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Multiple Bacteriophage Selection Strategies for Improved Affinity of a Peptide Targeting ERBB2

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Abstract

Due to the heterogeneity of ERBB2-expression between tumors and over the course of treatment, a non-invasive molecular imaging agent is needed to accurately detect overall ERBB2 status. Peptides are a highly advantageous platform for molecular imaging, since they have excellent tumor penetration and rapid pharmacokinetics. One limitation of peptides however, is their traditionally low target affinity, and consequently, tumor uptake. The peptide KCCYSL was previously selected from a bacteriophage (phage) display library to bind ERBB2 and did so with moderate affinity of 295 nM. In order to enhance tumor uptake and clinical utility of the peptide, a novel phage microlibrary was created by flanking the parent sequence with random amino acids, followed by reselection using parallel strategies for high affinity and specific ERBB2 binding in an attempt to affinity mature the peptide.

One limitation of traditional phage display selections is difficulty in releasing the highest affinity phages from the target by incubation of acidic buffer. In an attempt to recover high affinity second-generation peptides from the ERBB2 microlibrary, two elution strategies, sonication and target elution, were undertaken. Sonication resulted in an approximately 50-fold enhancement in recovered phage per round of selection in comparison to target elution. Despite the differences in elution efficiency, the affinities of phage-displayed peptides selected from either strategy were relatively similar. Although both selections yielded peptides with significantly improved affinity in comparison to KCCYSL, the improvements were modest, most likely because the parental peptide binding cannot be improved by additional amino acids.

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Conflict of Interest

The authors have no conflicts of interest to report.

Statement of Human Rights

This article does not contain any studies with human participants performed by any of the authors.

Statement of the Welfare of Animals

This article does not contain any studies with animals performed by the authors.

Keywords

Affinity Maturation; ERBB2; Peptides; Phage Display

Introduction

As medicine trends towards a more personalized approach, targeted therapies have become a powerful tool for the treatment of cancer. Antigens, such as the ERBB2 receptor (ERBB2) present in high numbers on the surface of certain carcinoma cells, while absent or at much lower levels in normal tissue, can be inhibited for effective therapeutic response to malignancy (Baselga et al. 1996; Di Fiore et al. 1987). ERBB2 is of particular interest, as it is overexpressed in a number of cancers, including breast, prostate and ovarian carcinomas (Yu and Hung 2000). Due to its importance in cancer progression, two therapeutic antibodies, trastuzumab and pertuzumab are used clinically to treat ERBB2 over-expressing cancers (Baselga et al. 2010). These therapeutic antibodies have been successful in extending mean survival rates, however over 50% of patients will either fail to respond to the treatment, or develop resistance (Kute et al. 2004). One potential reason for a lack of therapeutic success could be resultant from the inability to accurately monitor accessible ERBB2 in the tumor. Currently ERBB2 status is diagnosed by biopsy, which presents specific limitations (Cavaliere et al. 2005). Biopsies can be painful and are not ideal for repetitive monitoring of a tumor throughout the course of treatment. Biopsies also involve examining tissue *ex vivo*, which may not always be predictive of *in vivo* antigen expression and accessibility. In order to better predict and monitor effective treatment, complimentary imaging agents can be employed to identify and quantify the target non-invasively.

An ideal imaging agent would bind to the therapeutic target, providing clear diagnosis without affecting the therapeutic mechanism of inhibition. Additionally, the candidate would be rapidly cleared, non-immunogenic, and provide high target to background ratios for repeatable, real-time tracking of protein status. Small molecular weight peptides possess the rapid pharmacokinetics necessary for *in vivo* imaging applications, while providing the specificity necessary for detection of cancer-related antigens (Fischman et al. 1993). Combinatorial techniques, such as bacteriophage (phage) display, allow for selection of peptides that can hypothetically bind to any desired target, allowing for the production of a complimentary imaging agent for any therapeutic.

