A grafted peptidomimetic for EGFR heterodimerization inhibition: implications in NSCLC models.

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Supporting Information **Page**

MATERIALS AND METHODS

Peptide Synthesis. All the grafted peptides (**Table S1**) were synthesized via Fmoc based solid-phase peptide synthesis method described previously [1]. Briefly, linear peptide chain was performed out on an automated peptide synthesizer (Symphony; Protein Technologies, Inc.) on 2-chlorotrityl resin on a 0.25 mM scale. The sidechain protected peptides were cleaved off the resin using 10 x 5 mL 1% TFA in DCM (v/v) for 5 min. The eluate was then added to 150 mL of an aqueous solution of 50% ACN, 0.05% TFA. Using Vacuum, TFA and DCM were removed and the remaining solution was lyophilized. Crude sidechain protected peptides were dissolved in DMF and cyclized using HATU/DIPEA (5- and 10-mM final concentration, respectively) for 30 min at room temperature. Side chain protecting groups were removed by incubating the residue with a 4ml cocktail of TFA:EDT:Water:Triisopropylsilane (TIPS) (94:2.5:2.5:1 (v/v)) for 2.5 h at room temperature. The crude mixture was lyophilized, and the desired product purified by RP-HPLC. The crude peptide was dissolved in 15 mL of 50% ACN containing 0.1% TFA in water. Iodine solution (1 mg iodine in 1 mL of 50% ACN containing 0.1% TFA in water) was added by drop until the solution is in yellow color. It was kept for 1h under stirring. Then little of ascorbic acid was added to stop the reaction until the solution became colorless. The solution was then freeze dry to yield solid powder and finally purified by RP-HPLC method [1, 2].

HER2 Expression in Different Cancer Cell Lines Using Flow Cytometry. All the cells (Calu-3, A549, NCI-H1435, NCI-2122, SKLU-1, NCI-H522, NCI-H1975, BT-474, MCF-7) were grown to 75% confluency, and approximately 1x 10^6 cells were suspended in 2 mL of PBS. Then the cells were incubated with 2% BSA solution for 30 minutes to prevent nonspecific binding of the antibody. HER2 antibody conjugated with phycoerythrin (PE) fluorophore (Catalog # 324405, PE-CD340, Biolegend, CA) was added at a concentration of 5 μg/mL to the cell suspension and

incubated for 90 min at 4°C. Once the incubation is complete, the mixture was centrifuged at 3000 rpm for 10 min, and the pellets were transferred to flow cytometry tubes after resuspending with 2 mL of cold PBS. FACSCalibur flow cytometry instrument (BD Biosciences, San Jose, CA) operated with CellQuest Pro software (BD Biosciences) was used, and the data were further analyzed by using the FlowJo software (v10; FlowJo, Ashland, OR). Ten thousand cells were analyzed, and all samples were analyzed with FL1 fluorescence and side-scatter detectors for dot plots. Dot plots and histograms for cell populations, according to fluorescence (FL1), were plotted for different concentrations of PE-HER2 antibody (**Fig. S16)**. The shift in a number of cells with or without PE-HER2 affibody was monitored. Cells alone without PE-HER2 antibody were used as a negative control for the experiment. The experiments were repeated three times.

Table S1. Analytical data for peptides used in the study

Code	Sequence	Calculated average mass (Da)	Experimental mass m/z	Purity % by HPLC
Compound 18*	Cyclo(1,10)PpR-(***R***-X)FDDF-(***R***-X)R	1425.58	1425.60*	>95
SFTI-G1	Cyclo(C(***S***-X)RDR(***S***-X)CFDSIDF)	1750.95	1750.87	>90
SFTI-G2	Cyclo(C(***R***-X)RPpR(***R***-X)CFF)	1399.68	1399.73	>95
SFTI-G3	Cyclo(C(***R***-X)RPpR(***R***-X)CFDDF)	1629.86	1629.69	>95
SFTI-G4	Cyclo(C(***S***-X)-RPGR (***S***-X)CFDSIPPDF)	1984.26	1984.95	>95
SFTI-G5	Cyclo(C(***S***-X)RIPPR(***S***-X)CFPDDF)	1840.13	1839.73	>95
SFTI-G6	Cyclo(C(***R***-X)RIPPR(***R***-X)CFPDDF)	1840.13	1839.84	>90
Control	H_2N-K(***S***-(3-amino-3(biphenyl)propionic acid)F-OH	517.63	517.28	>95
Control, SFTI-1	Cyclo(GDPFCIPPISKTCR)	1513.78	1513.89	>90

*X is(3-amino-3-napthyl)-propionic acid; Uppercase letters represent L-amino acids; lowercase letters represent D-amino acids; Compound 18 was reported in Kanthala et al., Oncotarget 2017; 8(43): 74244-62. HR-MS reported for 18. MALDI-TOF mass spectral data is reported for all other peptides. Average mass is represented for calculated mass for SFTI series. The monoisotopic mass for the experimental MALDI data can be obtained by subtracting 1.007277 Da from the value in the mass spectrum.

Table S2. Combination index study. Erlotinib was at a constant concentration of 10 μM and concentration of SFTI-G5 was varied from nanomolar to micromolar concentration. Combination index was determined in NCI-H1975 lung cancer cell lines (EGFR double mutation). Then SFTI-G5 was at constant concentration of 3 μM and erlotinib concentration was varied from nM to micromolar range. Combination index was calculated. Similar studies with SFTI-G5 and lapatinib was carried out and combination index was calculated. SFTI-G5 exhibited synergistic effect with erlotinib and lapatinib in NCI-H1975 lung cancer cell lines.

