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Design of novel lipidated peptidomimetic conjugates for targeting EGFR heterodimerization in HER2 + cancer

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ABSTRACT

The human epidermal growth factor receptor (EGFR) family is known to be involved in cell signaling pathways. The extracellular domain of EGFR consists of four domains, of which domain II and domain IV are known to be involved in the dimerization process. Overexpression of these receptors is known to play a significant role in heterodimerization of these receptors leading to the development of cancer. We have designed peptidomimetic molecules to inhibit the EGFR heterodimerization interaction that have shown antiproliferative activity and specificity for HER2-positive cancer cell lines. Among these, a peptidomimetic, compound **5**, exhibited antiproliferative activity at low nanomolar concentrations in HER2-overexpressing cancer cell lines. To improve the stability of this peptidomimetic, we have designed and synthesized a novel conjugate of peptidomimetic compound **5** with a lipid, stearic acid. The antiproliferative activity of this conjugate was evaluated in HER2-positive cancer cell lines. Results suggested that the conjugate exhibited selective antiproliferative activity in HER2-overexpressing breast and lung cancer cell lines and was able to block HER2:HER3 heterodimerization. Also, the conjugate showed improved stability with a half-life of 5 h in human serum compared to the half-life of 2 h for parent compound **5**. The binding affinity of the conjugate to HER2 protein was evaluated by SPR analysis, and the mode of binding of the lipid conjugate to domain IV of HER2 protein was demonstrated by docking analysis. Thus, this novel lipid conjugate can be used to target HER2-overexpressing cancers.

The human epidermal growth factor (HER) family of transmembrane receptors is known to include potent mediators of normal cell growth and development. This epidermal growth factor family (transmembrane tyrosine kinase receptors) mainly consists four receptors–HER1 (EGFR1), HER2(EGFR2), HER3(EGFR3), and HER4(EGFR4).[1,2] Ligand binding to the extracellular region of EGFRs leads to conformational changes that are essential to generate receptor-mediated dimerization. Dimerization can be homodimerization (involves two molecules of the same receptor) or heterodimerization (two different HER2 receptors).[3] Among them, HER2:HER3 heterodimerization is known to be the most potent oncogenic unit because of its strong interactions, ligand-mediated tyrosine kinase phosphorylation, and downstream signaling. Therefore, the HER family became the major target for the development of novel anticancer agents.[4–7]

HER2 overexpression was found to be associated with 15–20% of breast cancer patients and 4–30% of non-small cell lung cancer patients with lower survival rates. Many therapeutic agents are available for HER2 targeting, including antibodies,[6] small molecules, and tyrosine kinase inhibitors.[8–10] Trastuzumab was the first anti-HER2 targeting humanized monoclonal antibody that binds to the extracellular domain of HER2 receptors. Its binding reduces HER2-mediated downstream signaling pathways and cell cycle prognosis.[11–13] Overall, considering the importance of HER2 receptor dimerizations with other HER family receptors and its role in breast and lung cancer pathogenesis, our interest is to design novel peptidomimetics that could block HER2-mediated heterodimerization and be therapeutically effective in inhibiting cell signaling and controlling cancer cell growth.

Peptides are a good choice as drug candidates because they offer several advantages such as potent bioactivity, specific receptor binding affinity, and can be easily synthesized in large amounts.[14,15] Compared to antibody drugs, peptides are generally non-immunogenic.[16,17] However, peptides are prone to rapid serum degradation and faster renal clearance in the human body.[18] Also, the hydrophilic nature of most peptide drugs limits their membrane permeability.[19] Several chemical modification techniques have been employed to reduce these disadvantages. Modifications of peptides include lipidation, backbone modification using cyclization, D-amino acid incorporation, conjugation with lipids, and conjugation of polyethylene glycol (PEGylation).[20–22]

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Among these techniques, lipidation of biological peptides has shown to improve stability, bioavailability, and extended permeability through biomembranes without loss of activity.[23] Lipidation also leads to changes in the peptide secondary structures through hydrophobic interactions between the fatty acid chain and peptide backbone or side chain[24] without affecting specificity and binding affinities of peptides. Moreover, lipidation reduces peptide renal clearance and elevates plasma stability.[25,26]

Previous studies have shown that peptides with fatty chain amino acids can alter the pharmacokinetic activity profile of lipidized peptides.[24,27] Also, the chain length of fatty acids has an influence on the potency of lipidized peptides. For example: (a) the long-chain fatty acid lipidation analogs of Phe-Asp-Cys-amide (FDC-amide) were more active than the corresponding short-chain fatty acid lipidation analogs in the inhibition of melittin-induced hemolysis[28] and (b) stearic acid-modified hirudin peptidomimetic showed increased pharmacokinetic and anticoagulant properties.[29] Some examples of lipidized peptide drugs that have been approved by the FDA and are on the market for human use are antibiotics such as polymixin B and daptomycin and anti-diabetic human glucagon-like peptide-1 (GLP-1) analogs liraglutide (Victoza®, NN2211)[30,31] and insulin detemir (Levemir®, ($N^{\varepsilon,B29}$-tetradecanoyl)-des-(B30)-insulin).[32]

Therefore, we believe that the lipidation approach could be used as a novel tool in developing therapeutic peptides. In an earlier report, we showed that peptidomimetics designed for targeting HER2 have antiproliferative activity against HER2-positive lung and breast cancer cell lines. One of the linear peptidomimetics that we designed, called compound **5**, was shown to bind to the HER2 extracellular domain and inhibit HER2:HER3 dimerization.[33–38] The specificity of compound **5** to HER2 protein and its antiproliferative activity (Fig. 1A) in HER2 positive cancer cell lines made it a good choice for conjugation of this compound to a lipid. In this study, we have chosen lipidation strategy to modify our parent compound **5** to improve stability and membrane permeability.

