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ORIGINAL ARTICLE

Cysteine co-oxidation process driven by native peptide folding: an example on HER2 receptor model system

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Abstract Synthetic models of receptors that have relevant biological roles are valuable tools for studying receptors itself and the corresponding ligands. Their properties can be validated at first by their capacity to fold in solution under native-like conditions and to assume conformations structurally and functionally equivalent to those in the native receptor. In this context, a new strategy to prepare the two-fragments synthetic receptor model HER2-DIVMP, an independent structural and functional motif of HER2, has been developed and the folding properties have been investigated. The strategy is based on a one-step cysteine co-oxidation procedure in slightly alkaline aqueous buffers, whereby the two separate peptide chains are allowed to self-assemble in solution. Under these conditions, the two chains spontaneously form the expected heterodimer with the correct pattern of disulfide bridges. To gain insights on the folding mechanism, we investigated the folding of two scrambled variants of the constituent peptide chains.

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Introduction

The introduction of disulfide bridges represents a valuable approach to improve the biological activity and stability of natural or de novo designed synthetic polypeptides. In fact, disulfide bonds play an important role in the folding and structural stabilization of many natural peptides and proteins (Buchner and Moroder 2009; Annis et al. 1997; Thornton 1981; Creighton 1988; Pace et al. 1988; Matsumura et al. 1989; Wedemeyer et al. 2000; Raimondo et al. 2005).

When approaching a strategy to design and prepare new disulfide-containing polypeptides, advantages and disadvantages of chemically-driven oxidation methods, mediated by several reagents (Chan and White 2000; Andreu et al. 1994), such as iodine, thallium trifluoroacetate, potassium ferricyanide, dimethylsulphoxide (Kamber et al. 1980; Zhang et al. 2008; Engebretsen et al. 1997; Eritja et al. 1987; Tam et al. 1991), or spontaneous folding must be, therefore, carefully considered (Reinwarth et al. 2013; Steiner and Bulaj 2011; Kellenberger et al. 1995; Kudryavtseva et al. 1998). It is obvious that regioselective synthetic routes are needed to assemble non-native structures with predetermined disulfide patterns. On the contrary, native or native-like structures can be conveniently induced to spontaneously convert under native-like conditions, to the molecular regioisomer with the more conformationally stabilized pattern of disulfide bridges (Marasco et al. 2006; Calvanese et al. 2008).

We recently reported the design and synthesis of a simplified HER2 mimetic, named HER2-DIVMP, which mostly recapitulates the binding properties for the monoclonal antibody Herceptin, an approved drug that potently blocks tumor growth by inhibiting HER receptors signaling. This simplified domain binds Herceptin with a nanomolar dissociation constant (Monfregola et al. 2009), thus could be used as a synthetic HER2 surrogate for dosing the antibody or for structure–activity studies. We have also recently reported that HER2-DIVMP is efficiently recognized by a short peptide that mimics the CDR3 loop from the Herceptin light chain (Calce et al. 2013). HER2-DIVMP was designed on the basis of the previously published X-ray structure of the receptor extracellular region, alone and in complex with Herceptin (Cho et al. 2003), and contains two modified fragments of HER2 domain IV linked by two disulfide bridges. In particular, we selected two peptide segments, encompassing residues 557–580 (chain A) and 591–607 (chain B), that contain the Herceptin interacting loops but exclude the disordered region 581–590, not observed in the crystal structure. The two peptide chains were linked by a first disulfide bridge connecting Cys578 and Cys601, and by a second bridge linking intramolecularly Cys565 and Cys574 on the segment 557–580, both present in the native protein. The reported synthetic strategy for preparing HER2-DIVMP used orthogonal protections onto cysteine residues, so that oxidation was chemically achieved on selected sulfhydryl pairs. This approach led to the desired heterodimer; however, the overall yield was largely limited by the step of intramolecular cyclization between Cys565 and Cys574 performed with iodine, which also provided significative amounts of disulfide-mispaired oligomers (Monfregola et al. 2009).

With the aim of improving the yield of the final product and to also investigate the conformational features of this small mimetic, we have prepared HER2-DIVMP by a co-oxidation procedure. In particular the two chains, in their free thiol form, were allowed to spontaneously self-assemble in solution. The aim was to perform cysteine oxidation under conditions favorable to the native folding process, so that naturally directed sulfhydryl pairings could occur.

Materials and methods

Chemicals and equipment

Fmoc-protected amino acids, *N*-hydroxybenzotriazole (HOBT), *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland), piperidine and diisopropylethylamine (DIPEA) were purchased from Fluka (Milwaukee, WI, USA), Rink Amide MBHA resin and other solvents were purchased from Aldrich (St Louis, MI, USA) or Fluka (Milwaukee, WI, USA) and were used without further purification, unless otherwise stated. Sequence-grade porcine trypsin was from Sigma-Aldrich.

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 system using a C18 column, 250 × 4.6 mm ID (Phenomenex, Torrance, CA, USA) at a flow rate of 1.0 mL min^{-1} or on a C18 monolithic column 50 × 2 mm ID (Phenomenex, Torrance, CA, USA), operating at 0.6 mL min^{-1}.

Preparative RP-HPLC was carried out on a Shimadzu 8A chromatograph coupled with an UV detector, using a C18 column, 22 × 250 mm (Phenomenex Torrance, CA, USA) at a flow rate of 20 mL min^{-1}. For all the RP-HPLC procedures the solvent system used was: H_2O 0.1 % TFA (A) and CH_3CN 0.1 % TFA (B). Separations were achieved applying a linear gradient of B from 20 % to 80 % in 20 min and monitoring at 210 and 280 nm.

