1} Å^{-1}). Finally, the systems underwent fully unrestrained MD simulations of 2.5 ns (HER2-DIVMP) and 5 ns (HER2-DIVMP-Herceptin complex), the first 500 ps of each trajectory being discarded in final analyses. All MD runs were performed at constant pressure (1 atm) and temperature (310 K), using a timestep of 1 fs. Snapshots from production runs were saved every 1000 steps and analyzed with NAMD program. Model figures were made with CHIMERA program.[14]

3. Results and discussion

3.1. Peptide design

An inspection of the published X-ray structure of rat extracellular region of HER2 (sHER2) alone[9] seems to suggest that domain IV can fold as a standalone structural motif, owing to its limited, although not negligible in principle, interaction with the rest of the protein. In addition, with the exception of residues 581–590, this conformational motif persists after complexation with the monoclonal antibody Herceptin.[9] The aforementioned residues, in fact, are disordered in the complex (not even observed in the crystal structure). They correspond to a loop in the unbound sHER2 structure, apart from residues Gly581 and Val582, that belong to a short β-strand (Fig. 1A).

As a proof of concept, we decided to design a peptide mimicking the Herceptin-binding conformation of HER2 domain IV, to test if a 'bound' conformation of a receptor could be stabilized as a standalone molecule. Thus, we selected two peptide segments, corresponding to 557–580 and 591–607 sequences of human HER2, that contain the interacting loops but exclude the disordered region. These peptide sequences were bound by a disulfide bridge between Cys578 and Cys601, since this bond is the connection already present in the native protein, and all terminal residues have been protected by acetylation (N-terminus) or amidation (C-terminus) both to increase degradation resistance and to minimize potential end effects by charged terminations on overall fold. Finally, to reduce problems in the formation of the correct intramolecular disulfide bridge, Cys604 was replaced by a Ser residue, thus leading to the final peptide sequence, named HER2-DIVMP (i.e., HER2-Domain IV-mimicking peptide) shown in Figure 2.

As a structural motif corresponding to domain IV of HER2 has not yet been identified or classified as a standalone folding motif by searching in SCOP database,[11] a prediction of its stability, and of the potential role of residues 581–590 on the overall conformational properties of this region, was obtained by a computational approach based on Molecular Dynamics (MD). This simulation was also aimed at verifying that the interactions formed by the selected residues with the rest of HER2 did not contribute appreciably to the overall conformational stability of the domain encompassed by HER2-DIVMP. In fact, several such interactions were detected both in HER2 structure, both alone and complexed with Herceptin, among whom three H-bonds involving E544 and H567, E544 and A566, Q546 and A564.

A 2 ns MD production run on a model built by imposing the crystal structure of the extracellular domain of human HER2 in

