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Directed Evolution of Scanning Unnatural Protease Resistant (SUPR) Peptides for In Vivo Applications

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Abstract

Peptides typically have poor biostabilities and natural sequences cannot easily be converted into drug-like molecules without extensive medicinal chemistry. We have adapted mRNA display to evolve highly stable cyclic peptides while preserving target affinity. To do this, we incorporated an unnatural 21st amino acid in an mRNA display library which was subjected to proteolysis prior to selection for function. The resulting “SUPR peptide” (Scanning Unnatural Protease Resistant) showed ~500-fold improvement in serum stability ($t_{1/2}$ = 160 hours) and up to 3,700-fold improvement in protease resistance versus the parent sequence. We extended this approach by carrying out SUPR peptide selections against Her2-positive cells in culture. The resulting SUPR4 peptide showed low nanomolar affinity toward Her2, excellent specificity, and selective tumor uptake *in vivo*. These results argue that this is a general method to design potent and stable peptides for *in vivo* imaging and therapy.

Graphical abstract

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Two-stage directed evolution of mRNA Display libraries containing unnatural N-methyl amino acids results in highly stable cyclic peptides with antibody-like affinities and specificities. This technique was employed to select a nanomolar affinity unnatural cyclic peptide for the Her2 receptor which showed rapid tumor uptake and minimal non-specific background in mouse models of Her2-positive breast cancer.

Keywords

Directed Evolution; mRNA Display; Peptides; Unnatural Amino Acids

Introduction

Although peptides are ubiquitous and essential in biological systems, they are generally considered to be poor leads for developing new drugs. The rationale for neglecting peptides stems from two major problems: 1) peptides do not typically possess the chemical properties of drugs (e.g. stability and affinity)^[1] and 2) there is no general design algorithm to convert natural peptide sequences into more drug-like molecules while simultaneously maintaining their original function. Peptides typically fail as imaging agents for many of the same reasons, particularly short serum half-lives (seconds to minutes) that are incompatible with selective uptake in tissues of interest^[2]. This is unfortunate, as theoretical models suggest that stable, low molecular weight peptides with antibody-like affinities would exhibit highly favorable imaging properties^[3].

Here, we sought to develop a general method to select unnatural peptides that possessed both protease resistance and high affinity target binding. To do this, we adapted mRNA display, an *in vitro* directed peptide and protein evolution system^[4], to include a 21st amino acid^[5] in a covalently cyclized peptide library^[6]. We chose an *N*-methyl residue for our expanded genetic code because 1) many *N*-methyl residues are compatible with ribosomal peptide synthesis^[7], and 2) *N*-methyl residues are well-known to improve protease resistance^[7b, 8]. However, while *N*-methyl residues can dramatically improve peptide stability, they often alter the specificity and function of the original peptide^[8]. Indeed, loss of selectivity and binding affinity appears to be a general theme for many types of chemical modifications that improve peptide stability. To optimize both of these properties simultaneously, we designed an *in vitro* selection/evolution process where unnatural amino acid incorporation was coupled with a two-step selection—the first for proteolytic stability, the second for target affinity (Figure 1 a, b, c).

Results and Discussion

Our model peptide was a sequence termed cycGiBP which was previously selected to bind the signaling molecule Gαi1*GDP (Figure 1a)^[9]. Starting from this sequence, we created a focused library where each “wild type” position was mutagenized with nucleotides for the UAG stop codon to code for a 21st amino acid, *N*-methyl alanine. This approach allowed us to select the desired average number of *N*-methyl residues while allowing for substitution of wild-type positions with other natural amino acids (Figure 1a).

In each round, the library was first subjected to proteolysis followed by a binding selection (see Experimental Section). After 5 rounds, we observed a single dominant sequence that we denoted the Gα SUPR peptide for scanning unnatural protease resistant peptide (Figure 1c, d). The Gα SUPR sequence retains the original high affinity ($K_D = 60$ nM) of the parent cycGiBP sequence (Figure S1A). The pattern of G protein isoform selectivity remained essentially unaltered, although Gα SUPR showed significantly lower binding to Gαi2 and Gαs than cycGiBP (Figure S1B). Gα SUPR showed slightly higher binding to the GTP-bound form of Gαi1 compared to cycGiBP (Figure S1C) although, again, the pattern of state selectivity remained intact^[9].

Several features of the selected Gα SUPR peptide bear comment. First, only two out of the original ten residues from cycGiBP (Y5 and E6) were preserved in the final SUPR sequence. These positions were highly conserved in the original cycGiBP selection and were heavily favored in this selection due to low diversity at positions 5 and 6 (Figure 1a). Second, the protease resistant SUPR sequence has two unnatural residues, spaced evenly throughout the molecule at positions 4 and 8, consistent with the idea that each residue provides a window of protection to adjacent positions (Figure 1c)^[8a]. Finally, mutations seem to have favored residues with increased steric bulk at all positions except position 11 (T to S) (Figure 1d).

The Gα SUPR peptide sequence has numerous predicted cleavage sites for both chymotrypsin^[10] and proteinase K^[10] (Figure 2a). Remarkably, the Gα SUPR sequence shows ~400-fold improvement in chymotrypsin resistance and >3,700-fold improvement in proteinase K resistance compared with the parent cycGiBP peptide (Figure 2b,c). Preliminary kinetic analysis suggests that these enhancements occur through a combination of changes in both k_{cat} and K_M (Figure S2), a phenomenon observed in previous selections for protease resistance^[11]. Further, both cyclization (structure) and unnatural residues (sequence) play an important role in this stabilization (Figure 2b, c).