LC–MS data were obtained using a Finnigan Surveyor MSQ single quadrupole or LCQ DECA XP MAX electrospray ionization mass spectrometers, coupled with a Surveyor HPLC (ThermoFisher), operating in the full scan positive mode, between 200 and 2,000 *m*/*z*.

Peptide synthesis

Chain A, chain B, peptide (557–580)mut and peptide (591–607)mut were synthesized by the solid-phase method, with standard Fmoc procedure on a fully automated synthesizer. Appropriate Fmoc-amino acid derivatives were employed and a Rink Amide MBHA resin (0.28 mmol g^{-1} substitution; 42 μmol scale) was used as solid support, as it releases C-terminally amidated peptides upon acid treatment. All couplings were performed twice for 20 min using an excess (4 equiv) of each amino acid derivative. Amino acids were activated in situ by the standard HOBt/PyBOP/DIPEA protocol. Fmoc deprotection was performed with 20 % piperidine in DMF. Peptides were N-terminally acetylated by treatment with a solution of 4.7 % acetic anhydride and 4 % pyridine in DMF. Peptide cleavage from the solid support and simultaneous removal of all protecting groups was carried out by suspending the fully protected compound resins in TFA/H_2O/TIS (97:2:1) for 3 h followed by filtration. Solutions were then concentrated and crude products isolated by precipitation into cold diethyl ether. Each peptide chain was fully reduced with a 20 mM aqueous solution of TCEP-HCl for 20 min at room temperature.

Chain A: $[M + H]^+$ calculated 2,630.18 *m*/*z*

Chain B: $[M + H]^+$ calculated 1,969.93 *m*/*z*

Synthesis of HER2-DIVMP

Strategy A

In the first oxidation protocol (strategy A), we used chain A with Cys565 and Cys574 as free thiols; the counterpart chain B was instead used after activation of the Cys601 thiol function with 2,2-dithiobis(5-nitropyridine) (DTNP). This group on Cys601 was introduced as follows: DTNP (3–5 equiv) was dissolved in the minimum amount of acetic acid/water (3:1), then 1 equiv of chain B, dissolved in the same solvent system, was added and left under stirring (Rabanal et al. 1996).

The reaction was monitored by RP-HPLC and, at completion after 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 to remove (in the form of a yellow powder) the excess of DTNP which is insoluble in the aqueous solution. The desired compound was analyzed and identified by RP-HPLC and mass spectrometry, respectively.

The subsequent oxidation reaction was performed by mixing 1 equiv of chain B[Cys601(p-Npys)] (3-nitro-2-pyridinesulfenyl) in ammonium acetate buffer (1 M; pH 5.9) with 1.0 equiv of chain A previously dissolved in the minimum volume of the same buffer. The final concentration of the peptides was 0.62×10^{-4} M. The reaction was left at room temperature overnight and was easily monitored by the intense yellow coloration due to the release of 5-nitro-2-pyridinethiol. The course of the reaction was monitored by LC–MS analysis.

Strategy B

In the second oxidation protocol (strategy B), both peptide chains A and B were used in the free thiol form. For this reaction, 1 equiv of each peptide was dissolved in ammonium bicarbonate aqueous solution (0.1 M; pH 7–8) at the final concentration of 0.62×10^{-4} M. The reaction mixture was stirred at room temperature for 12 h and monitored by LC–MS analysis. The correct product was purified by semi-preparative HPLC purification and was characterized by mass spectrometry.

Titration of chain A with increasing amounts of chain B

0.50 mg (0.19 μmol) of chain A was dissolved in 8 mL of 0.1 M ammonium bicarbonate (pH 7–8), while 0.40 mg (0.20 μmol) of chain B was dissolved in 500 μL of water. Increasing amounts of chain B solution (125, 125, 250 μL) were added every 3 h, achieving the following mol/mol ratios of chain B/chain A: 0.25, 0.50, 1.0.

Synthesis of HER2-DIVMPmut

Strategy B was also employed to try to assemble the molecules HER2-DIVMP[(557-580)mut] and HER2-DIVMP[(591-607)mut]. For this purpose the separate chains were mixed in a 1:1 ratio at 0.62×10^{-4} M in ammonium bicarbonate pH 7.0–8.0 and the reaction was left at room temperature overnight. Aliquots were analyzed by LC–MS over time as described above.

Product quantification by HPLC peak integrals

Products were quantified by area integration of HPLC analytical peaks and normalization of the obtained values for the different molar absorptivity of Trp- and/or Tyr/Trp-containing peptides (Pace et al. 1995). For each peptide we took into account contributions from tyrosine and tryptophan present in the primary structure; in particular the average molar absorption values (ε_{280}) amount to 1.5 and 5.5 mM^{-1} cm^{-1} for Tyr and Trp, respectively (Pace et al. 1995). Therefore, molar absorption coefficients used were:

- ε_{280} (Chain A homodimer) = 3 mM^{-1} cm^{-1}
- ε_{280} (Chain B) = 5.5 mM^{-1} cm^{-1}
- ε_{280} (Chain B homodimer) = 11 mM^{-1} cm^{-1}
- ε_{280} (HER2-DIVMP) = 7 mM^{-1} cm^{-1}

Characterization of the assembled polypeptides by trypsin digestion and mass spectrometric analysis of resulting fragments