Phage display is a powerful technique used to screen large numbers of candidate peptides for binding to a specific target (Smith 1985). A typical phage display selection screens up to 10^9 unique, random peptide sequences for a specific function, such as binding to a target antigen. Following several rounds of selection, the subpopulation of phage remaining theoretically represents the fittest phages for the desired function. Each phage displays a foreign amino acid sequence of a potential target binding peptide. Generally, successful peptides selected by phage display bind to their targets with affinities in the low micromolar to high nanomolar range (Landon et al. 2004). These targets have included a number of cancer antigens, including ERBB2, plectin-1, Nrp-1 and several integrin subunits (Binetruy-Tournaire et al. 2000; Karasseva et al. 2002; Kelly et al. 2008; Koivunen et al. 1993). Some

of these peptides have been demonstrated to be sufficient for delineating human tumor xenografts in mice (Kelly et al. 2008; Kumar et al. 2007). Previously, our laboratory demonstrated the tumor targeting capabilities of an ERBB2-targeted peptide with the amino acid sequence KCCYSL (Kumar et al. 2007). The peptide was demonstrated to bind to ERBB2 with an affinity of 280 nM, and has been used for single photon emission computed tomography/computed tomography (SPECT/CT) and positron emission tomography (PET) imaging in human breast and ovarian cancer xenografted mice (Deutscher et al. 2009; Kumar and Deutscher 2008; Kumar et al. 2007). Although the affinity of KCCYSL was sufficient for tumor targeting, an improved affinity for ERBB2 may deliver higher tumor uptake and increase the clinical utility of the peptide for assessing ERBB2 status *in vivo*.

One method of improving the affinity of a peptide is through combinatorial evolution, a technique that has been used previously to enhance the affinity of a peptide that targeted the Thomsen-Friedenreich antigen (Landon et al. 2003)[Landon, Peletskaya, et al. 2003]. Using combinatorial evolution, the affinity of a peptide may be improved by an approach similar to the affinity maturation process that occurs in human B cells. In B cells, immunoglobulin gene sequences are subject to mutation in the hypervariable regions of the antibody in order to facilitate higher affinity binding to its antigen. The action of somatic hypermutation can be mimicked by the use of a phage microlibrary through the addition of variable regions adjacent to the target binding sequence (Hawkins et al. 1992). It was hypothesized that the affinity of KCCYSL for ERBB2 could be improved using phage display-directed affinity maturation. To mimic this *in vivo* B cell process, a KCCYSL microlibrary described previously was synthesized with an additional 5 random amino acids on its N-terminus and 4 random amino acids on its C-terminus (N'-nnnnnKCCYSLnnnn-C') (Larimer et al. 2014). This allowed for exploration of a significantly larger sequence space surrounding the core binding peptide in an attempt to further refine affinity and specificity.

In order to isolate peptides from the KCCYSL microlibrary with the highest affinity possible, an appropriate selection strategy was required. Traditionally, bound phage clones have been eluted by short duration incubation with acid. However, this method of elution has been suggested to be ineffective for removing the tightest binding peptides (Lunder et al. 2005). In order to overcome the potential limitation of acid-based elution, we compared two alternative elution strategies. One strategy was elution by addition of concentrated target protein, which provides a lower likelihood of non-specific elution in comparison to acid elution (Pande et al. 2010). The second strategy involved adding sonication to the acid elution, which has been demonstrated to remove the well-known streptavidin-binding motif HPQ, which acid alone failed to elute (Lunder et al. 2005). In order to compare the both the ability to remove bound phage and successful enrichment of target avid phages, both selection strategies were characterized for total population recovered and individual phages from each selection analyzed by phage enzyme linked immunosorbent assay (ELISA). Two peptides from each selection strategy were then synthesized and their ERBB2 affinity were compared to the parent KCCYSL peptide using fluorescent titration. Finally, each peptide was assayed for its ability to bind ERBB2 expressing cells *in vitro* using fluorescent microscopy.

Materials and Methods

Materials

Unless otherwise specified, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and cell culture materials were purchased from Invitrogen (Carlsbad, CA). The fUSE5 phage microlibrary was provided as a generous gift by George P. Smith from the University of Missouri.