We have designed and synthesized a novel lipid conjugate by N-terminal lipidation to compound **5**. Four different lipid conjugate analogs were synthesized, namely, stearic acid-lipid conjugate, palmitic acid lipid conjugate (Fig. 1B and C), stearic acid-lysine-lipid conjugate, and palmitic acid-lysine-lipid conjugate. The structure-activity relationships of the lipid conjugate analogs were examined using antiproliferative activity assay in HER2-positive breast cancer and lung cancer cell lines. The stearic acid lipid conjugate exhibited better antiproliferative activity than the other lipid conjugate analogs. Therefore, for further study, the stearic acid lipid conjugate was selected and investigated for inhibition of HER2:HER3 heterodimerization, stability assessment in human serum, and binding to the HER2 protein. Also, the mode of interactions between the lipid conjugate and HER2 protein was modeled in a docking study. These studies indicated that the lipidized conjugate binds to the HER2 protein and inhibits HER2:HER3 dimerization. Furthermore, the conjugate showed improved stability with a half-life of 5 h in human serum compared to the half-life of 2 h for parent compound **5**.

In our earlier studies, we have shown that compound **5** (Fig. 1A) binds specifically to the HER2 extracellular domain, particularly to domain IV, and inhibits EGFR heterodimerization. Compound **5** also exhibits antiproliferative activity in HER2 + breast cancer cell lines.[33] Compound **5** is a peptidomimetic with free N-and C-termini that are susceptible to degradation in serum and may have a short half-life in circulation. Attaching lipids to peptides is known to increase the half-life of peptides in the serum. Compound **5** was conjugated with lipids at the N-terminal of the peptide. Two types of lipid chains were used, stearic acid and palmitic acid (Fig. 1B and C). Both of these lipids are known to increase the half-life and pharmacokinetic properties of peptide-based drugs when conjugated. We did not use any linker to conjugate the lipid to the peptide as conjugation results in an amide bond between lipid and peptide. In the design of conjugates of lipid, we

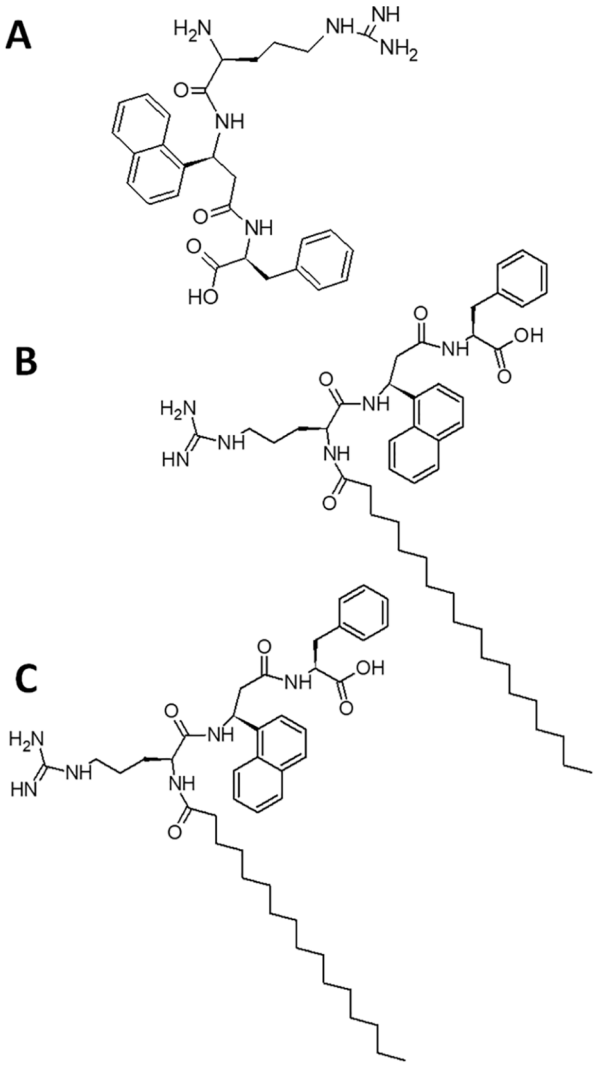

Fig. 1. Structures of A) compound **5**, B) stearic acid compound **5** conjugate (SA-5), C) palmitic acid compound **5** conjugate (PA-5).

used the N-terminal amine to conjugate the lipid. We also wanted to conjugate fluorescent labels to the lipidized peptide to study the binding of the conjugate to HER2 on the cell surface and possible internalization of the conjugate. Although compound **5** is known to bind to the HER2 protein extracellular domain, lipidization will allow the lipidized conjugate to permeate into the cell. Binding of peptide and internalization can be studied using fluorescent probes. Most of the peptides are attached with the fluorescent label at the free amino-terminal or side chain of Lys amino acid.

However, free N-terminal is not available for fluorescent conjugation as we conjugated the lipid with peptide using the free amino terminal. Lys was introduced in compound **5** by replacement of amino acid Phe in compound **5**. C-terminal Lys side chain can be used for conjugating fluorescent compounds. The resulting conjugates were SA-5K, PA-5K (Table 1). However, the fluorescent label 6-carboxyfluorescein (6-FAM) is a bulky functional group. Attachment of 6-carboxyfluorescein to Lys side chain was not successful. This could be due to stearic hindrance of long lipid chain to the C-terminal Lys side chain in SA-5K. Thus a linker consisting of Asp-Gly-Gly-Gly was used to conjugate 6-FAM. In our previous studies, we have also shown that peptidomimetic Arg-Anapa-Phe-Asp exhibited specificity for HER2 positive cancer cell lines.[38] Thus, Stearic acid-Arg-Anapa-Phe-Asp with a linker and C-terminal Lys was used for linking 6-FAM at the Lys side chain. The resulting compound was SA-5-6FAM- (Table 1). To evaluate

Table 1
The sequence of peptides and conjugates described in this study along with analytical data.