In order to characterize the pattern of disulfide bonds in the isolated HER2-DIVMP, HER2-DIVMP[(557-580)mut] and HER2-DIVMP[(591-607)mut], we treated the polypeptides with trypsin and analyzed the resulting fragments by LC–MS. Typically, 500 μg of purified product was dissolved in 500 μL of 50 mM Tris–HCl, pH 7.5 and was treated with 5 μg of porcine trypsin for 24 h at 37 °C (1:100 w/w). Samples were finally diluted with H_2O, 0.1 % TFA to block the enzyme and analyzed by LC–MS using a Biobasic C18 column 50 × 2 mm ID (flow rate 0.25 mL min^{-1}). The mass spectrometer was operated in full scan between 200 and 2,000 *m/z*, in positive mode. Gradients from 5 to 55 % solvent B (acetonitrile with added 0.05 % TFA; solvent A was water with 0.08 % TFA) were used to elute peptide fragments. To determine the relative amounts of correctly folded HER2-DIVMP, HER2-DIVMP[(557-580)mut], HER2-DIVMP[(591-607)mut] and the corresponding misfolded variants, tryptic fragments were identified by their MW and quantified by integrating peaks deriving from extracted ion chromatograms. Most intense multiply charged ions were selected for each fragment being quantified, and areas were

integrated. Peak areas of fragments coming from the same product were summed and the relative content of the product in the mixture was determined as percentage. We assumed that, by virtue of the quite similar aminoacidic compositions, ionizability by ESI was similar between the different tryptic peptides resulting from the differently assembled heterodimers.

Results and discussion

Synthesis and characterization of HER2-DIVMP

In order to conveniently prepare HER2-DIVMP in higher yields and in less time, we studied the synthetic process using two different co-oxidation protocols, both eliminating the iodine-promoted oxidation step previously reported (Monfregola et al. 2009).

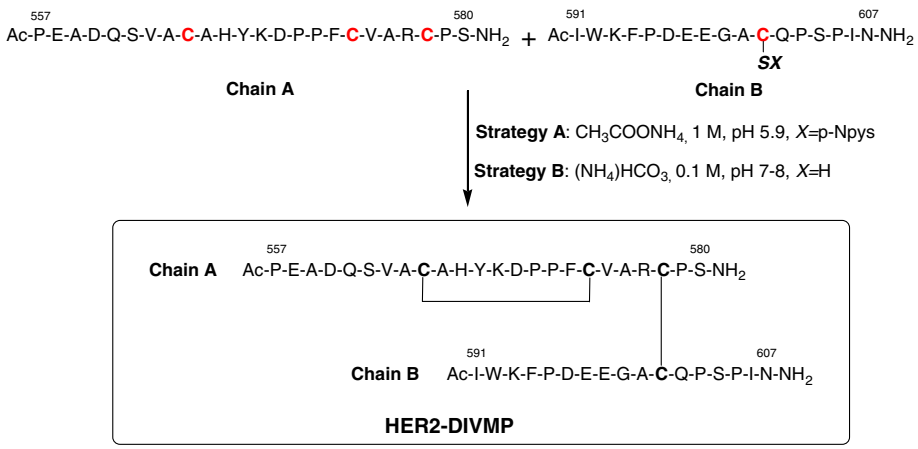
The first synthetic route (strategy A, outlined in Scheme 1) consisted in synthesizing both peptide chains, A and B, protected on cysteine residues with the same acid labile group. In a subsequent step, the Cys601 thiol function of chain B was pre-activated with 2,2-dithiobis(5-nitropyridine) (DTNP), and, after purification, was mixed with 1 equivalent of chain A, in the free thiol form dissolved in 1 M ammonium acetate aqueous solution, pH 5.9 (final concentration 0.62×10^{-4} M) (Rabanal et al. 1996).

As shown in Fig. 1, upper panel and also Figure S1a-f of Supplementary Material, the main products in the mixture were the unreacted chain B together with a high proportion of homodimeric chain B. Strikingly, the homodimers of the multicysteine-containing chain A were highly unfavored, or were likely distributed among the several combinations of dimers and oligomers (see also Scheme 2), as they were not detected in the reaction mixture.

We next explored the second co-oxidation protocol, whereby both peptide chains in the free thiol form were employed under native-like conditions (strategy B, outlined in Scheme 1). In this case, one equivalent of each peptide chain was dissolved in 0.1 M ammonium bicarbonate aqueous solution (final concentration 0.62×10^{-4} M, pH = 7–8), and the mixture was kept under stirring for one night at room temperature. As can be seen in Fig. 1 lower panel and also in Figure S2a-e of Supplementary Material, the product at 8.91 min with the MW of HER2-DIVMP formed in much higher amounts, while the chain B homodimer was still a quite abundant by-product. Under these reaction conditions, chain B was much more reactive and after 8 h nearly disappeared. Remarkably, the unreacted chain A was detected as the oxidizes species (Figure S2c of Supplementary Material). By integration of HPLC peaks and normalization for the tryptophan and tyrosine content (see "Materials and methods"), the following percentages were calculated: 8 % for the correct heterodimer and 25 % for the chain B homodimer, under strategy A; 39 % for the correct heterodimer and 46 % for the chain B homodimer, under strategy B.

It must be underscored that two additional mispaired heterodimers with the same MW of HER2-DIVMP can originate from the assembling of chain A and chain B (depicted in Scheme 2 and named MIS-1 and MIS-2). Therefore, to assess that the product eluting at 8.9 min (from both synthetic strategies A and B) was the correctly folded HER2-DIVMP domain, this product was isolated, digested with trypsin (see "Materials and Methods" section), and the resulting fragments were identified by LC–MS.