Phage Display Selections

Human ERBB2 (Sino Biological, Beijing, China) was prepared at 1 mg/mL in 0.1 M sodium bicarbonate buffer, pH 9.6. An aliquot of 100 μ L of ERBB2 was added to triplicate wells of an ImmulonTM 2HB 96-well microtiter EIA plate (ImmunoChemistry Technologies, Bloomington, MN) and allowed to adsorb overnight at 4°C. Following adsorption, plates were blocked with 250 μ L of BioF_x synthetic bock (BioF_x Laboratories, Owing Mills, MD) for 2 h at room temperature. A 100 μ L solution of 10^{12} tetracycline transducing unit (TU)/mL KCCYSL microlibrary phage diluted in Tris buffered saline (TBS) and 0.1% (v:v) Tween-20 (0.1% TBST) was added to preblocked wells and allowed to incubate for 1 h at room temperature. Following incubation, wells were washed 10 times with 0.1% TBST prior to elution. For ERBB2 elution, 100 μ L of a 10 mg/mL solution of ERBB2 was added to wells and incubated for 1 h at 37°C for 1 h. The ERBB2 phage solution was recovered and used to infect log-phase *Escherichia coli* (*E. coli*) K91BK. For sonication elution, 100 μ L of 0.1 M glycine pH 2.2 was added to wells and the plate was placed in a sonicator water bath (50 kHz) for 10 min. Following sonication, the eluent was neutralized in 100 μ L 1 M Tris, pH 8. The neutralized eluent was amplified by *E. coli* infection and purified for subsequent rounds of selection. Each selection strategy was repeated for a total of 3 rounds, with the number of washes increasing to 20 in round 2 and 25 in round 3. The final eluent from round 3 was plated and individual colonies chosen for binding analysis by phage ELISA.

ERBB2 Phage Supernatant ELISA

From the phage display selections, 20 clones were chosen to be examined for ERBB2 affinity and specificity in binding assays. Individual phages were amplified in 2 mL of NZY amine overnight and the supernatant containing the amplified phage was used for binding analysis. To ImmulonTM 2HB 96-well microtiter EIA plates, 100 ng purified ERBB2 diluted in sodium bicarbonate buffer (pH 9.6) was added and incubated overnight at 4°C. The ERBB2-immobilized plates were aspirated and subsequently blocked for 2 h at room temperature with BioF_x synthetic bock. Blocking buffer was removed, and phage supernatant was added to wells for incubation at room temperature for 1 h. Plates were washed 10x with 0.1% TBST using an ELx405 Microplate Washer (BioTek Instruments, Inc., Winooski, VT) and were then incubated 1 h at room temperature with polyclonal rabbit anti-phage antibody diluted 1:1000 in 0.1% TBST. The plates were again washed with 0.1% TBST, and polyclonal goat anti-rabbit horse radish peroxidase conjugated antibody (Santa Cruz Biotechnology, Dallas, TX) diluted 1:1000 in 0.1% TBST was added and incubated for 1 h at room temperature. After this final incubation, plates were washed with 0.1% TBST before the addition of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic-acid) to

visualize captured phage. Following a 15 min incubation, absorbance at 405 nm was analyzed by plate reader.

Peptide Synthesis

Corresponding foreign peptides from the selected phage were synthesized with an Advanced ChemTech 396 multiple peptide synthesizer (Advanced ChemTech, Louisville, KY) by solid phase Fmoc chemistry. All peptides were synthesized with an N-terminal GSG spacer covalently coupled to biotin for detection. Peptide sequences were as follows: E6 – Biotin-GSG-ASGTSKCCYSLPATT, E16 – Biotin-GSG-SEVLKKCCYSLHPIP, S13 – Biotin-GSG-VTQTTKCCYSLEFHQ, S16 – Biotin-GSG-ESVNWKCCYSLTLQE.

Immobilized ERBB2 Peptide Affinity ELISA

Immulon™ 2HB 96-well microtiter EIA plates were incubated with 100 ng ERBB2 at 4°C overnight. Wells were aspirated and then blocked with BioFx synthetic block. After incubating 2 h at room temperature, the blocking solution was removed and peptides serially diluted from 100 nM to 50 μM in 0.1% TBST were added to the plates. Following an incubation of 1 h at room temperature, plates were washed 10x with 0.1% TBST using an automated plate washer. Plates were incubated 1 h at room temperature with streptavidin-conjugated horseradish peroxidase diluted 1:1000 in 0.1% TBST and were thoroughly washed again. To visualize bound peptide, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) was added and developed for 15 min at room temperature before absorbance was measured by a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski VT) at 405 nm.