The combined selection versus a variety of different proteases (proteinase K, derived from fungus; trypsin, chymotrypsin and aminopeptidase from the gut) appears to have resulted in a SUPR peptide that is generally resistant to proteolytic and hydrolytic degradation. This can be seen most dramatically by the increase in human serum stability (Figure 2d), although no serum proteases were used in the selection. In this context, the Gα SUPR peptide gave a half-life of ~9,700 minutes at 37°C (~6.7 days), a >1,000-fold improvement over the natural linear peptide sequence. Similarly, the SUPR sequence has a half-life of ~900 minutes in liver microsomes bearing oxidative cytochrome P450 enzymes, almost a 10-fold improvement over cycGiBP despite never having exposed the library to these enzymes during the selection (Figure 2e). Finally, we observed an >35-fold improvement in *in vivo* stability in the mouse for the Gα SUPR peptide versus the parent cycGiBP compound (Figure 2f) with predominantly renal clearance of the intact SUPR peptide (data not shown). Taken together, these biochemical data suggest that SUPR peptides can survive intact for substantial periods *in vivo*.

Generally, step-wise modifications that improve peptide stability result in loss of function or altered specificity^[8]. Here, removal of either the *N*-methyl at position 4 or position 8 (or both), results in peptides that are orders of magnitude less stable than the natural parental

sequence, cycGiBP (Table 1). This argues that the Ga SUPR sequence could not have been discovered via systematic stepwise optimization of the starting peptide. We also note that the SUPR sequence contains changes at 80% (8 out of 10) of the natural amino acid positions, indicating that rational optimization would have required a leap of ~8 independent steps to achieve the combined stability and function of the evolved Ga SUPR peptide.

We sought to extend SUPR peptide selections to generate stable peptides that target the pro-oncogenic cell surface receptor Her2. Since Her2 is a large (>130 kDa) membrane protein, we elected to carry out selections against SK-BR-3 cells ($2-6 \times 10^6$ Her2 receptors per cell^[12]) to ensure that the target was present at high concentration in a natural biological context. To bias the selection toward peptides that bound the extracellular domain of the receptor, we used the CDRH3 loops of Her2-targeting Trastuzumab (Herceptin) and Pertuzumab (Perjeta) therapeutic antibodies as a starting point for library design (Figure 3a). In contrast to SUPR peptide display selections against Gai1, we chose *N*-methyl norvaline as the 21st amino acid because of its high incorporation efficiency and hydrophobic side chain that could contribute positively to binding. After translation and cyclization^[6], the libraries were incubated with SK-BR-3 cells in culture for 1 hour at room temperature followed by washing and library amplification. After four rounds of selection, the final pool was characterized by next-generation sequencing.

The final pool of sequences showed a high degree of conservation (Figure 3b). A significant majority of clones showed incorporation of *N*-methyl norvaline at position 5 along with the LY(D/E)(D/V) motif spanning positions 6-9. We chose the representative clone MVCvvLYDDK (Peptide D, v=*N*-methyl norvaline) for further analysis. To prevent confounding effects of oxidation and intramolecular disulfide bond formation, the *N*-terminal methionine residue was removed and the cysteine at position 3 was converted to a serine. The resulting peptide, designated SUPR4, was synthesized with a C-terminal propargylglycine for conjugation to Cy5 and biotin azides through the copper-catalyzed azide-alkyne cycloaddition reaction (Figure 4a).

SUPR4 bound to the recombinant Her2 ectodomain *in vitro* with an apparent $K_D = 76 \pm 30$ nM (Figure 4b) and to Her2-overexpressing BT-474 cells with an apparent $K_D = 13 \pm 2$ nM (Figure 4c). This affinity is comparable to that of Herceptin ($K_D \sim 10$ nM)^[16] and significantly higher than peptidomimetics previously obtained by rational re-design of Her2 antibody CDRH3 loops ($K_D > 300$ nM)^[13, 17]. SUPR4-Cy5 bound selectively to Her2-positive BT-474, SKOV3, and SK-BR-3 cells with minimal binding to Her2-negative MDA-MB-231 cells by flow cytometry (Figure 4d). This pattern of selectivity was analogous to that observed with an anti-Her2 monoclonal antibody and is consistent with cell line-specific Her2 expression (Figure S3). SUPR4-Biotin shows increased binding to recombinant Her2 ectodomain relative to the EGFR ectodomain by ELISA (Figure 4e) and binding of SUPR4-Cy5 to SKOV3 cells was significantly attenuated when Her2-expression was knocked down (Figure 4f), further confirming Her2 selectivity. The *ex vivo* serum half-life of the selected Peptide D sequence was found to be approximately 5600 minutes (Figure 4g), consistent with the serum stability observed for the Ga SUPR peptide.

We sought to determine if the favorable affinity, selectivity, and stability of SUPR peptides would make them suitable for *in vivo* imaging applications. Injection of SUPR4-Cy5 in mice bearing subcutaneous Her2-positive (SKOv3) or Her2-negative (MDA-MB-231) tumors revealed substantial tumor uptake within 4 hours only in the Her2-positive tumors (Figure 5a,b). Fluorescent signal was retained in SKOv3 tumors after 24 hours (Figure 5c) with very little signal observable in non-tumor tissue. As seen in Figure 5d, the SKOv3 tumor showed significantly higher fluorescence relative to the MDA-MB-231 tumor after 48 hours indicating Her2-selective tumor uptake and retention. Very little fluorescent signal is observed in non-tumor tissues with the exception of the gastrointestinal (GI) tract and the kidney (minor). Notably, the liver shows low fluorescence at 48 hours indicating that SUPR peptides show low hepatic uptake and retention, which distinguishes them from most Her2 imaging agents developed to date^[18].