As shown in Fig. 2, only the fragments expected from the proteolysis of the correctly assembled HER2-DIVMP were observed, whereas no peaks imputable to fragments from MIS-1 and MIS-2 were detected (see also Scheme S1, Table S1 and Figure S3 of Supplementary Material), indicating that chains A and B efficiently and selectively recognize and properly link each other through the two disulphide bridges. Especially diagnostics for identifying the correctly assembled polypeptide were the fragment



Scheme 1 Synthesis of HER2-DIVMP: schematic view of strategy A and B

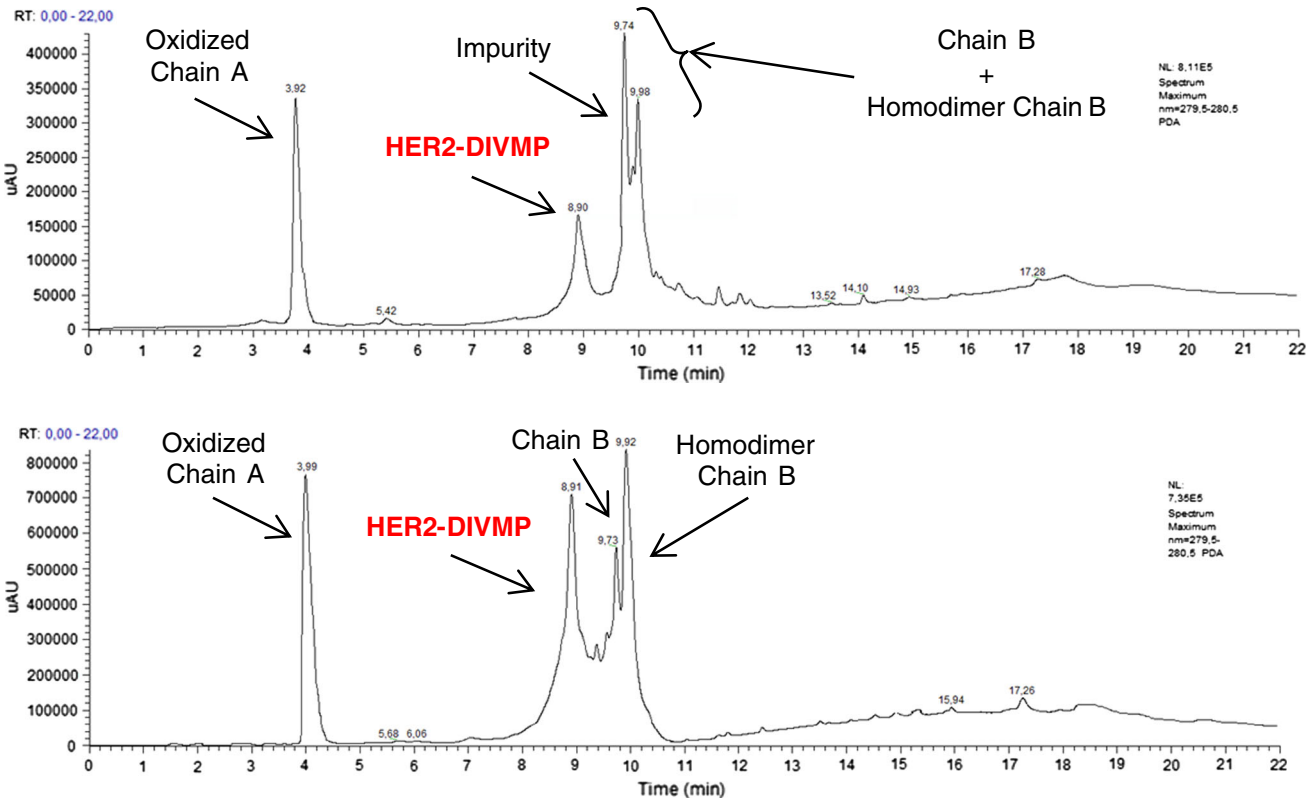
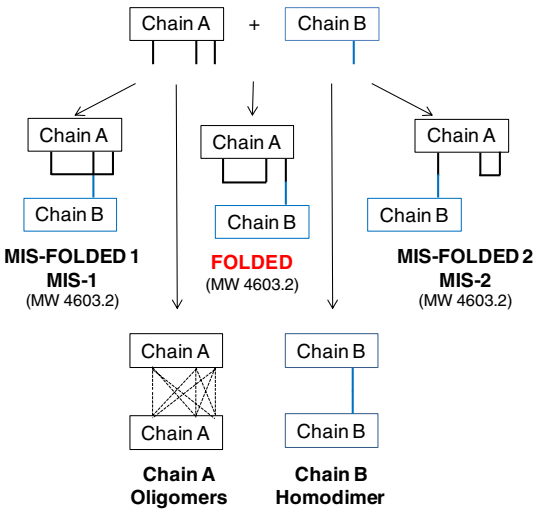


Fig. 1 HPLC profile of HER2-DIVMP crude product obtained under strategy A (*upper panel*) and strategy B (*lower panel*) conditions



Scheme 2 Possible combinations of disulfide bridges upon assembly of chain A and chain B

[578–580] + [594–607], detected as doubly charged ion *m/z* 903.2, the fragment [578–580] + [591–607], also detected as doubly charged ion *m/z* 1,137.9, and the fragment [557–577] containing the oxidized cysteins and detected as doubly (*m/z* 1,172.9) and triply charged ion (*m/z* 782.5) (see Supplementary Material Figure S3a,c, e).