Combination Index:

$$CI = \frac{C_{A,x}}{IC_{x,A}} + \frac{C_{B,x}}{IC_{x,B}}$$

Combinations		CI VALUES
COMBINATION I (SFTI-G5 & Erlotinib 10 μM)	0.247	0.76972678
COMBINATION II (SFTI-G5- 3μM & Erlotinib)	1	0.78151921
COMBINATION I (SFTI-G5 & Lapatinib 5 μM)	0.17	0.83441076
COMBINATION II (SFTI-G5- 3μM & Lapatinib)	0.285	0.75566229

(CI <1: synergistic effect, CI =1 : additivity, CI >1: antagonistic effect)

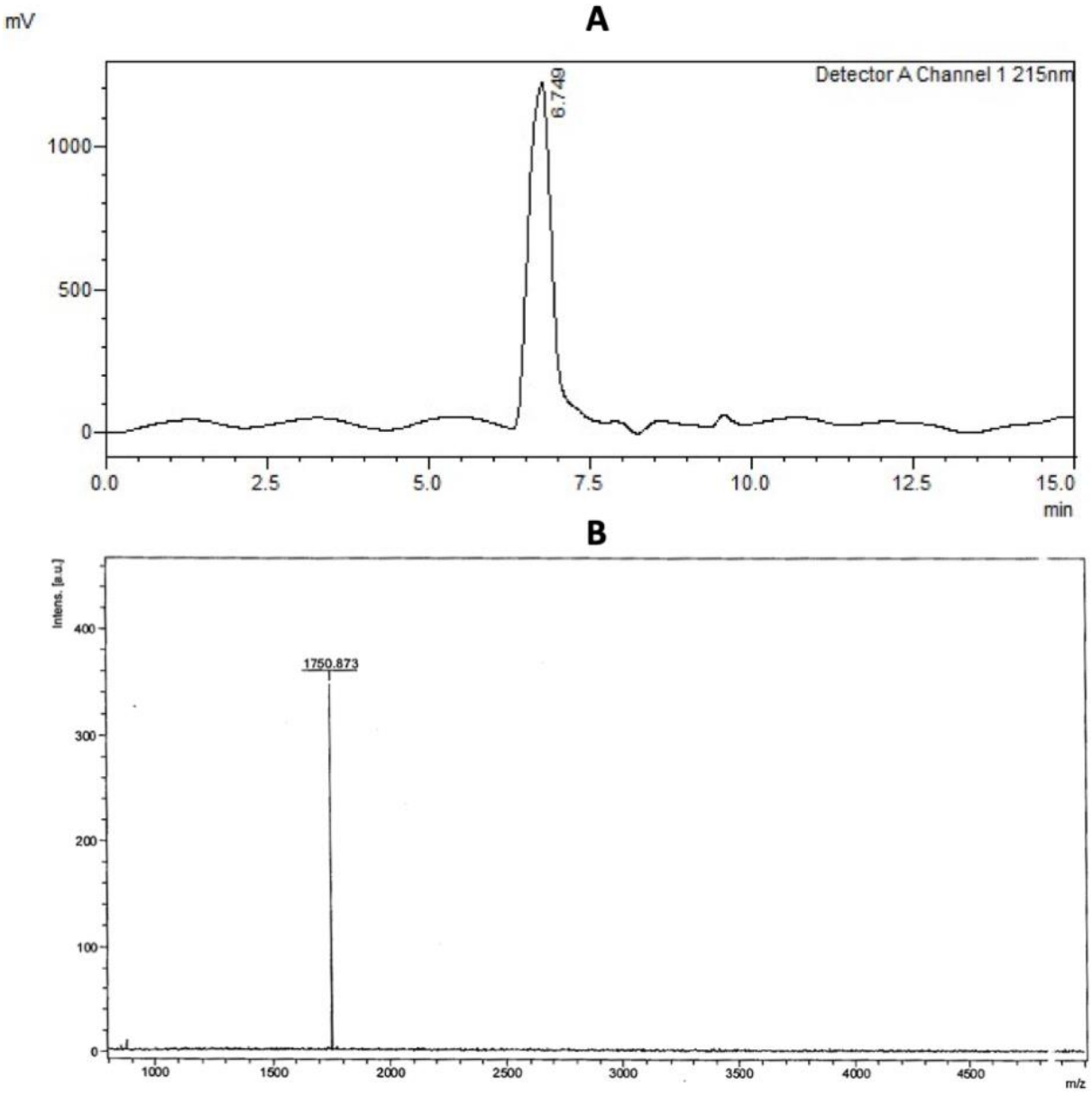

Figure S1. HPLC (A) and MALDI-TOF mass spectra (B) of SFTI-G1.