Fluorescence Titration

Effects of peptides on the intrinsic tryptophan fluorescence emission of ERBB2 (excitation at 290 nm) were studied using a Varian Cary Eclipse fluorescence spectrophotometer equipped with a Peltier thermocontroller (Varian Inc, IL) as previously described (Kumar et al. 2007). Briefly, peptides (KCCYSL, E6 or S13) were added to 0.6–0.9 ml of ERBB2 solution (50–150 nM) in Dulbecco's PBS (pH 7.4) in a quartz cuvette (wave pathway of 1 cm) at 25°C. Fluorescence emission spectra (excitation at 290 nm) were recorded in the range of 320–450 nm. The changes in the fluorescence emission at 334 nm (F) induced by peptides were calculated as $(F - F_{\min})$; where F and $F_{\min}$ are fluorescence emission after the addition of the indicated amount of the peptide and at saturation, respectively. The relative changes in the fluorescence emission $100 * F / (F_0 - F_{\min})$; where F_0 is the fluorescence emission of the protein without peptide, were plotted against concentration of the peptide ($[Peptide]$). The values of the apparent dissociation constants (K_d) were calculated as the concentration of the peptide, which induced half of the maximal quenching at 334 nm by fitting a hyperbolic equation ($F / (F_0 - F_{\min}) = [Peptide] / (K_d + [Peptide])$) to the data using SigmaPlot 12.0.

Fluorescent Cell Binding Microscopy

ERBB2 positive BT-474 human breast cancer and ERBB2 negative MDA-MB-468 cells were dried onto microscope slides overnight, 5% (w/v) bovine serum albumin (BSA) in TBS

was applied and samples were blocked for 2 h at room temperature. Phosphate buffered saline (PBS) was added to and aspirated from cells 3 times before 100 μ L of each appropriate biotinylated peptide diluted to 10 μ M with 0.1% TBST was added and incubated with the cells for 1 h at room temperature. Slides were washed 3 times with 0.1% TBST and incubated 1 h at room temperature with 50 μ L anti-biotin Cy3 diluted 1:1000 in 0.1% TBST. Finally, slides were washed in the same method, mounted with toluene, and secured with cover slip. Cells were visualized by an epifluorescent Nikon T1-SM inverted microscope (Nikon, Melville, NY).

Results

Phage Display Selections

Phage display selections with a KCCYSL microlibrary outlined in Figure 1 were performed in an attempt to affinity mature or improve the ERBB2 binding sequence. A microlibrary displaying the KCCYSL peptide flanked by random amino acids was used for parallel selections with ERBB2 as the target. Selections differed only in their method of elution, either excess target or sonication. Input and recovered phage were quantified by titration, and the percent recovery is shown in Figure 2a. The percent recovery for elution with ERBB2 antigen was 7×10^{-6} in round 1, 1.9×10^{-5} in round 2, and 1.1×10^{-4} in round 3. These recoveries resulted in 2.7 fold amplification for round 2 and 15 fold amplification for round 3 (Figure 2b). In comparison, sonication resulted in percent recoveries (Figure 2a) of 5.5×10^{-4} , 2.2×10^{-4} , and 7×10^{-3} for rounds 1–3, respectively. Amplification for round 2 of sonication (Figure 2b) was therefore 0.39, and 31 for round 3. The percent recovery for sonication was 78 times higher than for target elution in round 1. In round 2, a relatively low yield of recovery by sonication reduced the difference in total phage between the two strategies in round 2, however sonication remained 12 times higher than target elution. Round 3 percent recoveries were enhanced by over an order of magnitude for each selection strategy, however the sonication elution strategy was the superior method of elution, resulting in a final sonication percent recovery that was 64 times higher than target elution.

Phage ELISA

Following the final round of selection, individual phages from each selection were chosen arbitrarily for ERBB2 binding analysis. Unpurified supernatant containing amplified phage was analyzed for the ability to bind ERBB2 in a 96-well format. Twenty phages from each selection were chosen for binding in addition to a phage without a foreign displayed peptide as a control. The results of each selection are presented in Figure 3. The 2 phage clones which bound with the highest ratio of ERBB2 to an unrelated anti-epithelial cell adhesion antibody negative control protein were chosen for subsequent analysis as a biotinylated peptide outside of the phage scaffolding. For the sonication selection, phages S13 and S16 were chosen. Binding of S13 to ERBB2 was determined by colorimetric ELISA, yielding an absorbance of 0.37 ± 0.11 to ERBB2 an absorbance of 0.16 ± 0.02 to a control protein for a specificity ratio of 2.30. The second phage chosen from the sonication elution, S16, had a bound ERBB2 absorbance of 0.29 ± 0.1 and a control absorbance of 0.13 ± 0.01 , resulting in a specificity ratio of 2.11. The top two phages from the ERBB2 elution strategy were E6 and E16. E6 absorbance was measured at 0.29 ± 0.04 and 0.14 ± 0.01 and E16 at 0.30 ± 0.02 and