Altogether, the data show that both strategies A and B lead to a relevant conversion of reactants to final products compared to the site-directed chemical oxidation, and by far, they are also much more convenient, requiring less synthetic and purification steps. By comparing the ratio HER2-DIVMP:chain B homodimer, strategy B (1.0:1.2) resulted more convenient than strategy A (1.0:3.2) and more straightforward, requiring no chemical manipulation of the thiol groups.

Folding process analysis

To further optimize the conversion of HER2-DIVMP, trying at the same time to suppress the formation of the chain B homodimer, we investigated the folding reaction by performing a time course analysis of the two chains mixed in equimolar ratio. The formation of HER2-DIVMP was monitored over 16 h, looking at the appearance of its quadruply charged ion peak (*m/z* 1,151.8, see Supplementary Material Figure S4A), the disappearance of chain B doubly charged monoisotopic ion peak (*m/z* 986.3, see Supplementary Material Figure S4B) and the disappearance of chain A triply charged ion peak (*m/z* 878.0, see Supplementary Material Figure S4C). Also the formation of the oxidized cyclic chain A was monitored over the same time window, following the appearance of its triply

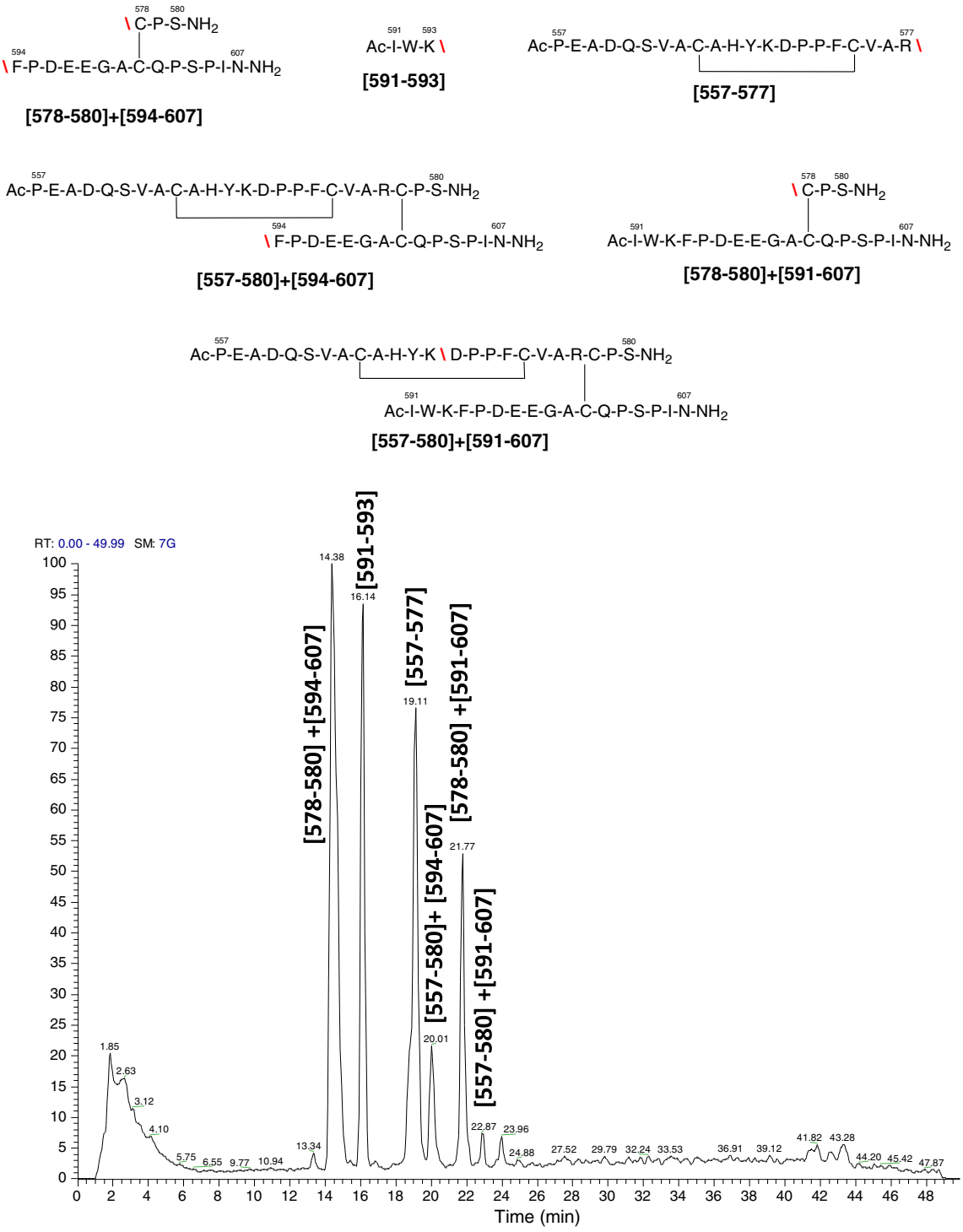


Fig. 2 LC–MS profile of tryptic peptide mixture obtained after digestion of wild type correctly folded HER2-DIVMP. All fragments expected on the basis of the right pattern of disulfide bonds were observed, together with a very small proportion of the uncut product. The peptide bond between Lys569 and Asp570 was expectedly not split (http://web.expasy.org/peptide_cutter/peptidecutter_enzymes.html), likely also due to poor accessibility of trypsin to peptide bonds in small cyclic peptides

charged ion peak (*m/z* 877.3, see Supplementary Material Figure S4C) over the corresponding fully reduced chain A ion peak (*m/z* 878.0). Both reaction progressions are reported in Fig. 3 left panel, while in Fig. 3 right panel are reported the disappearance of the two chains and the formation of chain B homodimer, as monitored by its triply charged ion peak (*m/z* 1,316.2, see Supplementary Material Figure S4D). As shown, a small amount of heterodimer