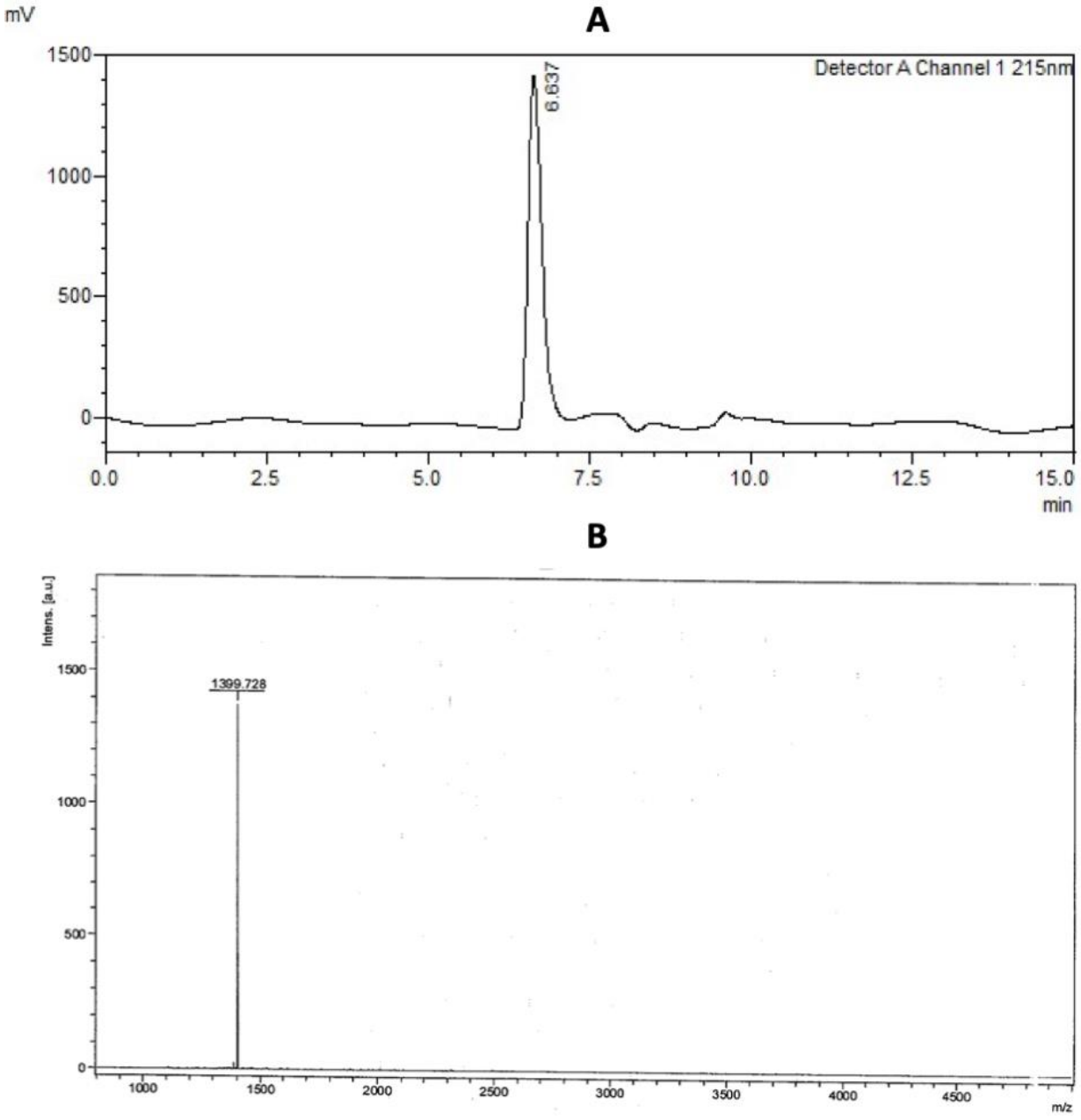

Figure S2. HPLC (A) and MALDI-TOF mass spectra (B) of SFTI-G2.

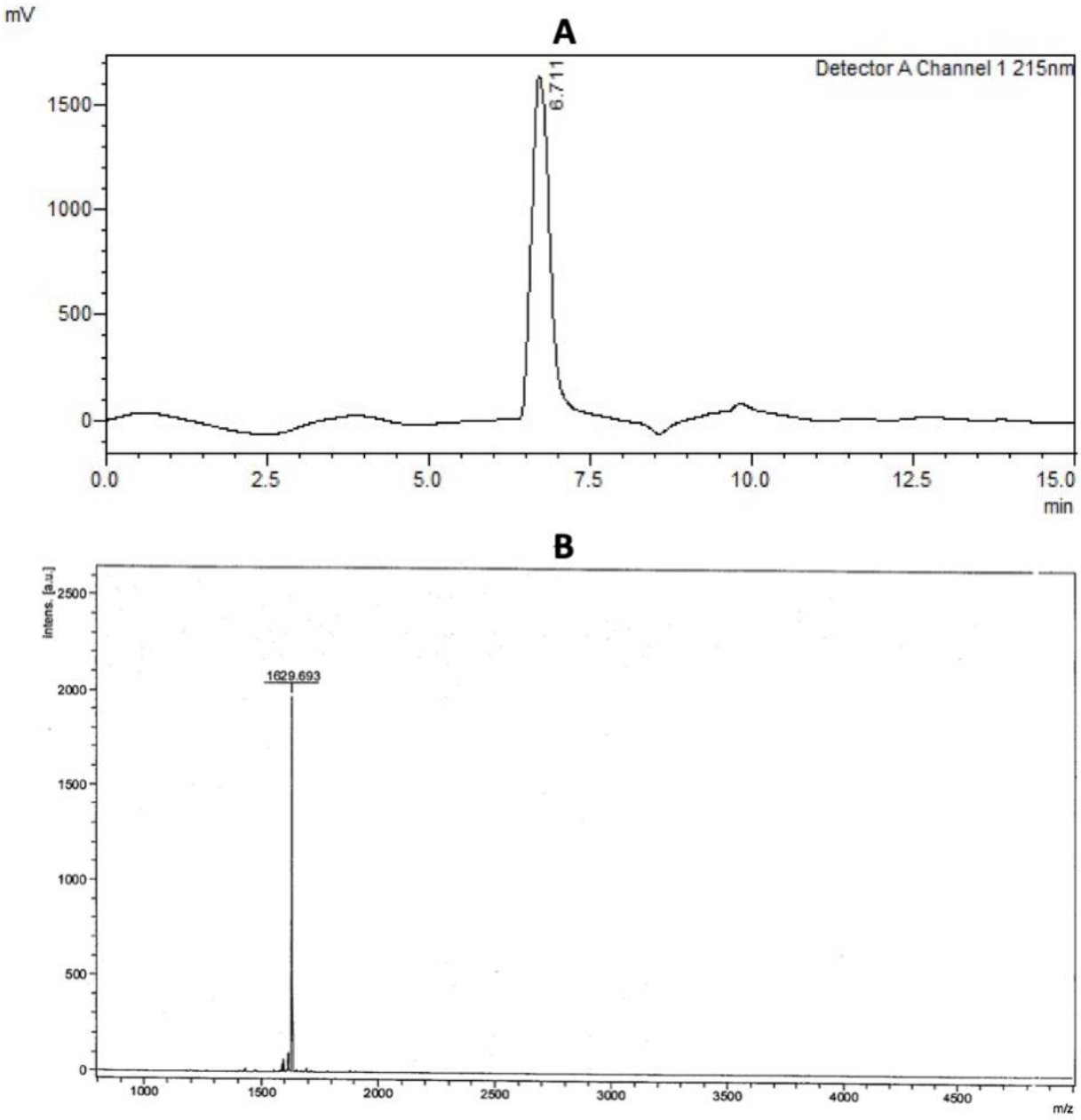

Figure S3. HPLC (A) and MALDI-TOF mass spectra (B) of SFTI-G3.