The SUPR peptide selections described here provide compelling evidence that the combination of *N*-methyl amino acids and cyclization in conjunction with selections for protease resistance yields highly stable compounds with antibody-like affinities and selectivities. While our prior work demonstrated that a dual selection for protease resistance and binding gave serum stable natural peptide sequences^[11], incorporation of an appropriate 21st amino acid results in peptides with substantially higher proteolytic stabilities that retain high-affinity target binding *in vitro* and *in vivo*. Moreover, SUPR peptide design is general and does not require specific structures or even *a priori* knowledge of the target structure in order to work. Finally, SUPR peptides demonstrate receptor-dependent tumor uptake with minimal non-specific background and rapid systemic clearance supporting their use as scaffolds for targeted molecular imaging and therapy^[3].

Experimental Section

Animal procedures were carried out with approval of MD Anderson Cancer Center's Institutional Animal Care and Use Committee.

Cell Culture

All cell lines were cultured in 5% carbon dioxide and 95% humidified air at 37°C. MDA-MB-231 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin. BT474 cells were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) high glucose media supplemented with 10% heat inactivated fetal bovine serum, 0.0036 mg/mL insulin, and 1% penicillin-streptomycin. SKOv3 cells were grown in McCoy's 5A Media supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin-streptomycin. Unless otherwise noted, SK-BR cells were grown in DMEM with 10% FBS.

Synthesis of *N*-methyl alanine-tRNA (NMA-tRNA) and *N*-methyl norvaline-tRNA (NMNVA-tRNA)

The syntheses of *N*-methyl, *N*-nitroveratrylcarbonyl alanine cyanomethyl ester (NVOC-NMA-CME) and *N*-methyl, *N*-nitroveratrylcarbonyl norvaline cyanomethyl ester (NVOC-NMNVA-CME) were carried out according to previously published protocols^[9]. The final

products were purified by silica gel chromatography in 3:1 EtOAc/hexanes. Product identity was confirmed by ESI-MS. Yield (NVOC-NMA-CME) = 187.5 mg (24%); (NVOC-NMNVA-CME) = 154 mg (20%). Conjugation to pdCpA was carried out as described previously [9] and product identities confirmed by ESI-MS. Yield (NVOC-NMA-dCA) = 0.5 mg (2.5%); (NVOC-NMNVA-dCA) = 0.7 mg (3.5%). Following ligation to THG-73 tRNA [19], deprotection of the nitroveratryloxycarbonyl group was effected by photolysis with a xenon lamp equipped with a 315-nm cutoff filter and set to a constant 400 watts for 5 min. Following deprotection the NMA-tRNA or NMNVA-tRNA was immediately added to the translation reaction.

Gαi1 Expression and Immobilization

Gαi1 was cloned into vector pDW363 and expressed with a C-terminal BirA tag in 2L BL21 (DE3) culture under standard conditions^[20]. Fifty mL aliquots were spun down at 6,000 rpm at 4°C and the pellets were washed with ice cold PBS and stored at -80 deg;C. Prior to each round of selection, a pellet was lysed with 1 mL of BPER. One hundred twenty μL of NeutrAvidin agarose beads were used for immobilization (Life Technologies, Carlsbad, CA). Immobilization was performed in binding buffer (25 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween-20, 1 mM EDTA, 5 mM MgCl₂, 10 μM GDP, and 0.05% (w/v) bovine serum albumin) with 1 mM PMSF for 1 hr at 4°C. The remaining biotin binding sites were quenched by the addition of 1 mM biotin for 30 min. The beads were stored in binding buffer at 4°C for up to one week.

Synthesis of *N*-Methyl Scanning Library for Gαi1

Single-stranded DNA template was obtained from Integrated DNA Technologies (Coralville, IA). The sequence 5′ - GGG ACA ATT ACT ATT TAC AAT TAC AAT GWW KWM STR GTA KKA RTW KKW GKM GKR SWM SAA ATC TGG AAG TGG AAG TGG A -3′ was synthesized using machine mixing for variable positions (W = A or T, K = G or T, M = A or C, R = G or A, S = G or C). The double stranded library was amplified with 6 cycles of PCR using 1 pmol of PAGE gel purified single-stranded library. PCR was performed under standard conditions utilizing the primers Gen-FP (5′ -TAA TAC GAC TCA CTA TAG GGA CAA TTA CTA TTT ACA ATT ACA-3′) and the reverse primer (5′ -CCA CTT CCA CTT CCA GAT TT -3′). The Round 0 mRNA pool was generated by T7 runoff transcription and purified by urea-PAGE. The purified mRNA was ligated to pF30P (5′ -pdA21[C9]3dAdCdC-P; C9 - tri-(ethylene glycol) phosphate (Glen Research, Sterling, VA); P - puromycin (Glen Research)), via an oligonucleotide splint (5′ - TTT TTTTTTTTT TCC ACT TCC ACT -3′). Ligations were purified by urea-PAGE and quantified via absorbance at 260 nm.