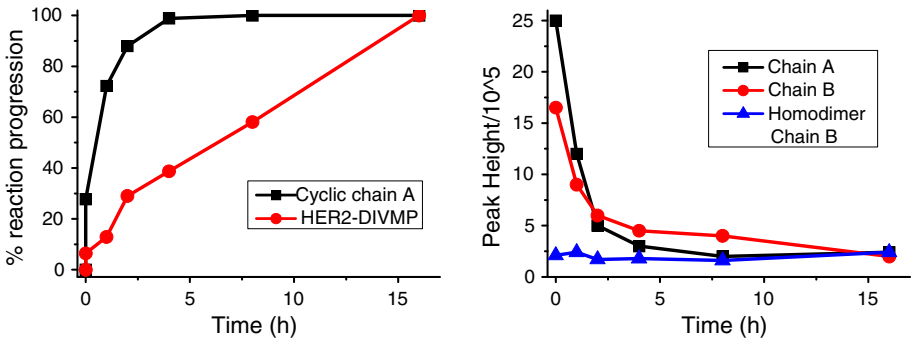


Fig. 3 Progression of the formation of HER2-DIVMP (expressed as percentage of reaction) and of oxidized cyclic chain A (*left panel*) over time; consumption of chain A and chain B (expressed as absolute value of the peak height changes), and formation of chain B homodimer over time (*right panel*)

was already formed after 1 h, and its appearance was paralleled by the loss of both fully reduced chain A and chain B.

Notably, the relative concentration of the two chains declined much more rapidly compared to the appearance of HER2-DIVMP, and reached the lowest value after 4 h, when the final product was only partially formed. We also observed that chain A consumption was paralleled by the occurrence of its oxidized cyclic variant, whose relative amount, compared to the reduced form, reached a plateau at 4 h and remained constant up to 16 h. This observation, together with the decrease of the absolute concentration of oxidized cyclic chain A, suggests that chain A rapidly undergoes oxidation and that this is the precursor species for forming, in a slower step, the heterodimeric final product. Notably, though the HER2-DIVMP curve appears to have not yet reached a plateau, both reactants are nearly consumed, prompting that chain A likely also forms some homo-oligomeric species which are not detected in our analysis. Moreover, chain B homodimer was already formed at the beginning of the folding reaction (Fig. 3, right panel), and remained constant over the time, indicating that it is either immediately generated in solution or already present in the starting material. However, it is seemingly not reactive toward other species in solution, therefore, while its presence contributes to lower the conversion of the final product, it does not interfere apparently with the reactions leading to HER2-DIVMP formation.

We next investigated the folding reaction by performing a titration of chain A with increasing amounts of chain B. Namely, chain A was dissolved in 0.1 M ammonium bicarbonate (pH 7–8) at 0.62×10^{-4} M, then increasing amounts of chain B were added every 3 h, achieving the following ratios of chain B/chain A: 0.25, 0.5, 1.0. Samples for LC–MS analysis were taken out from the mixture before the next peptide addition, and the solutions were then stirred overnight at room temperature. As shown in Fig. 4 (left panel), the conversion of the heterodimer reached a plateau when 0.25 equiv of chain B were allowed to react with 1 equivalent of the other chain, whereas the amount of chain B homodimer constantly increased

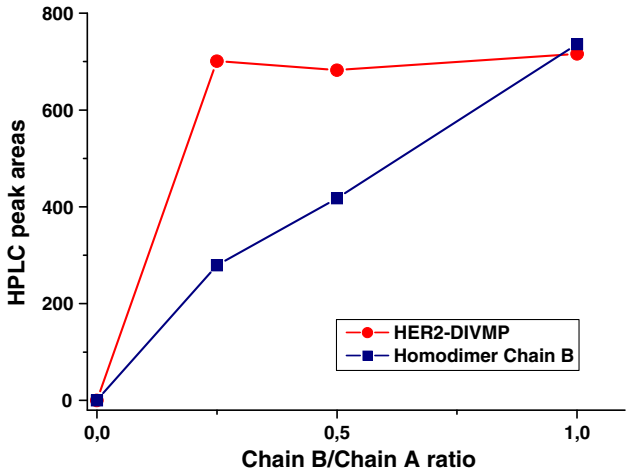


Fig. 4 HPLC peak areas of HER2-DIVMP (*red line*) and chain B homodimer (591–607) (*blue line*), respectively, as function of the different ratio values chain B/chain A (color figure online)

(Fig. 4, right panel). This further confirms our hypotheses that the chain B homodimer is either partially present in the starting material or it forms very quickly and that it is not converted to the heterodimer. Its formation is also likely in competition with chain B incorporation within the heterodimer, indeed the amount of folded heterodimer do not increase for chain B/chain A ratios beyond 0.2.