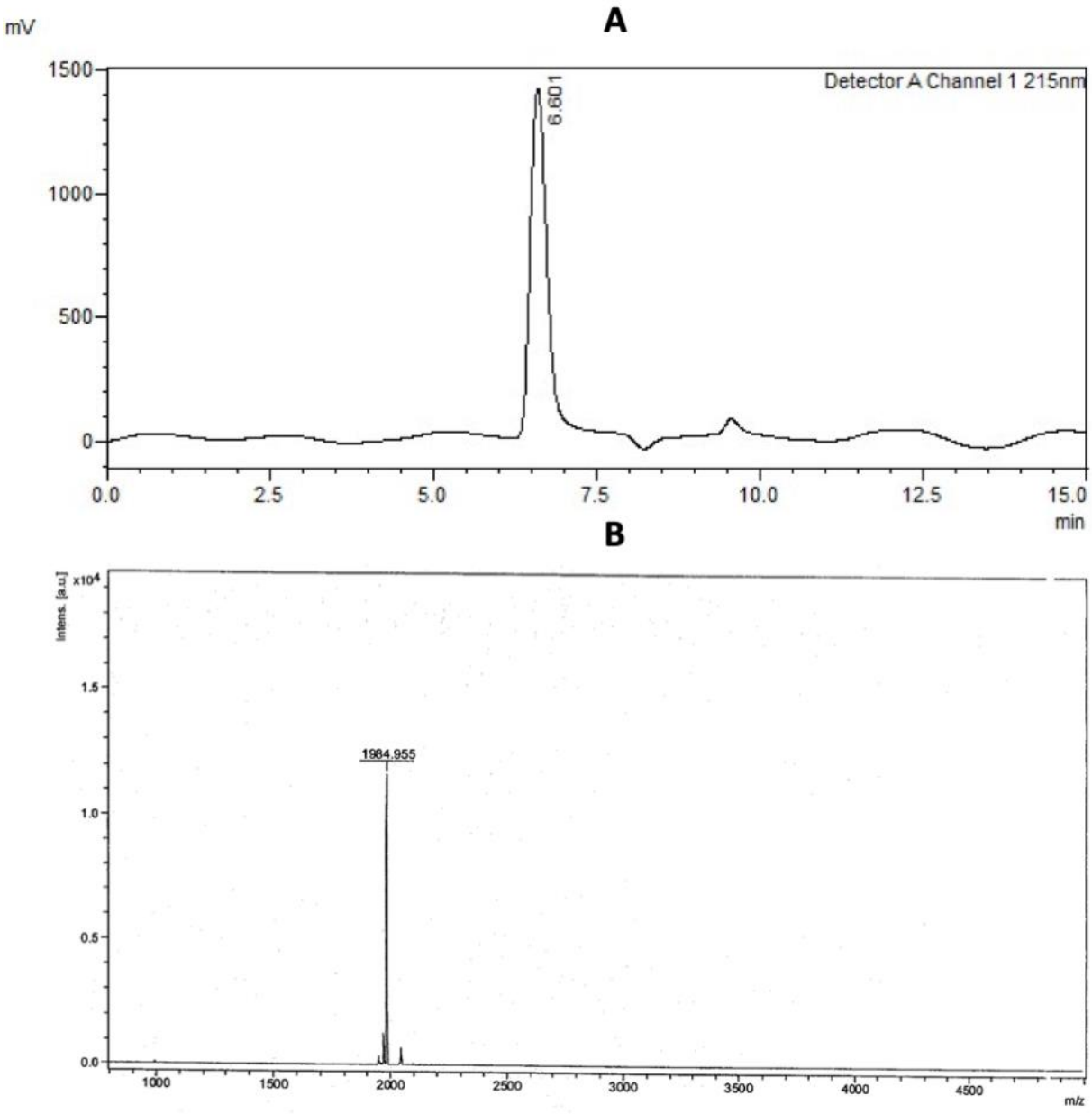

Figure S4. HPLC (A) and MALDI-TOF mass spectra (B) of SFTI-G4.