Compound	Sequence	Experimental Molecular Weight	Calculated Molecular Weight	Purity
SA-5	Stearic acid-Arg-(*S*)Anapa*-Phe-OH	785.53	785.06	> 95
PA-5	Palmitic acid-Arg-(*S*)Anapa-Phe-OH	757.50	757.01	> 95
SA-5K	Stearic acid-Arg-(*S*)Anapa-Lys-OH	766.97	766.06	> 95
PA-5K	Palmitic-Arg-(*S*)Anapa-Lys-OH	738.93	738.01	> 95
5*	NH_2-Arg-(*S*)Anapa-Phe-OH	519.40 [M + H] +	518.60	> 95
FITC-5*	FITC-aminocaproic acid-Arg-(*S*)Anapa-Phe-OH	1020.88	1021.14	> 90
SA-5-6FAM	Stearic acid-Gly-Arg-(*S*)Anapa-Phe-Asp-Gly-Gly-Gly-Lys-OH (FAM Lys side chain)	1614.94	1614.83	> 90
SA-Control-6FAM	SA-Gly-Arg-Ala-Ala-Leu-Gly-Gly-Gly-Lys-OH [FAM-Lys.Side Chain]	1410.91	1410.65	> 90
Control*	NH_2-Lys-(3-amino-biphenyl-propionic acid)-Phe-OH	517.28 [M + H] +	516.63	> 95

* Also reported in our earlier studies, Anapa, 3-amino-3-(1-naphthyl)-propionic acid.

the specificity of Arg-Anapa-Phe-Asp to HER2 protein binding, a control peptide with a random sequence and Stearic acid and Lys was designed. Arg-Anapa-Phe-Asp sequence was replaced with Gly-Arg-Ala-Ala-Leu, and Gly linker with Lys at the C-terminal was used for attaching a fluorescent probe 6-FAM (SA-control-6FAM). A control peptidomimetic that was previously shown not to exhibit antiproliferative activity in HER2 positive cell line was used as control (Table 1).[33] Conjugates were synthesized using a Tribute peptide synthesizer and standard Fmoc peptide chemistry protocol. Analysis of purified peptides by HPLC and MALDI-TOF showed that the conjugates had more than 95% purity as well as molecular ion corresponding to the calculated molecular weight (Table 1, Supporting Information).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.10.005.

We have evaluated the antiproliferative activity of lipid conjugates on HER2-overexpressed cancer cell lines such as BT-474, Calu-3, and A549 as well as the MCF-7 cell line, which does not overexpress HER2, and the non-cancerous breast epithelial cell line MCF-10A. Stearic and palmitic acid-lipid conjugates (SA-5, PA-5) showed antiproliferative activity at lower micromolar concentration and selectivity for HER2-overexpressing cancer cell lines BT-474, A549 and Calu-3 compared to MCF-7 cell line that does not overexpress HER2 (IC_{50}:39 μM) (Table 2). Expression of HER2 in different cell lines was evaluated using flow-cytometry. Calu-3, BT-474, and A549 cells showed expression of HER2 as indicated by a shift in the affibody labeled (Anti-ErbB2 Affibody FITC, Abcam Cambridge, MA) cell population to the right in the histogram compared to the cells without any affibody. For MCF-7 cells that were incubated with FITC-HER2 affibody, there was no change in the cell population between labeled and unlabeled cells indicating very low or no expression of HER2 (Supporting information). In MCF-10A cell lines, the antiproliferative activity was 39 μM. There was no significant difference in the antiproliferative activity of stearic acid and palmitic acid conjugates. However, stearic acid conjugates exhibited slightly higher antiproliferative activity compared to palmitic acid conjugates. Hence, detailed studies were carried out on stearic acid conjugates. A control peptidomimetic exhibited antiproliferative activity > 35 μM in different cancer cell lines.

We have performed competitive binding studies for determining the binding ability of lipid conjugate SA-5 on BT-474 cell line. Our previous study showed that FITC-labeled compound **5** has binding affinity to domain IV of HER2. Therefore, we used FITC-labeled compound **5** for assessment of the binding ability of lipid conjugates. The results showed that the addition of increasing concentrations of lipid conjugate caused dose-dependent binding inhibition of FITC-labeled compound **5** (Fig. 2A). This suggests that the lipid conjugate was able to bind to domain IV of HER2.

There was a drastic decrease in fluorescence upon addition of 1 μM SA-5 to BT-474 cells along with FITC-5 compound and then a dose response was observed in fluorescence intensity. This indicates that SA-5 could easily replace FITC-5 in the competitive binding to HER2 on the cell surface. A control compound was also used to competitively bind to HER2 protein on the cell surface of BT-474 cell lines. Results indicated that the control peptide did not show any dose-dependent binding to HER2 protein (Supporting information). On the other hand, when SA-5 was added to cells with fluorescently labeled SA-5-6FAM, there was a dose-dependent displacement of fluorescently labeled peptide conjugate as observed in Fig. 2B.

Competitive binding was also evaluated using fluorescence microscopy studies. Cells without the addition of SA-5-6FAM showed nuclear stain DAPI, and no green fluorescence was observed (Fig. 3A). When SA-5-6FAM was incubated with BT-474 cells and visualized under a fluorescence microscope, green fluorescence was observed (Fig. 3B). When unlabeled conjugate SA-5 was added to the cells at different concentrations (0.5, 5, 50 and 100 μM) to the cells with SA-5-6FAM at a constant concentration, the green fluorescence from labeled peptides was decreased as seen in Fig. 3C–F. As the concentration of unlabeled peptide was increased, the green fluorescence is decreased consistent with the competitive binding of unlabeled peptide replacing the labeled peptide.

The changes in the observed behavior of competitive binding of SA-5 with FITC-5 and SA-5-6FAM could be explained in terms of the structure of FITC-5 and SA-5-6FAM and attachment of fluorescent

Table 2
Antiproliferative activity of lipidized peptidomimetic 5 conjugate, compound **5** and control peptidomimetic in cancer cells BT-474 (HER2 + breast cancer cells), Calu-3, A549 (HER2 + lung cancer cells), and breast cancer cells that do not overexpress HER2 receptors (MCF-7) and non-cancerous cells (MCF-10A). Activity is represented as IC_{50} values in μM.

Compound	IC_{50} μM				
	BT-474	Calu-3	A549	MCF-7	MCF-10A
SA-5	1.0 ± 0.05	1.46 ± 0.07	1.17 ± 0.2	> 35	> 35
PA-5	1.4 ± 0.09	1.56 ± 0.04	1.01 ± 0.01	> 35	> 35
SA-5K	1.2 ± 0.06				
PA-5K	1.4 ± 0.02				
5*	0.895 ± 0.029	0.601 ± 0.02		16.9 ± 1.0	> 35
Control*	> 35	> 35	> 35	> 35	

* Also reported in our earlier publication.