0.14±0.01 for ERBB2 and negative control protein, respectively. These values resulted in a specificity ratio of 2.11 for E6 and 2.07 for E16. All four of the phage bound significantly higher ($P < 0.001$) to ERBB2 than wildtype phage.

In addition to individual phage analysis, the outputs from both the sonication and ERBB2 elution strategies were analyzed for ERBB2 binding in the same manner and compared as an indication of the global fitness of each selection. ERBB2 binding of the amplified third round eluate from sonication was measured at an absorbance of 0.22±0.09, and for excess target elution at 0.23±0.09. The measured absorbance of phage binding to the negative control was 0.14±0.02 for sonication and 0.14±0.01 for ERBB2 elution. Both selections resulted in significantly higher binding to ERBB2 than the negative control ($P < 0.001$) or wildtype phage to ERBB2 ($P < 0.05$).

Comparison of First and Second Generation Peptide Affinities for ERBB2

The amino acid sequence of the displayed peptides of the four phage chosen for further study by ELISA were determined, and each sequence was synthesized coupled to a biotin for detection. Each peptide was analyzed over several concentrations to determine an apparent binding constant for the ERBB2 extracellular domain. The binding affinity to ERBB2 of the sonication eluted phage displayed peptides was 1.6±0.4 μM for S13 and 2.7±0.6 μM for S16 (Figure 5). For the target eluted peptides, the affinities were 1.7±0.6 μM for E6 and 7.6±1.5 μM for E16 (Figure 5). For direct comparison of affinity matured peptides to parent KCCYSL using this technique, the binding affinity of KCCYSL was also investigated, and was determined to be 4.4±1.2 μM (Figure 5). One peptide each from sonication (S13) and ERBB2 elution (E6) bound with a significantly improved dissociation constant (K_D) ($P < 0.05$) than KCCYSL. S13 bound with 2.7 times higher affinity, while E6 had a 2.5 fold improvement over the first generation peptide. S16 had a slightly better dissociation constant (K_D) than KCCYSL, however it was not statistically significant. The binding of E16 was significantly worse than KCCYSL ($P < 0.05$). Although the ELISA results provided a means to directly compare parent and second generation peptides in terms of relative binding affinity and specificity, the difference in measured affinity between the ELISA-generated affinity and previously reported affinity of KCCYSL (295 nM) required a second, more accurate means of affinity measurement. Hence, only the two peptides which demonstrated statistically improved binding to ERBB2 in the ELISA were chosen for this additional binding characterization using fluorescence titration.

Second Generation Peptide Affinities Determined by Fluorescence Titration

The apparent K_D for the parent peptide KCCYSL (4.4±1.2 μM) determined by ELISA (Figure 5) was almost 15 fold higher than that observed previously ($K_D=295 \pm 56$ nM) using fluorescence titration (Kumar et al. 2007). In order to better evaluate the affinity of the second generation peptides for KCCYSL, fluorescence titration solution binding experiments were performed. Peptides induced a concentration-dependent quenching of the intrinsic tryptophan fluorescence emission of ERBB2 (Figure 6). The dependences of relative changes in the fluorescence emission at 334 nm (excitation at 290 nm) on the concentration of KCCYSL, E6 and S13 are shown in Figure 6; Inset. The apparent K_D values determined by the fluorescence titration were 180 ± 30 , 150 ± 20 and 120 ± 15 nM

for KCCYSL, E6 and S13, respectively (Figure 6, Inset). While the values of the apparent K_D for all three peptides were significantly lower than those estimated by the 96 well peptide ERBB2 ELISA assay (Figure 5), the affinity of the parent peptide determined under equilibrium conditions KCCYSL ($K_D=180 \pm 30$) was close to that observed previously ($K_D=295 \pm 56$ nM) (Kumar et al. 2007). These results demonstrate that measurements of affinities under equilibrium conditions with the original peptides could eliminate inherited limitations of solid phase assays.