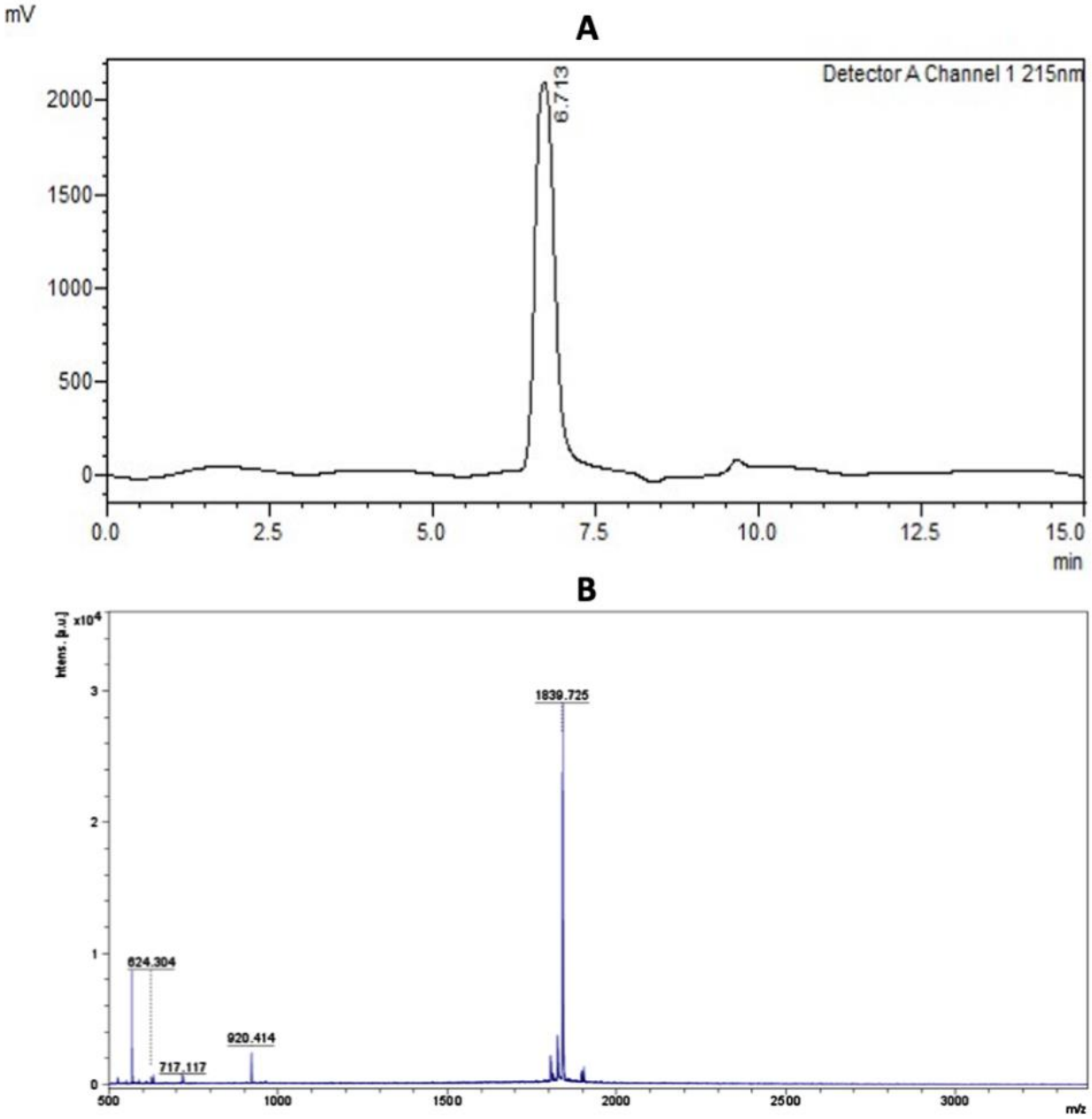

Figure S5. HPLC (A) and MALDI-TOF mass spectra (B) of SFTI-G5.

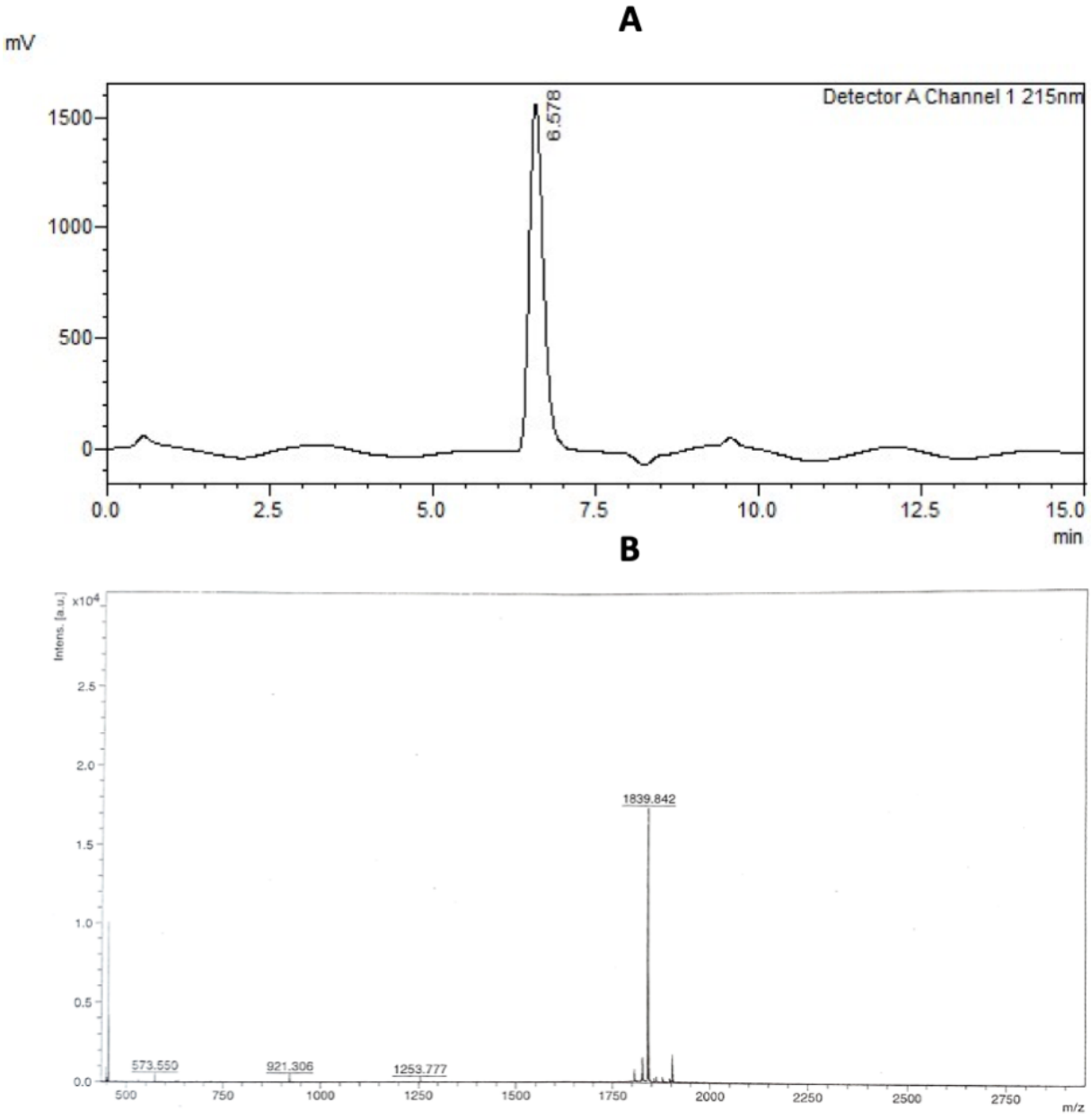

Figure S6. HPLC (A) and MALDI-TOF mass spectra (B) of SFTI-G6.