Translation and Cyclization of Gα SUPR peptide libraries

Fifty pmol of purified mRNA-F30P template was translated in 150 μL of rabbit reticulocyte lysate with final concentrations of 100 mM KOAc and 0.5 mM Mg(OAc)₂. The translation reaction was supplemented with 20 μg of *N*-methyl alanine (NMA)-charged tRNA in 1 mM NaOAc (pH = 4.5) and [³⁵S]-methionine. After 45 min at 30 °C, KOAc and Mg(OAc)₂ were added to a final concentration of 600 and 50 mM, respectively followed by incubation at room temperature for 15 min. Following purification on dT-cellulose and ethanol precipitation, the resulting libraries were cyclized by the addition of 50 μL of DSG (1

mg/mL in DMF) in 150 μ L of 12.5 mM phosphate buffer (pH=8.0). The cyclization reaction proceeded for 1 hour and the fusions were re-purified by ethanol precipitation prior to selection.

G α SUPR peptide selection

Following ethanol precipitation of the DSG-treated fusions, the pellet was dissolved in 80 μ L of dH₂O and reverse transcribed with Superscript II and diluted to 150 μ L in 50mM potassium phosphate buffer (pH = 8.0). The Round 0 fusion library was digested using 1 mg of immobilized trypsin, 1 mg of immobilized chymotrypsin, 1 mg of immobilized proteinase K, and 1 mg of immobilized aminopeptidase for 20 min at room temperature. Subsequent rounds used immobilized chymotrypsin and proteinase K for proteolysis. Fusions were purified from immobilized proteases by spin filtration.

Digested fusions were diluted into 1.5 mL of binding buffer (25 mM HEPES-KOH (pH % 7.5), 150 mM NaCl, 0.05% (v/v) Tween-20, 1 mM EDTA, 5 mM MgCl₂, 10 μ M GDP, and 0.05% (w/v) bovine serum albumin) containing 20 μ L of G α i1-NeutrAvidin agarose. Binding was carried out at 4 °C for 1 h followed by filtration and washing of the resin. The library was eluted with 0.15% (w/v) SDS at RT and the SDS was removed from the sample using SDS-Out (Life Technologies, Carlsbad, CA). Following ethanol precipitation, the library was amplified by PCR until a band was observed on a 4% agarose gel (18–24 cycles).

The round 5 pool was amplified by PCR and was subcloned into the TOPO-TA vector (Invitrogen, Grand Island, NY) followed by transformation into TOP10 competent cells (Invitrogen, Grand Island, NY). Individual clones were sequenced (Laragen, Culver City, CA) and candidate peptide sequences selected for synthesis.

Peptide Synthesis

All solvents were purchased from Sigma Aldrich (Carlsbad, CA). Amino acids and activators were purchased from P3 Bio (Louisville, KY). Peptides were synthesized at 0.15 mmol scale using Rink Amide MBHA resin (P3 Bio, Louisville, KY). Synthesis was performed on an automated microwave peptide synthesizer from Biotage (Charlotte, NC). Prior to initiating synthesis, the resin was swelled for 10 minutes in DMF (Dimethylformamide), heated to 65 °C, and washed. Fmoc (Fluorenylmethyloxycarbamate) protecting groups were removed by two successive reactions at 50°C for three minutes in 20% 4-Methylpiperidine in DMF. Amino acid couplings were carried out with 5 equivalents of amino acid delivered at 0.4 M, 4.98 equivalents of HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) delivered at 0.5 M, and 10 equivalents of NMM (4-Methylmorpholine) delivered at 2 M. Couplings were carried out at 65 °C for 7 minutes.

Peptide Cyclization

Following completion of the amino acid sequence on-resin, glutaryl-cyclized peptides were completed by coupling glutaric anhydride to the N-terminus. Briefly, the resin was treated with 15 equivalents glutaric anhydride and 30 equivalents NMM for 15 minutes at 65 °C.

The C-terminal MTT-protected lysine was deprotected by washing the resin with DCM (Dichloromethane) followed by treatment with 5% TFA (Trifluoroacetic acid) and 1% TIS (Triisopropylsilane) in DCM for 30 minutes. Before proceeding into cyclization, any remaining TFA was neutralized by washing the resin sequentially in DCM, DMF, 10% NMM (w/v) in DMF, and DMF. On-resin cyclization was performed by two successive coupling reactions using 10 equivalents PyAOP ((3-Hydroxy-3H-1,2,3-triazolo[4,5-b]pyridinato-O)tri-1-pyrrolidinyl-phosphorus hexafluorophosphate) and 20 equivalents of NMM at 65 °C for 20 minutes.

Peptides were cleaved from resin for three hours using a cocktail of scavengers that included 2.5% (v/v) EDT (1,2-Ethanedithiol), 1.25% (v/v) dH₂O (deionized water), 1.25% (v/v) Thioanisole and 1.25% (v/v) Phenol in TFA. Cleaved peptide was ether precipitated, dried and filtered. The crude product was purified on a Vydac C-18 reverse phase column using gradient elution (0% B for 5 min, 20-60% B in 35 min. Solvent A: H₂O with 0.1% (v/v) TFA, Solvent B: CH₃CN with 0.1% (v/v) TFA). Lyophilized solid was reconstituted in DMSO and quantitated by absorbance at 280 nm. The identities of all peptides and conjugates were confirmed by ESI-MS or MALDI-TOF MS.