Fluorescent Microscopy of Peptide Cell Binding

In addition to affinity for ERBB2, each peptide was tested for the ability to discriminate between ERBB2-expressing BT-474 human breast cancer cells and ERBB2-negative MDA-MB-468 cells. Peptide binding to each cell line was visualized by fluorescent microscopy (Figure 6). The parent peptide KCCYSL bound BT-474 cells and did not bind MDA-MB-468 cells. Peptides E6 and S13 appeared to have stronger binding intensities than KCCYSL to BT-474 target cells, in addition to minimal MDA-MB-468 binding. E16 did not bind either BT-474 or MDA-MB-468 cells. S16 demonstrated strong fluorescent binding to BT-474 cells, but also the highest amount of binding to MDA-MB-468 cells.

Discussion

Targeted therapies such as trastuzumab and pertuzumab have altered the diagnosis of ERBB2 positive breast cancer (Baselga et al. 2010). Currently, screening of patient candidates for this therapy is accomplished by biopsy, which poses problems for repeated monitoring, in addition to characterizing metastases, which may not have the same ERBB2 status as the solid tumor (Cavaliere et al. 2005). Molecular imaging of ERBB2 would alleviate the problems associated with biopsies. Peptides are highly advantageous *in vivo* molecular imaging agents, due to their rapid pharmacokinetics and excellent tissue penetrating abilities (Fischman et al. 1993). A radiolabeled diagnostic peptide would provide rapid and quantitative characterization of ERBB2 status in not only solid tumors, but also metastases. The peptide KCCYSL binds ERBB2 and has been used to image human breast and ovarian cancer xenografts in mice (Deutscher et al. 2009; Kumar et al. 2007). Its tumor uptake is moderate, at $\sim 0.7\%$ ID/g and it was hypothesized that increasing the affinity of KCCYSL could improve the tumor uptake and make the peptide a more attractive clinical imaging agent.

In an attempt to enhance the affinity of KCCYSL, a process similar to B cell hypervariation was implemented using phage display (Hawkins et al. 1992). In short, the KCCYSL sequence was flanked by random amino acids on both the N- and C- termini, providing, in essence, hypervariable regions for selection. This expanded sequence space would permit sampling of novel sequences with the potential for higher ERBB2 affinity due to interactions outside of the KCCYSL binding site. One of the keys to selecting high affinity peptides is the strategy used for elution of bound phage from their target. Traditional selections implement acid elution, and the parent KCCYSL peptide was selected in this manner. Newer strategies for elution include excess ligand, excess target, or sonication [Lunder, Bratkovic, et al. 2008, Pande, Szewczyk, et al. 2010]. Excess ligand and target have the benefit of

promoting target-specific elution. Unfortunately, ERBB2 is not thought to have a ligand, so that elution strategy was not possible for this selection (Cho et al. 2003). Instead, excess target elution was chosen because it offers a highly specific method of elution. One drawback, however, of target elution is the highest affinity peptides may not be competed off by excess target due to a potentially low dissociation rate constant (k_{off}). In order to compensate for this drawback, elution by sonication, which could potentially provide a high shearing force capable of removing bound phage mechanically, was also explored as a strategy. In order to explore this selection strategy in the context of affinity maturation, it was included as a second elution method.

The total amount of phage extracted by each elution strategy was monitored and normalized as a percent yield of total phage added in order to directly compare the efficiency of each strategy for removing bound phage. The percent yield of each strategy was tracked after each round, and as was to be expected, elution by sonication resulted in higher yields of recovered phage at every round of selection, due to the non-specific manner of phage elution. Interestingly, there was a decrease in the total percentage of recovered phage in the second round of selection. This could have resulted from the increased stringency of the washes removing non-specific and low affinity phages captured in the first round. The same trend was not seen in the ERBB2 elution, most likely because phages selected by this strategy were more likely to be specific and thus not affected as strongly by the wash stringency. Both strategies resulted in a large increase in recovered phage in the third round, indicating a significant enrichment for target specific phages.