Synthesis and characterization of HER2-DIVMP[(557-580)mut] and HER2-DIVMP[(591-607)mut]

To further investigate which residues drive the folding process, two receptor model systems mutated in their primary structure on several positions were designed and allowed to self-assemble under strategy B conditions. They were prepared by scrambling specific residues within both chain A and chain B, so that the resulting mutated systems had both the same molecular mass as the native molecule. Specifically, HER2-DIVMP[(557-580)mut] was prepared by exchanging selected peptide dyads in chain A while leaving unaltered chain B. As shown in Fig. 5, the dyad

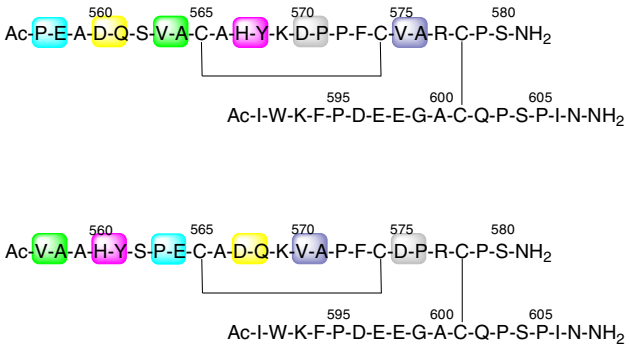


Fig. 5 Amino acid sequence of HER2-DIVMP (*upper panel*) and of HER2-DIVMP[(557-580)mut] (*lower panel*). Exchanged residues are highlighted with different *colors* (color figure online)

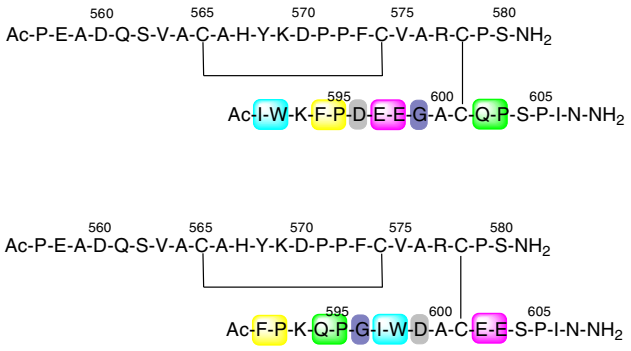


Fig. 6 Amino acid sequence of HER2-DIVMP (*upper panel*) and of HER2-DIVMP[(591-607)mut] (*lower panel*). Exchanged residues are highlighted with different *colors* (color figure online)

P557E558 was exchanged with V563A564; H567Y568 was exchanged with D560Q561; D570P571 was exchanged with V575A576. It must be underscored that these changes were chosen to facilitate the subsequent characterization by trypsin digestion, since Lys569 and Arg577 were kept on original positions and fragments resulting from enzyme proteolysis of the correctly assembled molecule would have the same molecular weight as in the wild type HER2-DIVMP.

In the second system HER2-DIVMP[(591-607)mut], chain A was left unaltered, while within chain B, I591W592 was moved to positions 597-598, F594P595 to positions 591–592, E597E598 to positions 602–603, and Q602P603 to positions 594–595. D596 was finally exchanged with G599 (Fig. 6). Again, to facilitate the characterization of fragments deriving from trypsin splitting, Lys593 was left on the original position.

As shown in Fig. 7 (upper panel), the co-oxidation reaction between peptide (557–580)mut and chain B still produced, beside a relevant amount of chain B homodimer, a heterodimeric regioisomer characterized by the same molecular weight of HER2-DIVMP (4,601.2 amu). In Figure S5c-e of the Supplementary Material mass spectra of the most relevant peaks are reported.

This product was isolated by HPLC purification and then digested by trypsin to identify the pattern of disulfide bridges. Notably, mass spectrometric analysis of the tryptic fragments revealed that the product was not homogeneous and that three main species were clearly identified: one was the correctly folded mutated product (44 %), whereas the others were the two related misfolded variants (MIS-1 and MIS-2, see Scheme 2), which have the same molecular weight. MIS-1 was identified by the following diagnostic fragments: disulfide linked fragments $[557–569]_{mut}$ + $[578–580]_{mut}$, $[570–577]_{mut}$ +[591–607], and [570–577] + [594–607] (see Scheme S2, Table S2b and Figure S7c, g, l of the Supplementary Material). In a similar manner, MIS-2, present for about 34 %, was identified by the occurrence of the diagnostic fragments [570–580], [557–569] + $[594–607]_{mut}$ and [557–569] + [591–607] (see Scheme S3, Table S2c and Figure S7e, f, i of the Supplementary Material).

When we co-oxidized the wild type chain A and the chain (591–607)mut, a relevant amount of the peptide (591–607)mut dimer still formed (Fig. 7, lower panel), though it was largely altered in its sequence. In Figure S6c-e of the Supplementary Material mass spectra of the most relevant peaks are reported. Nonetheless, an intense peak appeared at about the retention time of HER2-DIVMP and with the same molecular weight. The material eluting at about 9 min was collected in one single fraction and, after identification by trypsin digestion and LC–MS analysis, we found it was unequivocally composed of the correctly assembled (>98 %) mutated heterodimer. Indeed, only the following diagnostic fragments were found: the disulfide linked fragments [578–580] + $[594–607]_{mut}$ and $[557–577]_{mut}$ (see Scheme S1, Table S3a and Figure S8d,e of Supplementary Material).

The shoulder at R_t 8.98 min in Fig. 7, lower panel, is likely due to a conformationally different mutated heterodimer, which has the same primary structure and the same pattern of disulphide bridges, but can have a slightly different chromatographic behavior.