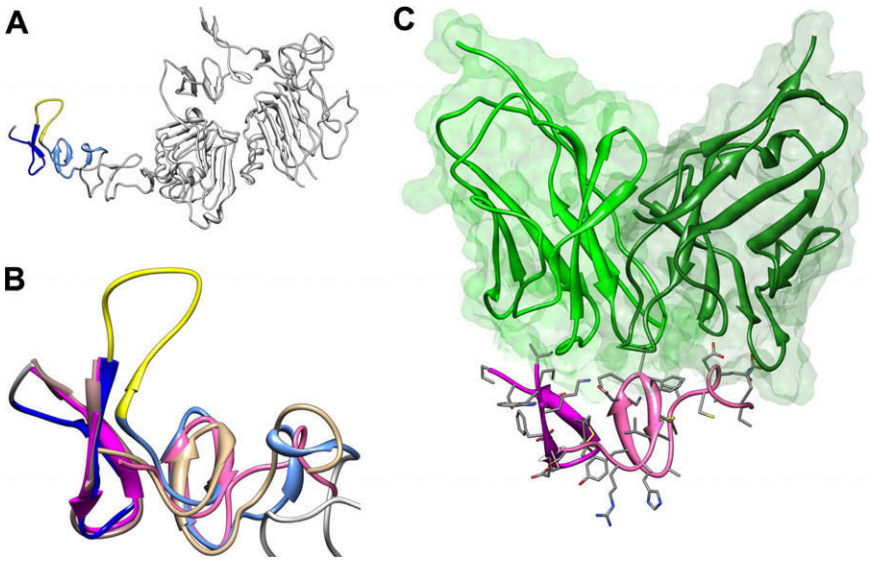


Figure 1. Molecular representations of peptides and proteins involved in HER2-DIVMP design. (A) Ribbon representation of rat HER2 crystal structure. The sequences corresponding to the two HER2-DIVMP chains are colored medium and dark blue, the 581-590 cut out sequence is painted yellow and the rest of the protein is white. (B) Superimposition of final MD frame of unbound HER2-DIVMP (tan and light brown), final MD frame of Herceptin Fab chains-HER2-DIVMP complex (light and dark magenta) and crystal structure of unbound rat HER2 C-terminus (medium and dark blue, yellow and white). Ribbons are used for all peptides, and the two shades of colors are used for regions corresponding to the first (lighter) and the second (darker) chains of HER2-DIVMP, while yellow and white are used accordingly to panel A. (C) Final frame of the MD simulation of Herceptin Fab chains-HER2-DIVMP complex. The two Herceptin Fab chains are shown as medium and dark green ribbons enclosed in transparent solvent accessible surfaces, while HER2-DIVMP is represented as ribbons (light and dark magenta for the two chains) and sidechain sticks colored by atom types.

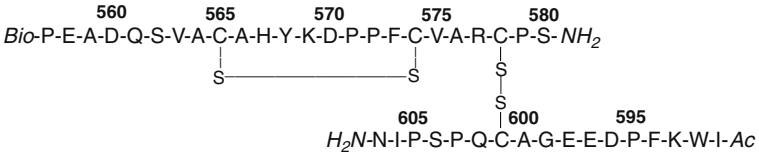


Figure 2. Amino acid sequence of the HER2 receptor fragment Biot-HER2-DIVMP.

complex with Herceptin Fab (PDB entry: 1N8Z) to the corresponding HER2-DIVMP residues showed that both the secondary structure and the overall three-dimensional fold are well-preserved (r.m.s.d. 1.38 Å at level of backbone atoms encompassing 563–605 residues), and, apart for an expected disorder at the very N-terminus of the peptide, no large β-sheet or tertiary structure perturbation derived from missing interactions with the rest of HER2 (Fig. 1B).

An important result for future application of our design strategy is the substantial preservation of the Herceptin-bound conformation in isolated HER2-DIVMP, showing that we succeeded in stabilizing a receptor-bound form in a model subreceptor peptide.