From this enriched third round of selection, 20 individual phages were arbitrarily chosen from each selection strategy for analysis of apparent affinity and specificity for ERBB2. The top 2 phages from each selection method were chosen to have their displayed peptide synthesized for further characterization. All four of the phages, S13 and S16 from the sonication elution and E6 and E16 from the target elution, bound approximately two times higher to ERBB2 than to the control protein, a target to background binding ratio which has been demonstrated to be sufficient for selection of target-specific peptides (Liu et al. 2013; Wang et al. 2013). Interestingly, the binding of these top phages were not vastly different than the entire enriched population of each selection strategy. Sonication and target elution resulted in enriched populations that bound approximately 1.6 times higher to ERBB2 than the control protein. This indicated that the selection as a whole was successful, however the range of peptide affinities was likely very small.

Upon examination of each peptide outside of the phage scaffolding, one peptide from each of the selection strategies, S13 and E6, exhibited a decreased K_D in comparison to KCCYSL. However, the degree of enhanced binding was small, only an approximately 2.5 fold improvement for each peptide. Fluorescence quenching was performed to obtain more precise affinities for the two peptides that appeared to bind better to ERBB2 than KCCYSL. The trend was the same but fluorescence quenching yield slightly higher affinities than ELISA. The equilibrium dissociation constant values were found to be 180 ± 30 , 150 ± 20 and 120 ± 15 nM for KCCYSL, E6 and S13 respectively. These values are of the same magnitude as the affinity previously reported for KCCYSL using a similar fluorescence quenching titration approach (Karasseva et al. 2002). The difference in the values of K_D

obtained for the parent peptide KCCYSL with ELISA and under conditions of fluorescence titration, demonstrate the limitations of non-equilibrium methods for estimation of the affinity. Biotinylation, immobilization of the target, nonspecific interactions, which include both matrix and immobilized ERBB2, fast dissociation of the peptide, avidity, or steric hindrance could all individually or collectively affect interactions between ERBB2 and peptide and, therefore, K_D . The enhanced affinities appeared to correlate well with cell binding, as both peptides appeared to bind better to BT-474 ERBB2 over-expressing breast cancer cells than the first generation peptide. Notably, biotinylated E16, for which binding to ERBB2 was detected by ELISA (Figure 5), did not bind ERBB2 expressed on BT-474 breast cancer cells. Therefore, these results demonstrate that binding to ERBB2 at the cell surface could differ from that of recombinant extracellular ERBB2, either in solution or immobilized.

The lack of significant improvement in binding strength of the peptides selected from this affinity maturation may be a result of a binding site which is not amenable to improvement through the additional sequence space. This idea is consistent with the phage display selection results that were originally performed to obtain KCCYSL (Karasseva et al. 2002). Of all of the phage clones eluted and analyzed in terms of sequence over 70% were the exact amino acid sequence KCCYSL. While a winning sequence is often found, such a high percentage is unusual and may be indicative of the binding fitness of this sequence. Although Landon et al. successfully improved the affinity of a linear peptide 5 fold for the Thomsen-Friedenreich antigen (Landon et al. 2003), the strategy was slightly different, as the core 6 amino acid sequence was selected from a 15 amino acid library whereas the KCCYSL peptide was selected from a 6 amino acid library. It may be that the KCCYSL peptide was chosen for binding to a small pocket amenable to 6 amino acids, and the additional amino acids have very little effect on the affinity of the peptide. If this is the case, the KCCYSL peptide may not benefit from the addition of extra sequence space. Further selection in this manner may never result in a higher affinity peptide, and the evidence presented in this work and the previous work support that despite selection resulting in successful target binding, the affinity of second-generation peptides remains only minimally improved at best (Karasseva et al. 2002; Kumar et al. 2007; Larimer et al. 2014). In an attempt to further elucidate the binding mechanism of KCCYSL to ERBB2, co-crystallization trials have been initiated, which may allow a more definitive explanation for the apparent lack of improvement on the parent KCCYSL peptide.

Conclusion

Two methods for phage display affinity maturation of an ERBB2 targeted peptide were tested, and each strategy successfully selected a peptide with improved affinity for ERBB2. However, the increase in affinity was modest, and taken in combination with previous ERBB2 affinity maturation attempts, this may suggest the KCCYSL sequence cannot be drastically improved. Both of the higher affinity peptides bound to ERBB2-overexpressing BT-474 human breast cancer cells, which warrant further investigation as to the *in vivo* capabilities of each peptide. For example, radiolabeled versions of the peptides would permit characterization of additional critical properties of the peptides, including biological half-life, tumor specificity and retention. *In vivo* analysis, in addition to crystallographic

structural analyses could help elucidate the novel hypotheses generated by the results of this work.