Measurement of G α SUPR peptide protease resistance

Two hundred fifty nmol of synthetic peptide in DMSO were added to 50 mM sodium phosphate buffer (pH = 8.0) with a final DMSO concentration of 2%. Sixty U of immobilized chymotrypsin or 6 U proteinase K (Sigma) were added and the digestion was allowed to proceed at room temp (chymotrypsin) or 4°C (proteinase K). Aliquots were taken at various time points, filtered to remove protease, and injected onto a C-18 reverse phase column and separated by gradient elution (15–90% B in 25 min. Solvent A: 0.1% TFA in water. Solvent B: CH₃CN with 0.05% (v/v) TFA). The area under the starting material peak was quantitated using the 32 KaratGold Software package (Beckman, Brea, CA). The plotted values represent the mean of two experimental values, and the error bars represent the standard error of the mean. The graph was generated by fitting the data to a one phase exponential decay equation (GraphPad Prism 5.0).

Measurement of SUPR peptide stability in human serum

Delipidated/lyophilized human serum was purchased from Thermo Scientific (Carlsbad, CA) and reconstituted as per manufacturer's instructions. Two hundred fifty nmol of peptide in 50 μ L sodium phosphate buffer (pH = 8.0) and 10% DMSO were added to 1 mL of reconstituted serum and incubated at 37 °C. One hundred μ L aliquots were taken at various time points and quenched in 300 μ L of acetonitrile. Samples were spun down and decanted to remove precipitate followed and diluted in 1.5 mL water. Analysis of intact peptide was performed by HPLC as described above.

G α SUPR peptide stability in microsome preparations

Human microsomes containing cytochrome P450's were obtained from Xenotech (Lenexa, KS). One hundred μ L of microsomes were added to 500 μ L of PBS buffer (1% (v/v) DMSO) containing 250 nmoles of peptide. Fifty μ L aliquots were removed at various time points and analyzed as described above for human serum.

Synthesis of the N-Methyl Scanning Library for Her2 Selection

Two single-stranded DNA templates were ordered from Integrated DNA Technologies (Coralville, IA). The sequences (5' - GGG ACA ATT ACT ATT TAC AAT TAC AAT GNN SKR SKA KKR KTW STA KKM SNN SAA AAG TAG TGG TAG CAG CGA TTA CA -3' and 5' - GGG ACA AAT ACT ATT TAC AAT TAC AAT GNN SYM KYA KKM KYA STW KNN SAA AAG TAG TGG TAG CAG CGA TTA CA -3') were synthesized using machine mixing for variable positions (W = A or T, K = G or T, M = A or C, R = G or A, S = G or C, Y = T or C). The double stranded library was produced and amplified by performing 6 cycles of PCR using 1 pmol of PAGE gel purified single-stranded library. PCR was performed under standard conditions utilizing the primers Gen-FP (5' -TAA TAC GAC TCA CTA TAG GGA CAA TTA CTA TTT ACA ATT ACA-3') and the reverse primer (5' - TGT AAT CGC TGC TAC CAC TAC TTT T -3')

The Round 0 mRNA pool was generated by T7 runoff transcription and purified by urea-PAGE. The purified mRNA was ligated to F30P (5' - dA21[C9]3dAdCdC-P; C9 - tri-(ethylene glycol) phosphate (Glen Research), P - puromycin (Glen Research, Sterling, VA)), via an oligonucleotide splint (5' - TTT TTTTTTTTTT TTG TAA TCG CTG C -3'). Ligated products were purified by urea-PAGE and quantified via absorbance at 260 nm.

Selection for Her2 SUPR peptides

Following ethanol precipitation of the round 0 DSG-treated fusions, the pellet was dissolved in 100 μ L of dH₂O and reverse transcribed with Superscript II under standard conditions. Following reverse transcription, the library was diluted 2-fold into 50 mM potassium phosphate buffer (pH = 8.0). Round 0 fusions were subjected to 0.1 mg of proteinase K for 30 sec. Round 1 was proteolyzed with 1 mg of proteinase K for 30 sec. Rounds 2 and 3 fusions were proteolyzed with 1 mg of proteinase K for 5 min. Finally, round 4 fusions were digested with 1 mg of proteinase K, 1 mg of chymotrypsin, and 1 mg of trypsin for 5 min. All proteolysis treatments were performed at room temperature.

Two days prior to selection, 150,000 SKBR-3 cells per well were seeded in a 96 well plate and grown in D-10 media (5% CO₂, 37°C). The cells were washed and incubated with proteolyzed fusions in 400 μ L D-10 media. After 1 hr at room temperature, the fusions were removed and cells were washed 4 times with D-10 media. After washing, the cells were treated with immobilized proteinase K for 20 minutes (0.5 mg/well) to remove bound fusions. Following filtration (Spin-x) to remove cells and immobilized protease, the Her2 libraries were regenerated by PCR.

The round 5 pool was amplified by PCR using Taq polymerase and sub-cloned into the TOPO-TA vector (Invitrogen, Grand Island, NY) followed by transformation into TOP10 competent cells (Invitrogen, Grand Island, NY). Individual clones were sequenced (Laragen, Culver City, CA). The most promising peptide sequence (SUPR4) was synthesized as described above and conjugated to Cy5 or Biotin-PEG-N₃ for further evaluation.

Synthesis of SUPR4-Cy5

SUPR4-alkyne (2 mg, 1.65 μ mol), Copper(I) Iodide (2.3mg, 15.5 μ mol), Ascorbic acid (7.2mg, 54 μ mol), and TBTA (1.4mg, 2.8 μ mol) was dissolved in DMF (642 μ L), deionized water (250 μ L), and DIPEA (7.9mg, 61 μ mol). Cy5-Azide (Lumiprobe, 1.7mg, 2.8 μ mol) was dissolved in DMF (100 μ L) and added to the peptide solution and rotated for 4 hours at room temperature. The solution was purified via Reverse Phase HPLC-MS with a reverse phase Vydac C-18 semi-prep column (Grace) and dried into a blue film using a Biotage V-10. Characterization by ESI-MS showed $[M+2Na]^{2+} = 921.8$, Predicted $[M+2Na]^{2+} = 922.05$.