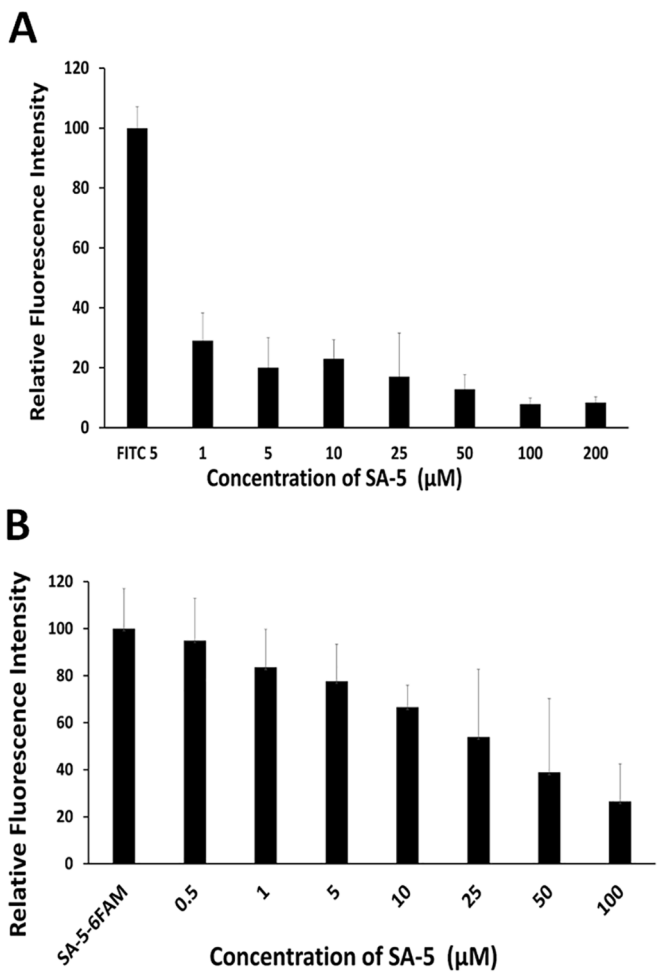

Fig. 2. Competitive binding of the conjugate to HER2 + cells. A) Competitive binding of fluorescently labeled conjugate 5 with compound SA-5. BT-474 cells were incubated with FITC-5 and unlabeled lipidized peptide conjugate SA-5 at different concentrations. FITC-5 was known to bind to HER2 domain IV. There was a significant decrease in the intensity of fluorescence after the addition of SA-5, indicating that SA-5 binds to the same site on HER2 protein as does FITC-5. B) Competitive binding of fluorescently labeled conjugate SA-5-6FAM with SA-5. BT-474 cells were incubated with SA-5-6FAM and unlabeled lipidized peptide conjugate SA-5 at different concentrations. There was a stepwise decrease in the intensity of fluorescence, indicating that SA-5 competes its fluorescently labeled conjugate SA-5-6FAM.

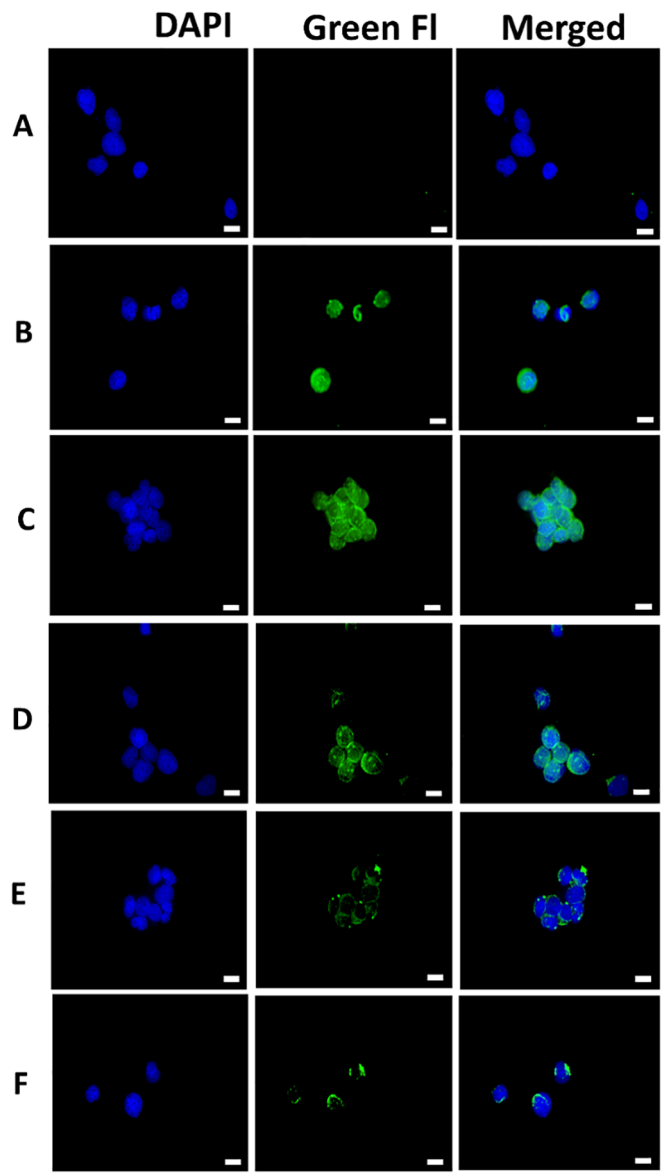

Fig. 3. Competitive binding of conjugate SA-5-6FAM with unlabeled SA-5 studied by fluorescence microscopy in HER2 (+) BT-474 cell line. Different amount of unlabeled conjugate was added to cells with a constant amount of SA-5-6FAM (50 μM). A) BT-474 cells without conjugate. B) Cells with only SA-5-6FAM, C) 0.5, D) 5, E) 50 and F) 100 μM SA-5. Nuclei are shown stained blue with DAPI. Scale bar 10 μm. Magnification 40×. Green Fl, Green fluorescence.

probe (Table 1). In FITC-5, compound **5** with three amino/non-amino acids and attached to bulky FITC by a small linker (5 carbons attached to N-terminal of the peptide). The bulky group of FITC might create stearic hindrance of peptidomimetic part binding to HER2 protein on cell surface resulting in relatively weak binding to HER2 compared to compound **5** or SA-5. With the long-sized linker in SA-5-6FAM (Gly-Gly-Gly and Lys side chain length, the fluorescent label is nearly 15 heavy atoms away from the peptidomimetic part that binds to HER2) the stearic hindrance from fluorescent label is minimized, thus allowing SA-5-6FAM to bind to HER2 with relatively high affinity compared to FTIC-5. However, overall, these experiments indicate that SA-5 binds to HER2 on the binding site that is similar to compound **5** binding site, near the C-terminal of domain IV of HER2 ECD.