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List of Abbreviations

ELISA	Enzyme linked immunosorbant assay
Fmoc	Fluorenylmethyloxycarbonyl
GSG	Gly-Ser-Gly
K_D	Dissociation Constant
TBS	Tris buffered saline
TBST	Tris buffered saline and tween
TU	Tetracycline transducing units

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Fig. 1.
A depiction of the parallel strategies used for affinity maturation of KCCYSL by phage display.

Fig. 2.

Phage display selections utilizing excess ERBB2 or sonication as elution strategy were performed in parallel. A) The number of phage recovered after each round was quantified and the percent recovery was expressed as the total phage recovered/input phage. Black bars represent sonication eluted phage recoveries, while gray bars represent the recovery by ERBB2 elution. B) The amplification of each round, as calculated by dividing the total recovered phage of the current round by the total recovered phage of the previous round is shown.

Fig. 3.

Individual phage supernatants were incubated with either ERBB2 or a negative control protein, and total bound phages were quantified by ELISA. From each selection strategy, 20 phages were chosen and their absorbance are plotted above, with black bars representing ERBB2 bound phage and white bars representing phage bound to control protein. Phages represented by an “S” were selected by sonication, while those with an “E” were selected by elution with excess ERBB2.

Fig. 4.

The total phage recovered from round 3 of each selection strategy were analyzed for ERBB2 binding by phage ELISA and compared to insertless wildtype phage. Black bars represent ERBB2 binding, white bars negative control protein. Error bars represent a mean of three separate assays. Both ERBB2 elution and elution by sonication resulted in significantly higher binding to ERBB2 than negative control, in addition to higher ERBB2 binding than wildtype phage. * - $P < 0.05$; *** $P < 0.001$

Fig. 5. Peptide binding curves of the four affinity matured peptides: E6, E16, S13 and S16 in addition to the first generation peptide KCCYSL were determined by incubating concentrations of peptides ranging from 100 nM to 50 μ M with ERBB2 protein. Bound biotinylated peptides were detected by the addition of horseradish peroxidase-conjugated streptavidin. Bars represent the mean of triplicate wells, and the K_D of each peptide is averaged from three separate experiments

Fig. 6.

Peptide-induced tryptophan fluorescence quenching of ERBB2. Changes in the fluorescence emission spectrum (320–450 nm) of 80 nM ERBB2 in DPBS (excitation at 290 nm) in the presence of increasing concentrations (0 – 6.65 μ M) of S13. Spectra were corrected for Raman scattering. **Inset:** Binding isotherms for interactions of ERBB2 with peptides. ERBB2 (5–15 μ g/ml) was titrated with KCCYSL ($\circ$), E6 ($\square$) and S13 ($\triangle$) in DPBS. F were calculated as $\frac{F - F_{\min}}{F_0 - F_{\min}}$, F , and $F_{\min}$ are fluorescence emission at 334 nm (excitation at 290 nm) after the addition of peptide, and at saturation, respectively. F_0 corresponds to the fluorescence emission prior to addition of the peptide. Solid lines represent the best fit ($r^2 > 0.97$) of the hyperbolic equation $\frac{F}{F_0 - F_{\min}} = \frac{[\text{Peptide}]}{K_d + [\text{Peptide}]}$, where $[\text{Peptide}]$ is the concentration in nM and K_d is the apparent dissociation constant, to the data using SigmaPlot 12.0. The values of K_d , which correspond to the concentration of the peptide, which induced half of the maximal quenching of the fluorescence emission, were 180 ± 30 , 150 ± 20 and 120 ± 15 nM for KCCYSL, E6 and S13, respectively

Fig. 7.

Fluorescent microscopy was used to assay the binding of biotinylated peptides to either ERBB2-overexpressing BT-474 cells or ERBB2-negative MDA-MB-468 cells. Peptide bound to the 4% paraformaldehyde fixed cells was visualized by the addition of Cy3-conjugated anti-biotin antibody