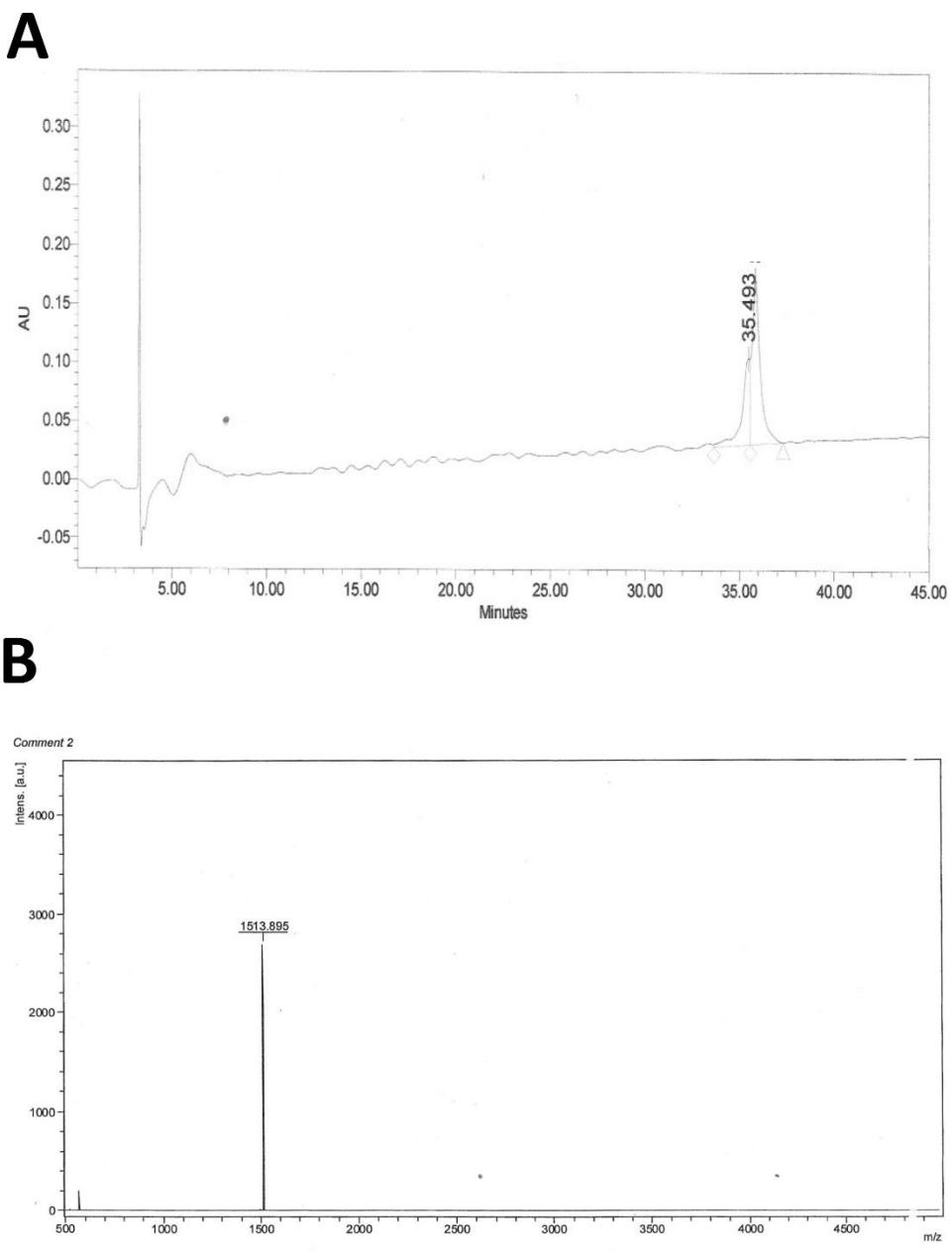

Figure S7. HPLC (A) and MALDI-TOF mass spectra (B) of control SFTI-1.

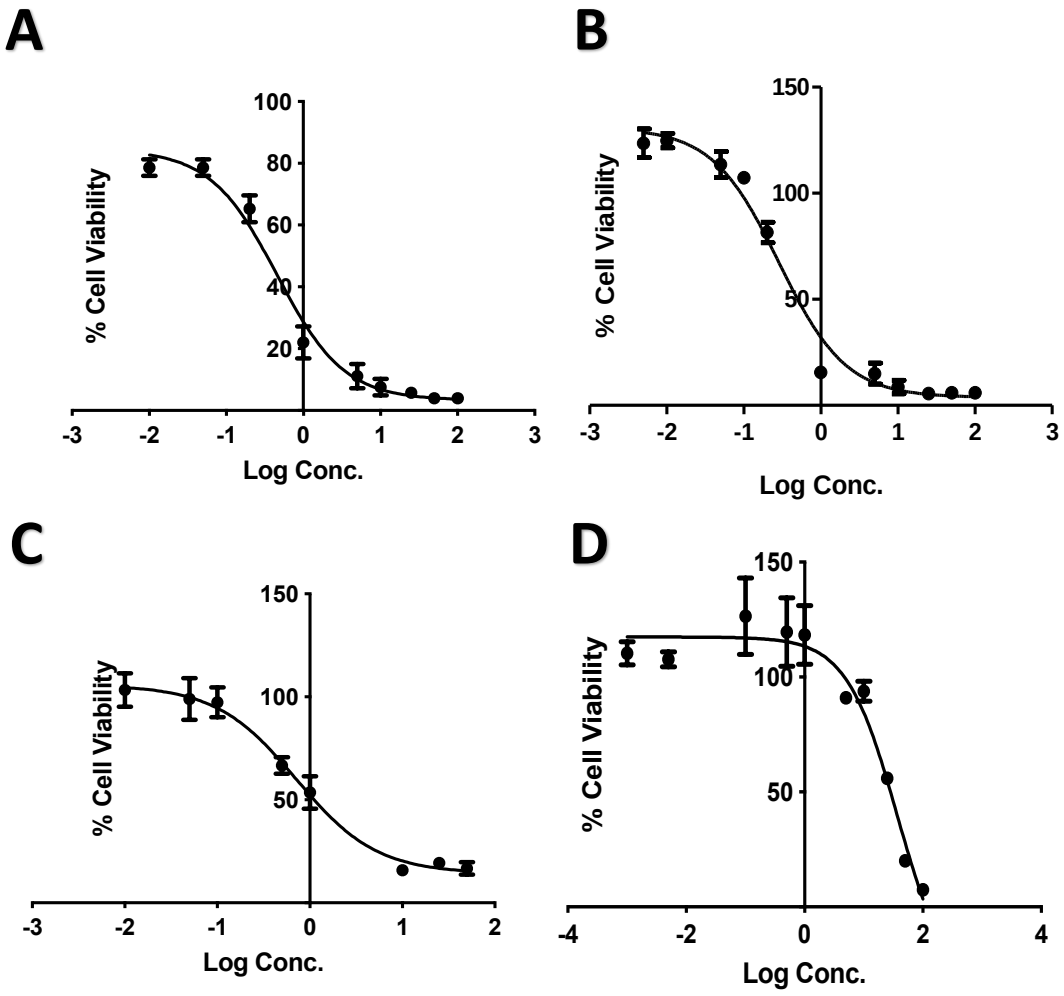

Figure S8. Dose-response curve for SFTI-G5 in different cell lines A) BT-474, B) Calu-3, C) A549, and D) MCF-7. IC_{50} value was calculated from dose response curve.