Synthesis of SUPR4-Biotin

~800 nmol (1 mg) of SUPR4-alkyne was dissolved in 95 μ L DMSO and 40 μ L dH₂O. 2.3 μ mol (1 mg) Biotin-PEG3-N₃ (Sigma) dissolved in 20 μ L DMSO was added along with 880 nmol TBTA in 10 μ L DMF. 85 μ L of Copper Sulfate solution (160 mg CuSO₄ hydrate, 600 mg L-ascorbic acid in 5 mL dH₂O) was added and the reaction was allowed to proceed overnight at room temperature. The reaction was diluted in 1:1 dH₂O:CH₃CN and purified by C18 RP-HPLC. The lyophilized product was dissolved in DMSO and quantitated by A280 (55% yield). Characterization by ESI-MS (Waters Acquity) showed $[M+Na]^+ = 1721.96$. Predicted $[M+Na]^+ = 1721.77$.

ELISA-based Analysis of Binding Affinity of SUPR4-Biotin for Her2

Human IgG1-Fc-Her2 ectodomain (ThermoFisher Life Technologies) was immobilized on a Protein G-coated 96 well plate (ThermoFisher) for 1 hour at room temperature (5 pmol/well). The plate was washed with Tris-buffered saline (TBS) + 0.05% (v/v) Tween-20 followed by application of SUPR4-Biotin in TBS + 0.05% (v/v) Tween-20 (0-1 μ M). Following overnight incubation at 4 °C, the plate was washed with TBS + 0.05% (v/v) Tween-20 and incubated with Streptavidin-conjugated horseradish peroxidase (Strep-HRP, ThermoFisher Life Technologies) at 4 °C for 1 hour. After washing, the plate was developed with 1-step Ultra TMB-ELISA substrate (ThermoFisher Life Technologies), quenched with 2M H₂SO₄, and read at 450 nm on a SynergyH4 plate reader (BioTek). The resulting absorbance values were corrected for background (no ligand). Five samples (n=5) for each concentration were used. The resulting data was analyzed by non-linear regression in GraphPad Prism.

Cell-based Analysis of Binding affinity of SUPR4-Cy5 for Her2

The binding affinity of SUPR4-Cy5 for Her2 was determined by incubating 2.5×10^5 BT474 cells with various concentrations (0 – 90 nM) of SUPR4-Cy5 in triplicate (n=3). Cells were incubated at 4 °C for 30 min followed by three washes with cold PBS. Cells were analyzed using a BD FACSCalibur flow cytometer and the binding affinity (K_D) was determined using non-linear regression in GraphPad Prism 6.

Cell-based Analysis of SUPR4-Cy5 Binding Specificity

SUPR4-Cy5 was incubated with various Her2-positive cell lines (SK-BR-3, BT-474 and SKOV3) and a Her2-negative cell line (MDA-MB-231). Cells (2.5×10^5) were placed in

Eppendorf tubes and incubated with 25 nM SUPR4-Cy5. After incubation at 4 °C for 1 h, cells were washed 3 times with cold PBS and analyzed using a BD FACSCalibur Flow Cytometer and analyzed in GraphPad Prism 6. The mean of triplicate experiments is shown along with the standard deviation. Statistical significance was obtained by unpaired two-tailed t-test in GraphPad Prism (**, $p < 0.01$).

ELISA-based Analysis of Binding Specificity of SUPR4-Biotin

Human IgG1-Fc, IgG1-Fc-Her2 ectodomain, or IgG1-Fc-EGFR ectodomain (ThermoFisher LifeTechnologies) were immobilized on a Protein G-coated 96 well plate (ThermoFisher) for 1 hour at room temperature (5 pmol/well). The plate was washed with Tris-buffered saline (TBS) + 0.05% Tween-20 (v/v) followed by application of C-terminally biotinylated SUPR4 in TBS + 0.05% Tween-20 (200 nM). Following overnight incubation at 4°C, the plate was washed with TBS + 0.05% Tween-20 and incubated with Streptavidin-conjugated horseradish peroxidase (Strep-HRP, ThermoFisher LifeTechnologies) at 4 °C for 1 hour. After washing, the plate was developed with 1-step Ultra TMB-ELISA substrate (ThermoFisher Life Technologies), quenched with 2M H₂SO₄, and read at 450 nm on a SynergyH4 plate reader (BioTek). The resulting absorbance values were corrected for background (no ligand). Triplicate samples for each condition were used (n=3). The resulting data was analyzed by non-linear regression in GraphPad Prism. Statistical significance was determined by unpaired, 2-tailed t-test (*, $P \leq 0.05$. **, $P < 0.01$. GraphPad).