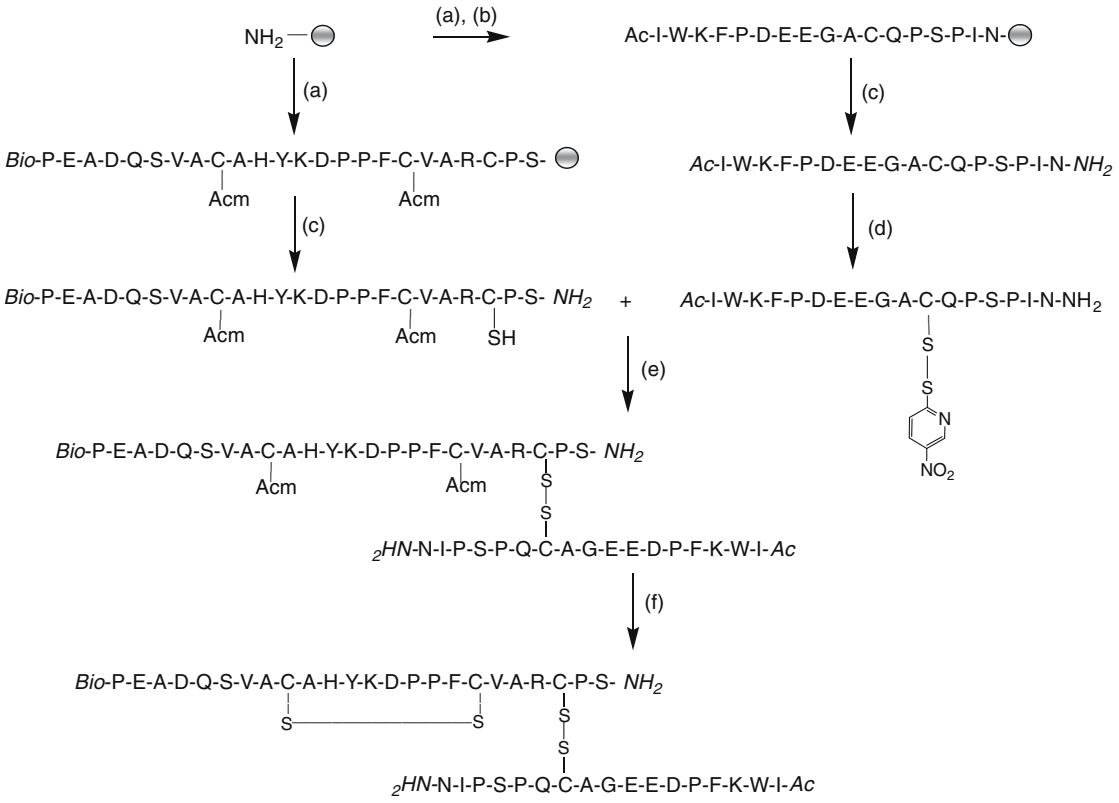
As HER2-DIVMP was designed to mimicking HER2 domain IV in its bounded form, we also evaluated the stability of its complex with Herceptin Fab. A 4.5 ns MD production run of the complex, built on the crystal structure of the corresponding complex between human HER2 and Herceptin Fab (PDB entry: 1N8Z), confirmed the stability of the overall complex structure and of HER2-DIVMP fold too, also in comparison with the MD trajectory of HER2-DIVMP alone (Fig. 1C).

3.2. Peptide synthesis

The N-terminus of HER2-DIVMP (557–580) peptide chain was chosen to introduce a biotine, which was necessary to immobilize HER2-DIVMP on the streptavidin-coated biosensor for SPR analyses. The whole synthetic strategy is shown in Scheme 1.

Solid-phase peptide synthesis of the two peptide chains, using Fmoc strategy on fully automated peptide synthesizer, gave a high yield of the linear peptides.

The peptide chain (557–580), after high percentage of trifluoroacetic acid treatment, was cleaved from the resin and freed from all protecting groups except the Acm groups, stable under acidic conditions, that protect the side chains of the residues Cys565 and Cys574. It allowed the selective disulfide bridge formation between Cys578 and Cys601. In particular, the formation of this asymmetric disulfide bond was performed by activation of the thiol function of the peptide (591–607), followed by the addition of the second peptide (557–580) in the free thiol form. This approach was necessary in order to promote the formation of the desired heterodimer, otherwise unwanted homodimeric molecules could be obtained in appreciable yields. One of the most widely used methods employs aromatic sulfenyl protecting/activating groups. In particular we decided to employ the 2,2′-dithiobis(5-nitropyridine) (DTNP) to activate the thiol function of Cys601. Hence, the peptide (591–607), after cleavage from the resin and removal of all protecting groups, was allowed to react with an excess of DNTP in acetic acid/water for 4–5 h. This synthetic procedure provided the desired crude product in excellent yield. A subsequent simple workup yields this crude product pure enough to be used in the next reaction step. It consisted of the heterodimer formation which was performed by dissolving the Cys(*p*-Npys) containing peptide (591–607) in an ammonium acetate aqueous solution, while the free thiol peptide (557–580), having been