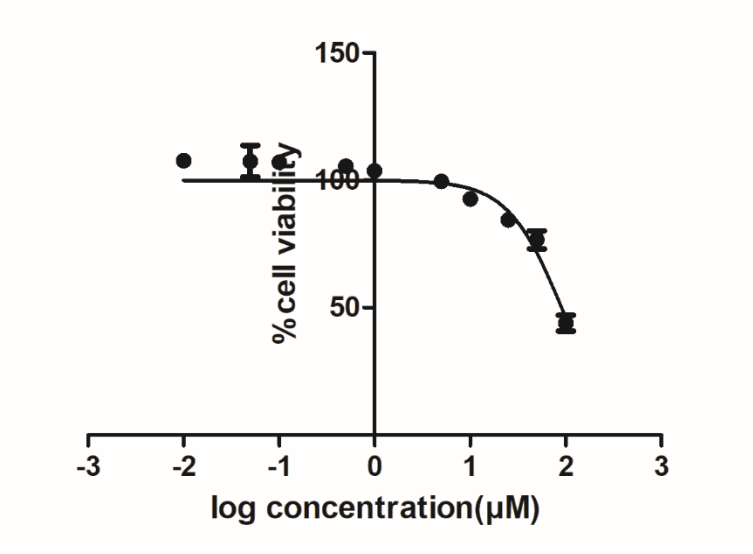

Figure S9. Dose-response curve for SFTI-G5 in different MCF-10A cells. IC_{50} value was calculated from dose response curve.

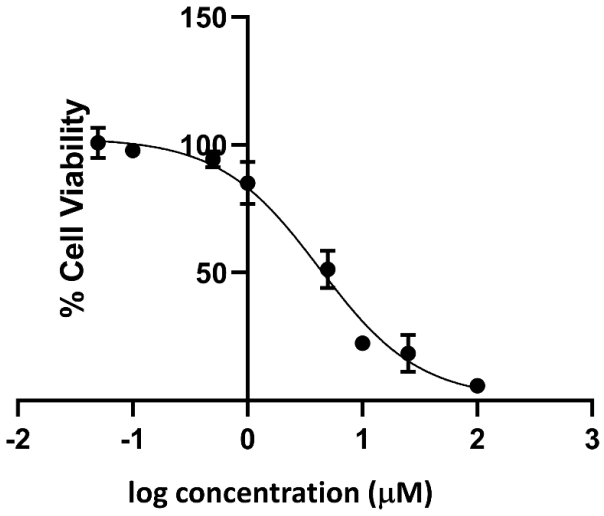

Figure S10. Dose-response curve for SFTI-G5 in different NCI-H1975 cells. IC_{50} value was calculated from dose response curve.

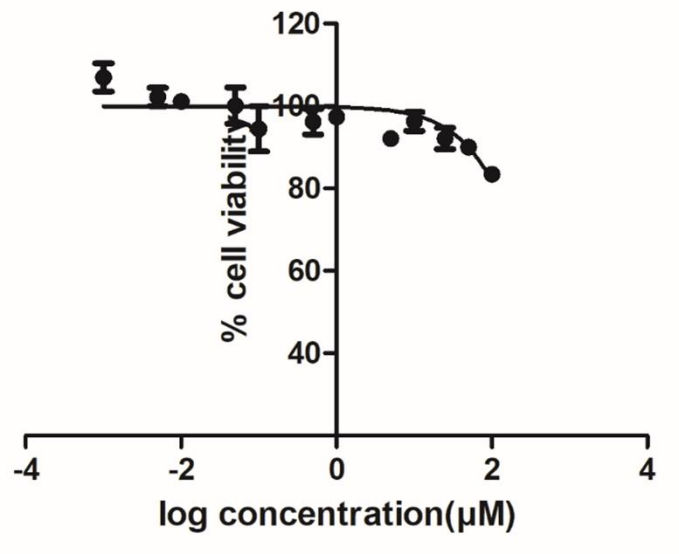

Figure S11. Dose-response curve for SFTI-G5 in human lung fibroblast cell lines (HLF).