The binding affinity of lipid conjugate SA-5 to HER2 protein extracellular domain was examined by surface plasmon resonance analysis. HER2 protein ECD was immobilized on a CM5 chip, and lipid conjugate SA-5 was used as analyte. Upon addition of SA-5, there was an increase in sensorgram response, suggesting that SA-5 binds to HER2 ECD (Fig. 4A). Addition of different concentrations of SA-5 showed concentration-dependent binding to HER2 -ECD. The kinetics of association and dissociation were calculated based on a 1:1 Langmuir interaction model and the Kd value was calculated by curve fitting. The Kd for SA-5- HER2 ECD was 1.16 μM. Similar experiments were conducted on a control peptide. The control peptide did not show any binding to HER2- ECD (Supporting Information). These results clearly indicate the specific binding of SA-5 to HER2 -ECD. A fluorescently labeled conjugate SA-5-6FAM also showed the binding ability to HER2 -ECD (Fig. 4B).

We assume that the designed lipid conjugate SA-5 binds to the HER2 extracellular domain and inhibits heterodimerization of HER2, in particular, HER2:HER3. To evaluate this inhibition, we used proximity ligation assay (PLA). In PLA, two proteins that are in proximity can be labeled with primary antibodies from particular species. Secondary antibodies with DNA fragments are directed to these primary antibodies. If the two proteins of interest are in proximity (distance ≤ 40 nm), DNA fragments hybridize, and this hybridized DNA

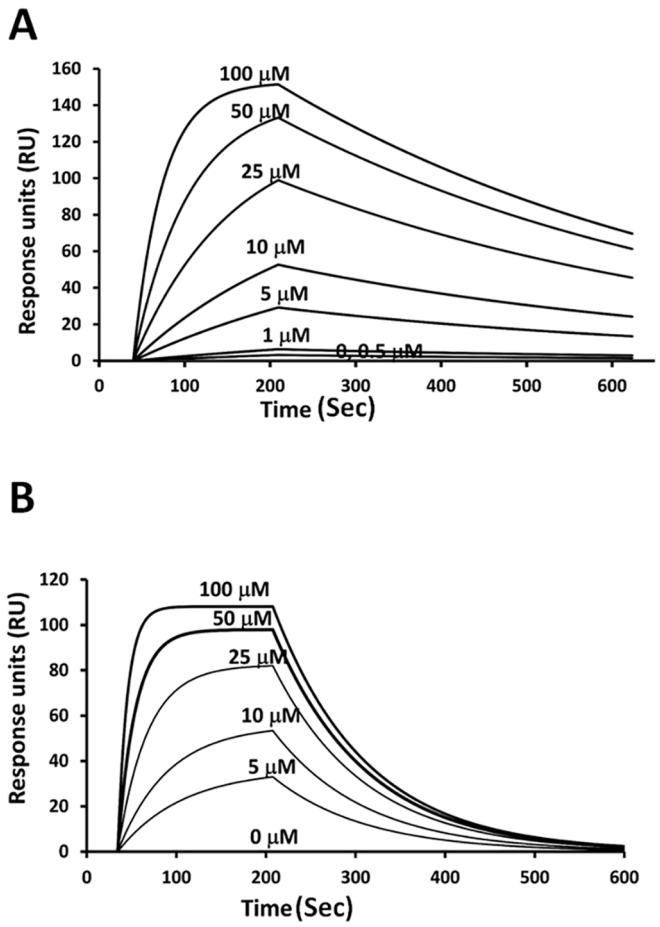

Fig. 4. SPR analysis of binding of conjugates to HER2 ECD. A) Concentration-dependent binding of conjugate SA-5 to HER2 protein was seen. Experiments were repeated twice, and a representative sensorgram is shown here. B) SPR sensorgrams for the binding kinetics of SA-5-6FAM with HER2 protein extracellular domain. Concentration-dependent binding of conjugate SA-5-6FAM to HER2 protein was seen. Experiments were repeated twice, and a representative sensorgram is shown here.

can be amplified and detected using DNA fluorescence probes. Traditional immunocytochemistry displays only co-localization of proteins, whereas the proximity ligation assay helps to detect and visualize protein-protein interactions using a fluorescence probe in a native state of the cells. This assay is carried out on protein in cancer cells in the native state and hence the data obtained is more reliable.[39–42] In this particular case, species-specific primary and secondary antibodies and probes are employed to stain HER2:HER3 heterodimerization as fluorescent red dots. The presence of red fluorescent dots around cells indicate heterodimerization of HER2:HER3. PLA assay was carried out on BT-474 cells (that overexpress HER2 protein and known to have HER2:HER3 dimers) in the absence of conjugate SA-5. Presence of red fluorescence dots in the microscopic image indicated a protein-protein interaction between HER2 and HER3 (Fig. 5A). As a control when only secondary antibodies are added, no red fluorescence dots were appeared (Fig. 5B). When PLA was carried out on BT-474 cells in the presence of conjugate SA-5 at a concentration of 1 and 5 μM (Fig. 5C and D), the microscopic images showed a significant reduction in the number of red fluorescent spots, suggesting that SA-5 inhibits HER2:HER3 heterodimerization compared to controls (Fig. 5D).