Scheme 1. Peptide synthesis according to the Fmoc solid phase protocol. Reagents and conditions: Rinkamide resin, loading 0.5 mmol g^{-1}; (a) protected amino acids, PyBop/HOBt/DIPEA in DMF, 20 min; (b) Ac_2O (4,7%), Pyridine (4%) in DMF, 30 min; (c) TFA/H_2O/TIS (tris-iso-propylsilane) (97:2:1), 3 h; (d) CH_3COOH/H_2O (3:1), 2,2[I]-ditiobis(5-nitropyridine), 5 h; (e) CH_3COONH_4 1 M, 2 h; (f) I_2, CH_3COOH/H_2O (4:1) 1 h.

previously dissolved in the minimum volume of the same buffer, was added in several portions. The yellow color observed of the freed 5-nitro-2-pyridinethiol allowed to monitor the reaction in real time.

After having completed the assembly of the two peptide chains, it was possible to perform the second disulfide bridge formation between Cys565 and Cys574. This oxidation reaction took place by the treatment of Biot-HER2-DIVMP[Cys^{565}(Acm), Cys^{574}(Acm)] with a solution of iodine dissolved in a mix of acetic acid and water.

The final compound Biot-HER2-DIVMP was obtained in good yield and with high purity grade (>95%) after RP-HPLC purification. It was fully characterized for its identity by mass spectrometry. CD spectra carried out on HER2-DIVMP peptide showed an α-helix and β-sheet content of 0% and 39%, respectively, as estimated by neural network-based CD fitting model. This secondary structure content is very close to that detected in the corresponding region of HER2 crystal structure.

3.3. Binding studies

Surface plasmon resonance analysis was our method of choice for characterization of HER2 receptor fragment-antibody binding, in order to develop a SPR-based screening method for rapid and accurate determination of the affinity of drug candidates toward HER2-DIVMP, a fragment of the entire HER2 receptor used as a model system.

In Biacore experiments, one of the interacting molecules (termed the ligand) is immobilized on the sensor surface, and the other interactant (termed the analyte) in solution phase is continuously flown over that surface in a micro-flow cell. For our purposes, HER2-DIVMP was chosen as ligand, while the antibody Herceptin was chosen as analyte and passed across ligand-derivatized surface.

Streptavidin-coated sensor chip (SA chip) was used to perform the SPR binding experiments in order to create homogenous binding surface by a site-directed immobilization of biotinylated HER2-DIVMP to the sensor surface. The HER2-DIVMP N-terminus was chosen to introduce a biotine, since the immobilization on this peptide sequence position does not compromise the recognition between the ligand and the analyte, as suggested by modeling studies. Moreover, the intensity of the sensorgrams signal shown in Figure 3 revealed a high surface density of ligand (the biotinylated peptide), which enhances the sensitivity in detection. This result also allowed to conclude that the N-terminal peptide position

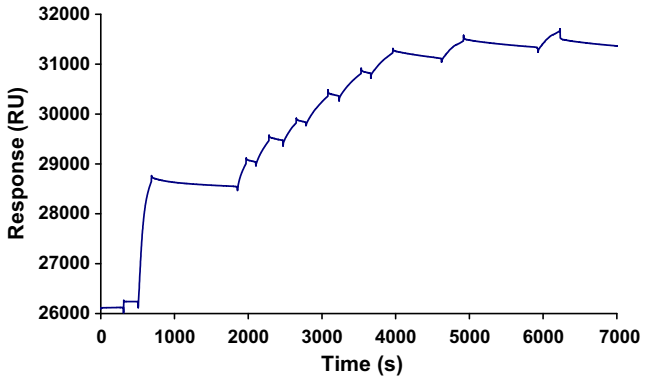


Figure 3. Maximum immobilization level of Biot-HER2-DIVMP on the SA chip. After nine injections of the ligand, at the concentration of 10 mg/μL, the chip surface was saturated.