Generation of SKOV3 Her2 Knockdown and Measurement of SUPR4-Cy5 binding

SKOV3 cells were plated on six-well plates (3×10^5 cells per well) and were allowed to adhere overnight. Lipofectamine 2000 (cat #1529502, Invitrogen, Carlsbad, CA) was used for the transfection following the manufacturer's protocol. Cells were transfected with either Her2 29mer shRNA constructs in lentiviral GFP vector or negative control GFP vector (TL 320342 Kit, Origene, Rockville, MD). Transfected cells were then cultured in a 5% CO₂ incubator for 72 hrs before harvesting and protein analysis. Three days after transfection, transfected cells were passaged into growth medium containing 0.3 µg/ml puromycin (L1609, Santa Cruz Biotechnology, Dallas, TX) to select for cells that integrated the Her2 shRNA plasmid. Knockdown was confirmed by western blot (Figure S4). Binding of SUPR4-Cy5 to each variant was measured by flow cytometry. The error bars show the standard deviation (n=3). Statistical significance was determined by an unpaired two-tailed t-test (**, $P < 0.01$, GraphPad).

In vivo Near-infrared (NIR) Optical Imaging of SUPR4-Cy5

Female athymic nude mice were subcutaneously injected with 1.5×10^6 SKOV3 or MDA-MB-231 cells near the shoulder or rear flank. After ~12 weeks, mice were intravenously injected with 5 or 10 nmol of SUPR4-Cy5 via the tail vein. Mice were imaged using an IVIS Lumina XR at 4 or 24 hours post injection. After imaging, tumors and organs were removed for further imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Lipinski CA, Lombardo F, Dominy BW, Feeny PJ. *Advanced Drug Delivery Reviews*. 1997; 23:3–25. b Veber DF, Johnson SR, Cheng HY, Smith BR, Ward KW, Kopple KD. *J Med Chem*. 2002; 45:2615–2623. [PubMed: 12036371]
- Kim EM, Park EH, Cheong SJ, Lee CM, Kim DW, Jeong HJ, Lim ST, Sohn MH, Kim K, Chung J. *Nuclear medicine and biology*. 2009; 36:371–378. [PubMed: 19423004] b Okarvi SM. *Medicinal research reviews*. 2004; 24:357–397. [PubMed: 14994368]
- Schmidt MM, Witttrup KD. *Mol Cancer Ther*. 2009; 8:2861–2871. [PubMed: 19825804]
- Roberts RW, Szostak JW. *Proc Natl Acad Sci USA*. 1997; 94:12297–12302. [PubMed: 9356443]
- Li S, Millward S, Roberts RW. *J Am Chem Soc*. 2002; 124:9972–9973. [PubMed: 12188645]
- Millward SW, Takahashi TT, Roberts RW. *Journal of the American Chemical Society*. 2005; 127:14142–14143. [PubMed: 16218582]
- Bain JD, Diala ES, Glabe CG, Wacker DA, Lyttle MH, Dix TA, Chamberlin AR. *Biochemistry*. 1991; 30:5411–5421. [PubMed: 2036409] b Frankel A, Millward SW, Roberts RW. *Chemistry & biology*. 2003; 10:1043–1050. [PubMed: 14652071] c Kawakami T, Murakami H, Suga H. *Chemistry & biology*. 2008; 15:32–42. [PubMed: 18215771] d Merryman C, Green R. *Chemistry & biology*. 2004; 11:575–582. [PubMed: 15123252]
- Fiacco SV, Roberts RW. *Chembiochem*. 2008; 9:2200–2203. [PubMed: 18780385] b Haviv F, Fitzpatrick TD, Nichols CJ, Swenson RE, Bush EN, Diaz G, Nguyen A, Nellans HN, Hoffman DJ, Ghanbari H, et al. *J Med Chem*. 1992; 35:3890–3894. [PubMed: 1433199]
- Millward SW, Fiacco S, Austin RJ, Roberts RW. *ACS Chem Biol*. 2007; 2:625–634. [PubMed: 17894440]
- Keil, B. *Specificity of Proteolysis*. Springer-Verlag; Heidelberg-New York: 1992.
- Howell SM, Fiacco SV, Takahashi TT, Jalali-Yazdi F, Millward SW, Hu B, Wang P, Roberts RW. *Scientific reports*. 2014; 4:6008. [PubMed: 25234472]
- Ekerljung L, Lennartsson J, Gedda L. *PloS one*. 2012; 7:e49579. [PubMed: 23166716]
- Park BW, Zhang HT, Wu C, Berezov A, Zhang X, Dua R, Wang Q, Kao G, O'Rourke DM, Greene MI, Murali R. *Nature biotechnology*. 2000; 18:194–198.
- Cho HS, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney DW Jr, Leahy DJ. *Nature*. 2003; 421:756–760. [PubMed: 12610629]
- Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. *Cancer cell*. 2004; 5:317–328. [PubMed: 15093539]
- Troise F, Cafaro V, Giancola C, D'Alessio G, De Lorenzo C. *The FEBS journal*. 2008; 275:4967–4979. [PubMed: 18795950]
- Nakajima H, Mizuta N, Sakaguchi K, Fujiwara I, Yoshimori A, Takahashi S, Takasawa R, Tanuma S. *Breast cancer*. 2008; 15:65–72. [PubMed: 18224397]
- Baum RP, Prasad V, Muller D, Schuchardt C, Orlova A, Wennborg A, Tolmachev V, Feldwisch J. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine*. 2010; 51:892–897. b Lub-de Hooge MN, Kosterink JGW, Perik PJ, Nijhuis H, Tran L, Bart J, Suurmeijer AJH, de Jong S, Jager PL, de Vries EGE. *Brit J Pharmacol*. 2004; 143:99–106. [PubMed: 15289297]
- Saks ME, Sampson JR, Nowak MW, Kearney PC, Du F, Abelson JN, Lester HA, Dougherty DA. *The Journal of biological chemistry*. 1996; 271:23169–23175. [PubMed: 8798511]