Binding of SA-5-6FAM to HER2-overexpressing BT-474 cells was investigated by fluorescence microscopy. A time-dependence study of binding was carried out. When SA-5-6FAM conjugate was incubated with BT-474 cells for 30 min and visualized under a microscope, green fluorescence was observed, indicating that the conjugate binds to HER2 on the cell surface (Fig. 6, first row). A control peptide conjugate with 6FAM was designed with the replacement of amino acid Phe by Ala and beta amino acid Anapa by Ala in the peptide conjugate (Table 1). The control conjugate did not show any green fluorescence, suggesting that the control did not bind to HER2-overexpressed BT-474 cells (Fig. 6, second row). For comparison, an affibody that is known to bind to the HER2 extracellular domain was also incubated with BT-474 cells, and a fluorescence image was obtained. Affibody exhibited green fluorescence indicating the binding of affibody to the cell surface (Fig. 6, third row). The conjugate was incubated for 2 h and monitored for cellular uptake by LysoTracker. Green fluorescence of cells indicated that apart from binding to cell surface conjugate may have internalized (Fig. 7, first row). When the green fluorescence was merged with images of cells from LysoTracker (red) (merged image yellow color) indicated that some conjugate also internalized in the cell (Fig. 7, first row). On the other hand, the control compound did not show any fluorescence (Fig. 7, second row) suggesting that conjugate is highly specific for HER2. Compound **5** is known to bind to HER2 ECD, in particular to domain IV. It is clear from these studies that the lipid conjugate of compound **5** binds to the HER2 protein extracellular domain first. Since the compound binds to the extracellular domain and inhibits PPI of EGFR and HER2, it was retained on the cell surface for some time. Thus,

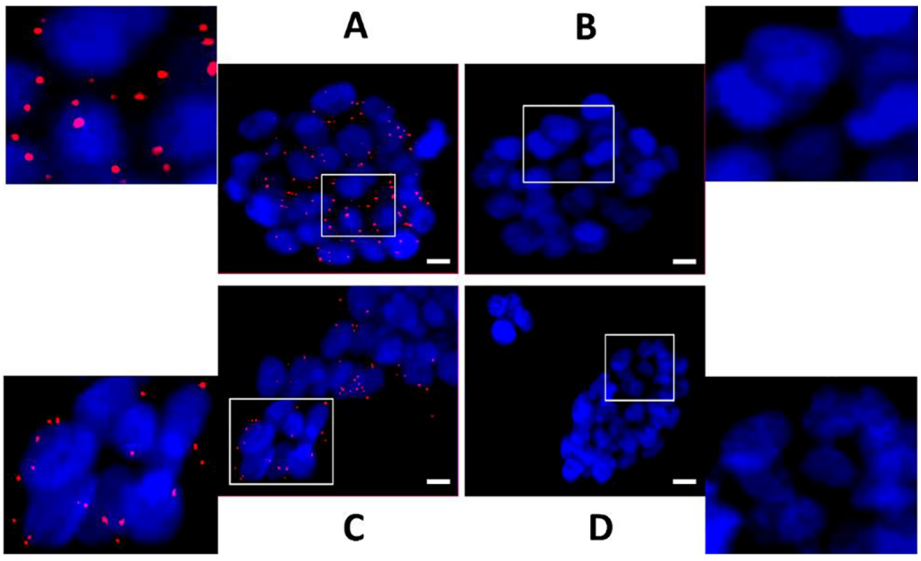

Fig. 5. Proximity ligation assay (PLA) on HER2-positive BT-474 cell lines to study PPI inhibition by the conjugate. A) Control without the addition of conjugate. Red dots indicate the PPI of HER2:HER3. Zoomed areas of the cells show red dots, indicating the PPI of HER2:HER3. B) Control. C) BT-474 cells in the presence of 1 μM concentration of SA-5 conjugate. D) BT-474 cells in the presence of 5 μM concentration of SA-5 conjugate. Expanded regions are shown for clarity. Nuclei are shown stained blue with DAPI. Scale bar 10 μm. Magnification 40×.

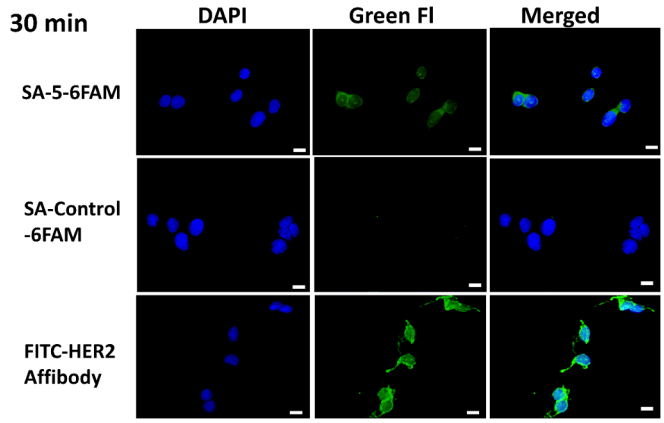

Fig. 6. Binding of fluorescently labeled peptide conjugate SA-5-6FAM and SA-control-6FAM conjugate to HER2 + BT-474 cells. BT-474 cells were incubated with SA-5-6FAM or control conjugate for 30 min, and the cells were washed and observed under a microscope. After washing the cells, green fluorescence was observed in cells treated with SA-5-6FAM, indicating specific binding of peptide conjugate to HER2 on BT-474 cells (first row). SA-Control-6FAM conjugate did not show any binding (second row). For comparison, BT-474 cells incubated with HER2 extracellular domain binding affibody is also shown (third row). SA-5-6FAM specifically binds to HER2 protein compared to control. From the images, it is clear that most of the fluorescence due to SA-5-6FAM is on the surface of BT-474 cells. Nuclei are shown stained blue with DAPI. Scale bar 10 μm. Magnification 40×.

conjugate SA-5 binds specifically to the HER2 protein extracellular domain. After 2 h, the conjugate can enter the cell either directly or indirectly by HER2 internalization. Since the peptide is attached to the lipid, we anticipate that the conjugate can insert into the lipid bilayer and reach the inside of the cell. Evaluation of the internalization of the compound into the cell via EGFR trafficking[43,44] requires detailed experiments that involve tracking the proteins with fluorescent labels.

Analysis of binding of SA-5 by SPR and inhibition of dimerization of HER2:HER3 by PLA suggested that SA-5 binds to HER2 protein ECD. In this study, docking was performed using AUTODOCK[45] software to determine the binding mode of the lipid conjugate on HER2 protein. Structures of conjugates of SA-5 and PA-5 were generated using Insight II (BIOVIA, San Diego CA) and subjected to energy minimization. The energy-minimized structures were docked to HER2 domain IV of ECD (Fig. 8A–C). The low energy docked structures were clustered around the C-terminal of domain IV of HER2 ECD. Detailed analysis indicated that the lowest energy structure of SA-5 was −4 kcal/mol. Two possible binding modes of lowest energy docked structures are shown in Fig. 8B). Docked pose 1 (magenta sticks) was stabilized by three hydrogen bonds between the conjugate and HER2 protein. Anapa backbone carbonyl and amide formed hydrogen bonds with Ala576 NH and Arg577 C═O. The peptide bond between peptide and lipid formed hydrogen-bonding interactions with Arg577 side chain guanidine group. Phe of the conjugate was involved in hydrophobic interactions with Phe573 and His542, Pro543. The lipid portion of the conjugate was stabilized by a hydrophobic pocket formed by Val582, Lys583, and Trp592. The second pose (green sticks) with the lowest energy docked