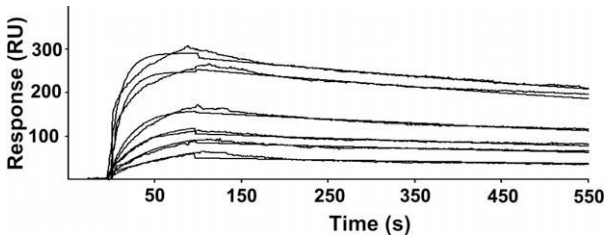


Figure 4. Sensorgram of kinetic response of Biot-HER2-DIVMP to Herceptin antibody. Concentrations of Herceptin were 2200, 1900, 1100, 700, 500 and 200 nM, respectively, from the top to bottom.

Table 1
Kinetic and termodinamic parameters of Herceptin against Biot-HER2-DIVMP fragment

K_a (10^4 M^{-1} s^{-1})	K_d (10^{-4} s^{-1})	K_D (10^{-9} M^{-1})	x^2
3.33 ± 0.04	6.38 ± 0.04	19.2	0.306

is able to guarantee an optimum accessibility of the biotine to the SA.

After immobilization of Biot-HER2-DIVMP on the SA chip, injection of a series of Herceptin solution was carried out. Figure 4 shows the experimental sensorgrams obtained, association of the analyte with the ligand proceeded for 100 s and dissociation in analyte free buffer was typically 500 s. For each titration, a zero analyte surface as a control was subtracted from test sensorgram and the chip surface was regenerated in order to remove any remaining analyte.

The kinetic rate constants (K_a and K_d), as well as equilibrium dissociation constant (K_D), were estimated by global fitting analysis of the titration curves to the 1:1 Langmurian interaction model. The results are summarized in Table 1. Good fitting of experimental data to the calculated curves has been observed, suggesting the correctness of the used fitting model.

4. Conclusions

This study produced several interesting results both addressing the specific system under examination, and, more in general, the possibility of developing devices for SPR experiments based on synthetic peptides designed as modified target protein subdomains.

By analysis of HER2 receptor, we designed a peptide, HER2-DIVMP, whose sequence encompasses the so-called domain IV of HER2, already recognized as a possible target for smaller and more active HER2-binding molecules than Herceptin (Cho et al., 2003). Although the subdomain spanned by HER2-DIVMP sequence is not currently reported as a standalone structural motif, both our calculation, and CD spectra, and Herceptin Fab binding properties, all support a well-defined and stable fold, even in the absence of both Herceptin and HER2 adjacent regions. Deletion of residues 581–590, folded in HER2 alone and disordered in HER2-Herceptin complex, does not appear to negatively affect the conformational stability of the peptide, both isolated and complexed to Herceptin. This result seems to rule out a possible fold stabilizing role for the 581–590 region in monomeric HER2.

Thanks to its predicted conformational stability, HER2-DIVMP has been immobilized on a Biacore chip aimed at screening HER2-targeting molecules highly selective for domain IV by a high-throughput approach, in the absence of potential misleading contributions from the other HER2 domains. For the same reason, HER2-DIVMP could be also used as a mold to design totally new HER2-binding ligands. The whole set of SPR experiments provided values of the Herceptin—HER2-DIVMP complex dissociation constant in the nanomolar range, thus demonstrating that our HER2 fragment represents a reliable model system of HER2 domain IV in its bound state.

From a more general point of view, in this study we have successfully:

(a) Identified by a careful structural search a (non canonical) protein motif able to preserve its fold when synthesized as standalone molecule and endowed with interesting properties for high-throughput screening applications.
(b) Modified the selected motif to simplify its sequence, to allow its immobilization on a Biacore chip and to more specifically mimicking its 'bound' conformational state.
(c) Developed an efficient strategy for synthesis and immobilization of the designed peptide.
(d) Verified by ligand binding assays the efficacy and efficiency of the proposed approach.

The screening device so obtained is completely immune from interferences from receptor regions different from that covered by the design, thus decreasing the possibilities of selecting potential ligands with lower selectivity toward different subtypes of the targeted receptor families.

In this view, the present study represents a promising proof of concept for a synthetic receptor approach addressing specific regions and/or conformational states of the protein under investigation, and exploiting the simplification and cost reduction associated to efficient synthesis techniques.

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