These findings strongly suggested that sequence alterations in chain B, did not influence the folding process, nor its tendency to dimerize. Noticeably, no homodimers of the native chain A were observed, nor oligomers containing multiple copies of either chain A or chain B.

Altogether, these data showed that a major impairment of the folding process was induced by scrambling chain A (less than 50 % of the correctly assembled heterodimer was obtained, see Table 1), whereas scrambling of chain B very poorly affected the self-assembling process.

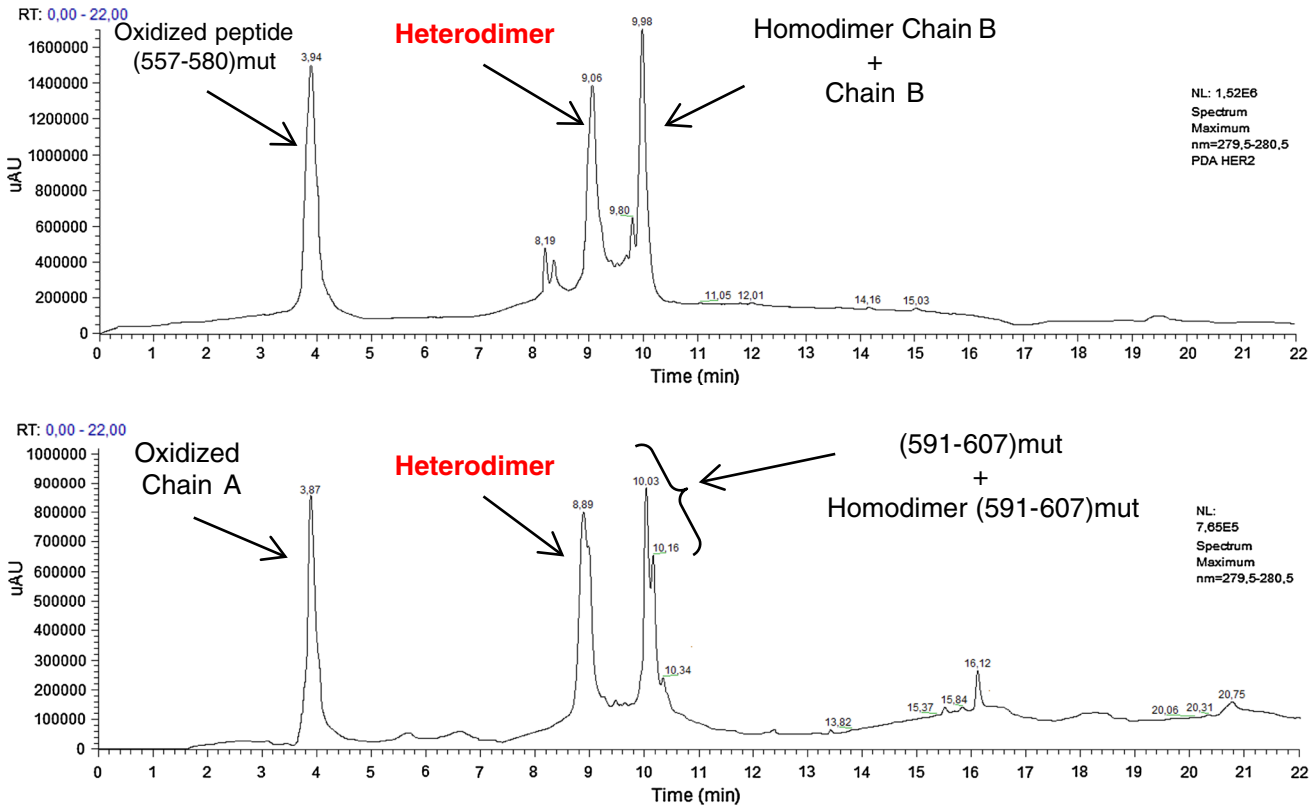


Fig. 7 HPLC profile of HER2-DIVMP[(557-580)mut] (*upper panel*) and HER2-DIVMP[(591-607)mut] (*lower panel*) crude products obtained under strategy B conditions

Table 1 Relative percentages of folded and misfolded isomers of the HER2-DIVMP (557-580)mut variant

Isomer	%
Folded	44.5
MIS-1	21.7
MIS-2	33.8

Conclusion

In summary, our studies demonstrated that naturally occurring peptide conformations, under the employed native-like reaction conditions (air co-oxidation in a buffered aqueous medium), drive the right sulfhydryl pairings, thus allowing a simplified and convenient synthetic procedure of HER2-DIVMP. Moreover, by studying this reaction with scrambled chain A and chain B, we have also investigated the mechanism of folding of the heterodimeric polypeptide. We indeed showed that the folding is essentially driven by chain A, which has a poor capacity to self-recognize and a strong tendency to form the internal bridge between Cys565 and Cys574. Therefore, the slow step is the formation of the intermolecular disulfide bond, which seemingly only forms when the intramolecular bridge on chain A is in place. On the other hand, it seems that chain B has a strong tendency to homodimerize, even when largely altered in its primary structure, and the homodimer is no longer reactive toward chain A.

In conclusion, the ability of HER2-DIVMP to self-assemble and fold under native conditions, further validates its design and provides a simpler and valuable alternative to its preparation by site-directed chemical approaches. This synthetic module, which is an effective mimetic of HER2, is a useful tool and a valuable scaffold to further study the domain IV of HER2 receptor and for the design of ligands for its selective targeting.

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Conflict of interest The authors declare that they have no conflict of interest.

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