20. Ja WW, Roberts RW. Biochemistry. 2004; 43:9265–9275. [PubMed: 15248784]

Figure 1. Selection of the Ga SUPR peptide

(a) Library design based on cyclic GiBP peptide (cycGiBP)^[9]. The wt. DNA codons were doped with TAG to enable insertion of *N*-methyl alanine via nonsense suppression and natural amino acids as shown. Selected amino acids are shown boxed. (b) A chemically cyclized mRNA display library was subjected to immobilized proteases and screened for binding to immobilized Gai1-GDP. (c) The selected Ga scanning unnatural protease resistant (SUPR) peptide sequence, with *N*-methyl alanine residues in red. (d) Structure of the Ga SUPR peptide with amino acid changes relative to cycGiBP indicated in blue (natural residues) and red (*N*-methyl alanine).

Figure 2. Relative stability of the Ga SUPR peptide

(a) Theoretical digest sites of the SUPR peptide with chymotrypsin (dotted red arrows) and Proteinase K (dotted black arrows). Observed cleavage sites are shown with solid red (chymotrypsin) and black (Proteinase K) arrows. The *in vitro* stabilities of SUPR peptides against (b) chymotrypsin, (c) proteinase K, (d) human serum (*ex vivo*), and (e) human liver microsomes. (f) Stability of the Ga SUPR peptide in mouse serum *in vivo*.

Figure 3. Selection of Her2 SUPR peptides

(a) The therapeutic antibodies Herceptin (Trastuzumab) and Pertuzumab (red and gray) recognize distinct epitopes on the Her2 ectodomain (green). The sequences of the CDRH3 loops of Herceptin and Pertuzumab (inset, red) were used to design focused SUPR peptide mRNA display libraries. We designed two mRNA display libraries with diversities of $> 16 \times 10^6$ sequences (Herceptin-derived, HMP Design) and $> 4 \times 10^6$ sequences (Pertuzumab-derived, PMP Design). The sequences of previously selected Her2 peptides (AHNP and HRAP) in the literature^[13]. The focused libraries were combined and translated with stop codons suppressed with N-methyl norvaline (underlined in red) and cyclized (black line). Following protease treatment and selection for binding to Her2-overexpressing SK-BR-3 cells in culture, the winning sequences were sequenced by next-generation sequencing. The Her2:Herceptin Fab structure is based on PDB structure 1N8Z^[14]. The Her2:Pertuzumab Fab structure is based on PDB structure 1S78^[15]. (b) Sequences obtained by next-generation sequencing from the final pool of selected clones. N-methyl norvaline (v) residues are shown underlined in red. A conserved LY(D/E)(D/V) sequence is shown in grey. One of the most promising sequences derived from the Herceptin library (Peptide D) was synthesized without the N-terminal methionine and with the cysteine at position 3 converted to serine to generate the high affinity SUPR4 peptide.

Figure 4. Binding and Selectivity of SUPR4

(a) Chemical structure of SUPR4 (VSvvLYDDK, v = N-methyl norvaline, red). (b) Binding of biotinylated SUPR4 to immobilized recombinant Her2 ectodomain by ELISA. (c) Binding of SUPR4-Cy5 to Her2-positive BT-474 cells by flow cytometry. (d) Binding of SUPR4-Cy5 to Her2-positive (SK-BR-3, BT-474, and SKOV3) and Her2-negative (MDA-MB-231) cells by flow cytometry. (e) Binding of SUPR4-Biotin to immobilized Her2 and EGFR extracellular domains by ELISA. (f) Binding of SUPR-Cy5 to SKOV3 cells transfected with control shRNA (shCtrl) or shRNA specific for Her2 (shHER2) as measured by flow cytometry. (g) Stability of Peptide D (MVCvvLYDDK) in human serum (*ex vivo*) at 37 °C.

Figure 5. Near-Infrared Fluorescence Optical Imaging with SUPR4-Cy5

(a) Two sets of mice with SKOV3 (Her2-positive) and MDA-MB-231 (Her2-negative) tumors were imaged with 5 nmol SUPR4-Cy5 to confirm Her2 specific uptake 4 hours post IV injection. (b) After 4 hours, resected tumors were re-imaged. (c) Mice with SKOV3 and MDA-MB-231 subcutaneous flank tumors were imaged with 10nmole SUPR4-Cy5 24 hours post-injection. (d) After 48 hours, tissues were removed for imaging. T=tumor; Lu=lung; S=spleen; H=heart; Li=liver; K=kidneys; GI= gastrointestinal tract. The scale shows the epi-fluorescence radiant efficiency.

Table 1

Protease resistance half-life data for GiBP, Gα SUPR, as well as single and double mutants of Gα SUPR.

Name	Sequence	Chymotrypsin		Proteinase K	
		Lin t _{1/2} (min)	Cyc t _{1/2} (min)	Lin t _{1/2} (min)	Cyc t _{1/2} (min)
GiBP	MITWYEFVAGTK	0.33	2.1	< 0.2	1.0
SUPR	MFY<u>A</u>YEY<u>A</u>QWSK	21	394	2800	5300
<u>A</u>4A	MFYAYEY<u>A</u>QWSK	< 0.2	< 0.2	< 0.2	0.71
<u>A</u>8A	MFY<u>A</u>YEYAQWSK	< 0.2	< 0.2	< 0.2	0.77
<u>A</u>4A, <u>A</u>8A	MFYAYEYAQWSK	< 0.2	< 0.2	< 0.2	< 0.2