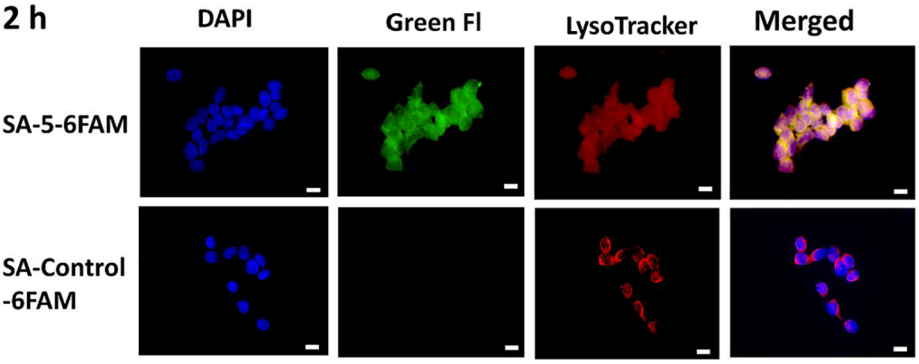

Fig. 7. Binding of fluorescently labeled peptide conjugate SA-5-6FAM and SA-control-6FAM conjugate to HER2 + BT-474 cells after incubating the conjugate for 2 h. BT-474 cells were incubated with SA-5-6FAM or control conjugate for 2 h and cells were washed and observed under a microscope. LysoTracker was added to observe the internalization of the conjugate. After washing the cells, cells were observed under a fluorescence microscope. Green fluorescence was observed in cells treated with SA-5-6FAM, indicating specific binding of peptide conjugate to HER2 on BT-474 cells (first row). SA-Control-6FAM conjugate did not show any binding (second row). Green fluorescence on the cells was distributed on the surface as well as within the cells compared to Fig. 6 above. Merged image of DAPI, green and LysoTracker red indicates that conjugate is also distributed inside the cell (yellow color due to the merging of green and red). Nuclei are shown stained blue with DAPI. Scale bar 10 μm. Magnification 40×.

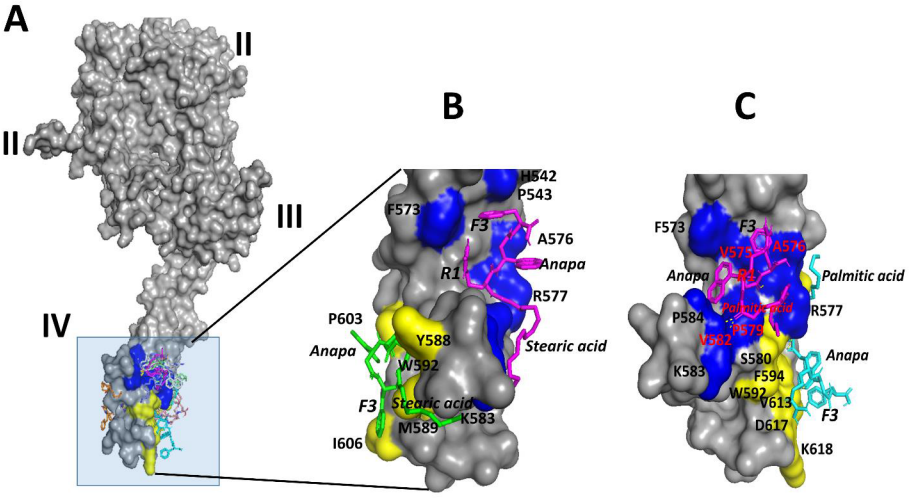

Fig. 8. Docking of conjugates to HER2 ECD domain IV. A) Crystal structure of HER2 extracellular domain (ECD) shown with domains I-IV with some low energy docked structures of conjugates of 5 bound to domain IV. B) Two docked poses of lowest energy docked structures of stearic acid conjugate are shown as magenta and green sticks. C) Two docked poses of lowest energy docked structures of palmitic acid conjugate are shown as magenta and cyan sticks. Amino acids are shown as single letter codes, and amino acids from conjugates are shown in *italics*. Anapa, 3-amino-3-(1-naphthyl)-propionic acid.

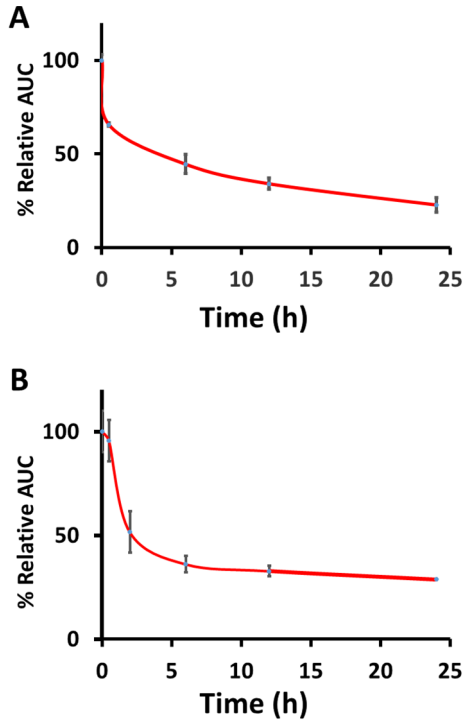

Fig. 9. Stability of the conjugate SA-5 and compound **5** in human serum (*in vitro*) analyzed by HPLC. A) conjugate SA-5, B) compound **5**. Each data point is from triplicate experiments.

structure has hydrogen-bonding interactions between Arg of the conjugate and Trp592 C═O, Anapa. Amino acid Phe of the conjugate formed a hydrophobic interaction with Pro603, Ile606. The lipid part of the conjugate was stabilized by a hydrophobic core formed by Met589, Tyr588, and Lys583.

The lowest energy docked structure of PA-5 had –5 kcal docking energy; the two lowest energy docked poses are shown in Fig. 8C. The docking position of PA-5 was similar to that of SA-5. In docked pose 1 (magenta sticks), hydrogen bonding was between Anapa C═O and Arg577, peptide bond between lipid and peptide with Lys583. Hydrophobic interactions occurred between Anapa, Phe and the lipid portion with Val575, Arg577, Ala576, Pro579, Val 582, Phe573, and Pro584. Docked pose 2 (cyan sticks) formed a hydrogen bonding interaction between lipid-peptide peptide bond with Arg577 and Ser580. Arg formed a hydrogen-bonding interaction with Asp617. The lipid part of the conjugate formed hydrophobic interactions with Tyr568, Arg577, and Phe 594. The Arg alkyl side chain of conjugate formed a hydrophobic interaction with Trp592. Anapa and Phe of the conjugate were stabilized by a hydrophobic pocket formed by Val613 and Lys618 side chain. Docking studies clearly indicated that the probable binding site of the conjugate on HER2 domain IV is the C-terminal part where the PPI interactions take place as observed in the homodimer crystal structure of EGFR.[46]

Our aim here was to design a lipid conjugate that is more stable in serum than compound **5** as compound **5** is a relatively small peptide with N-and C-termini susceptible to degradation. We have used the lipidation approach to increase the stability of peptidomimetics in serum. For stability assessment, the lipid conjugate was incubated with human serum and aliquots of sample were collected at different time points and analyzed using a Shimadzu HPLC system. The results showed that the lipid conjugate was stable for 24 h in human serum with a half-life of 5 h (Fig. 9A). These data were supported by mass spectrometry analysis (Fig. 10). Compound **5** has a half-life of around 2 h (Fig. 9B) as seen in the present studies as well as reported in our previous studies using mass spectrometry analysis.[38] Although, there was not significant improvement in the stability of the peptide in serum, overall, the stability of the compound was increased by conjugating the lipid into the peptide design.

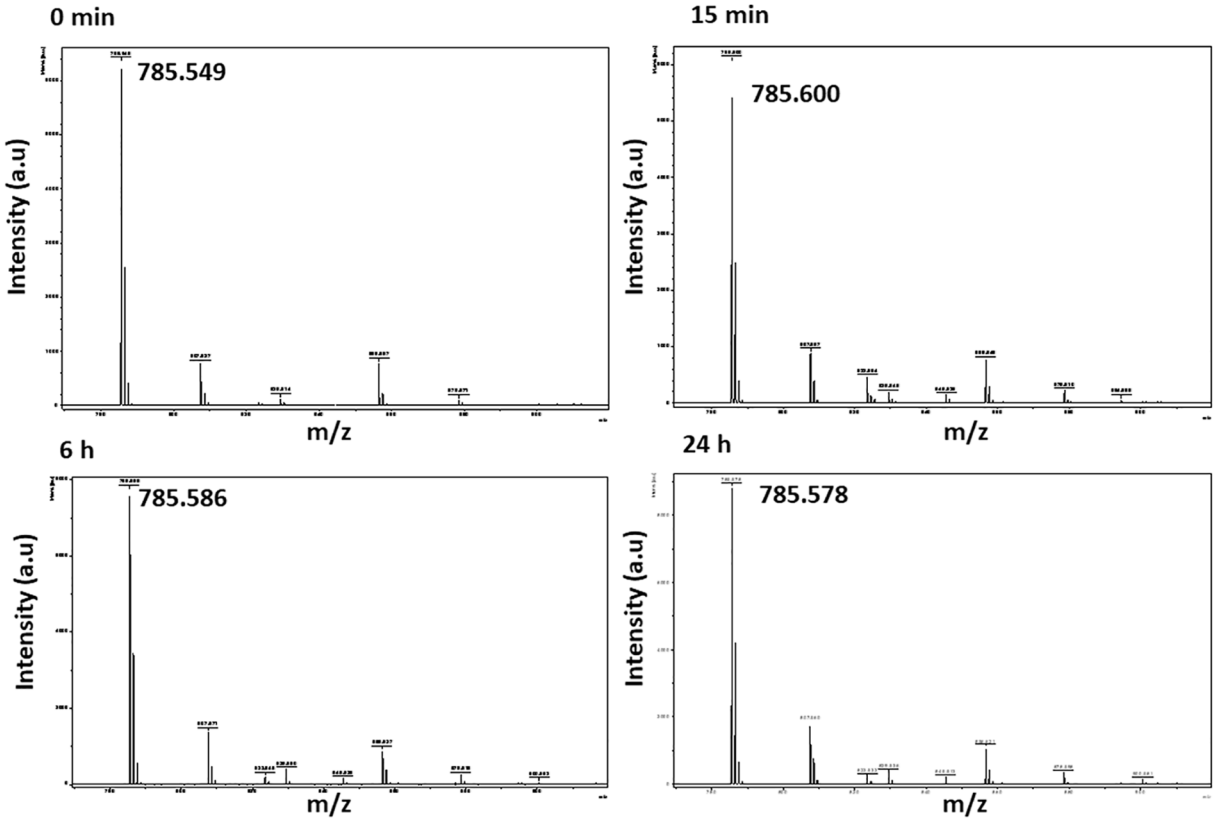

Fig. 10. Stability of the conjugate SA-5 in human serum (in vitro) analyzed by mass spectrometry. Incubation times 0 min, 15 min, 6 h and 24 h are shown.

A peptidomimetic that is specific for HER2 protein was conjugated with lipids via the N-terminal of the peptide using a peptide bond. Two types of lipids, stearic and palmitic acids, were conjugated to the peptidomimetic. The stearic acid-conjugated peptide was subjected to detailed cellular assay and binding studies. The results indicated that the stearic acid-conjugated peptide exhibited antiproliferative activity against HER2-positive breast and lung cancer cells. The peptide conjugate was also shown to bind specifically to HER2 protein domain IV and to inhibit HER2:HER3 dimerization in HER2-positive cancer cells. Compared to the peptide without lipid, the lipidized conjugate exhibited an improvement in serum stability in an in vitro stability assay. These results indicate that targeted peptides can be lipidized for improving stability and possibly pharmacokinetic properties.

Author contributions

SJ and HN designed the experiments and TG synthesized and purified the peptides and conjugates. SS performed the microscopy experiments. SJ, HN, SS, and TD contributed to the writing of the manuscript.

Declaration of interest

Conflicts of interest: none.

Acknowledgments

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