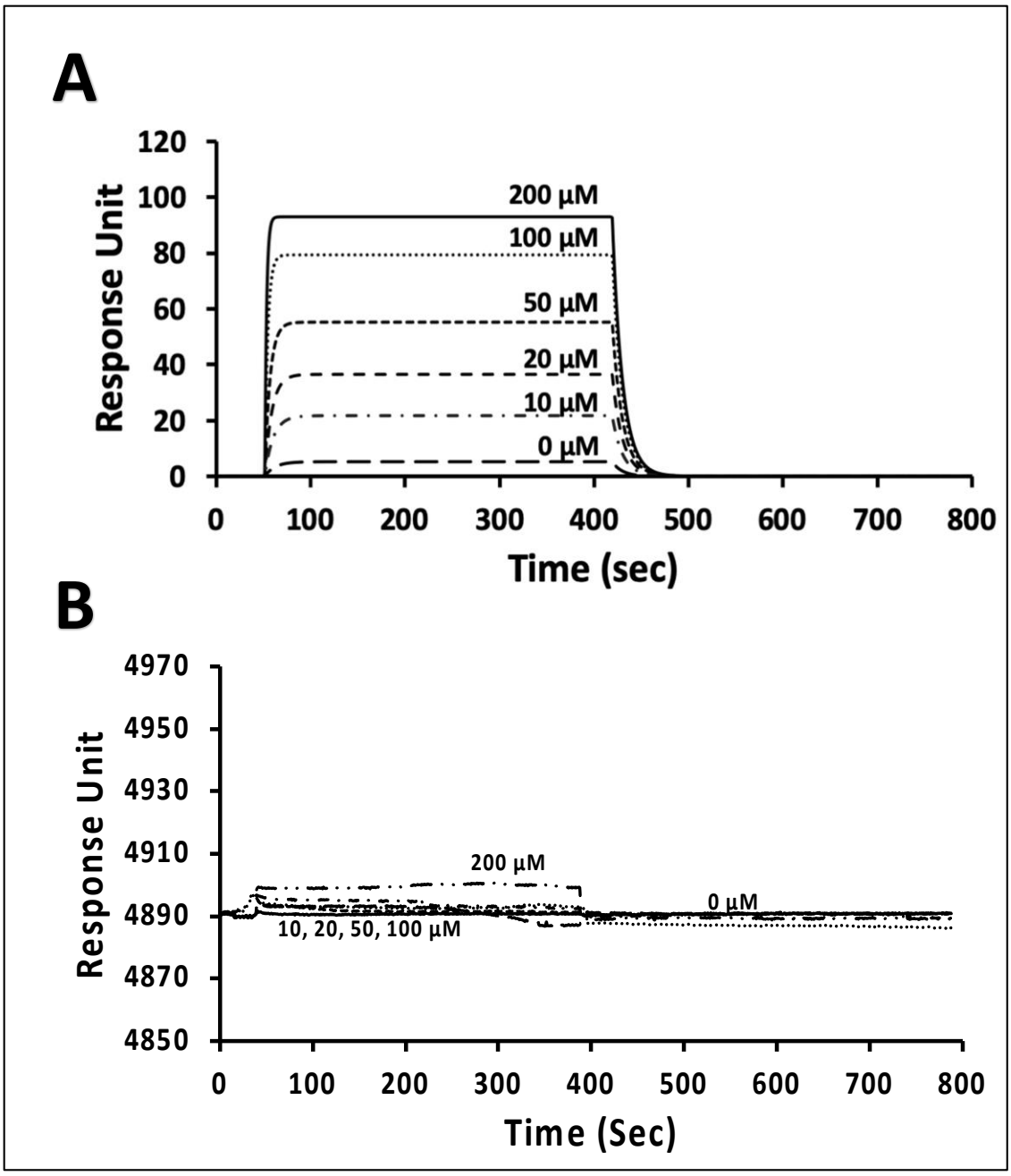

Figure S12. A) SPR analysis of SFTI-G5 binding to HER2 domain IV of extracellular domain. Kinetics of association and dissociation are shown with K_d value 3.46 x 10^{-7} M. B) SPR analysis of control compound binding to HER2 ECD domain.

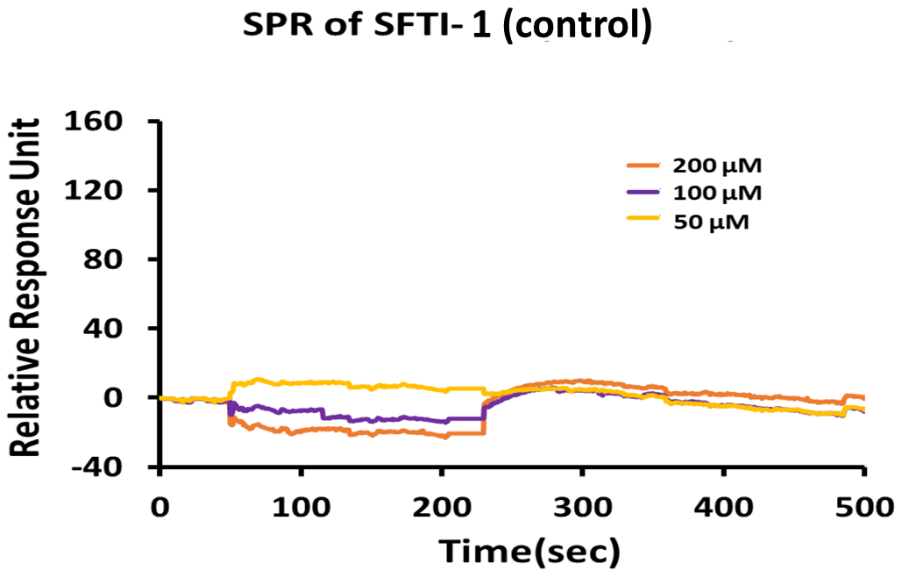

Figure S13. SPR analysis of control compound SFTI-1 binding to HER2 ECD.

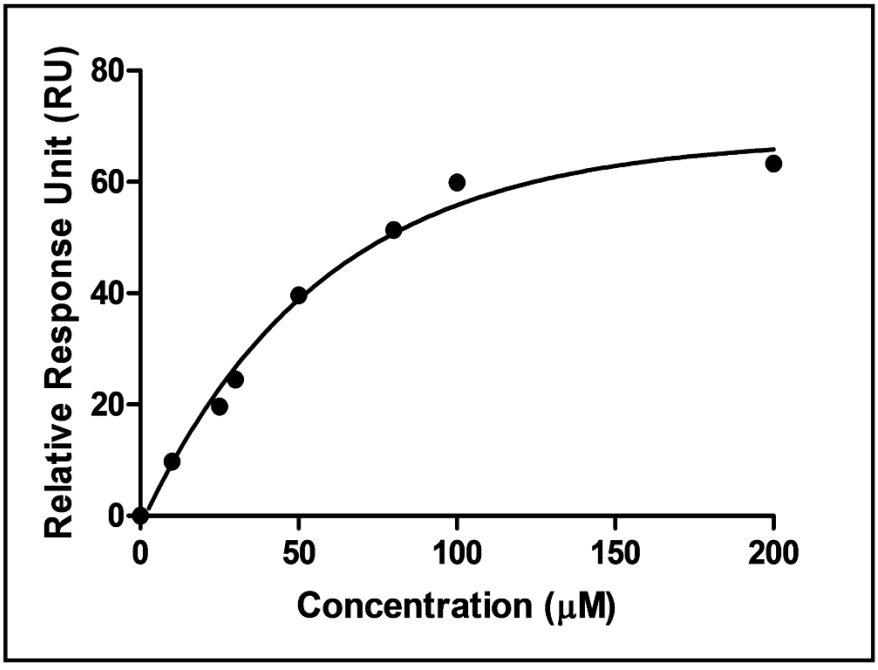

Figure S14. Saturation of response signal from SPR sensorgram for binding of SFTI-G5 to HER2 ECD.

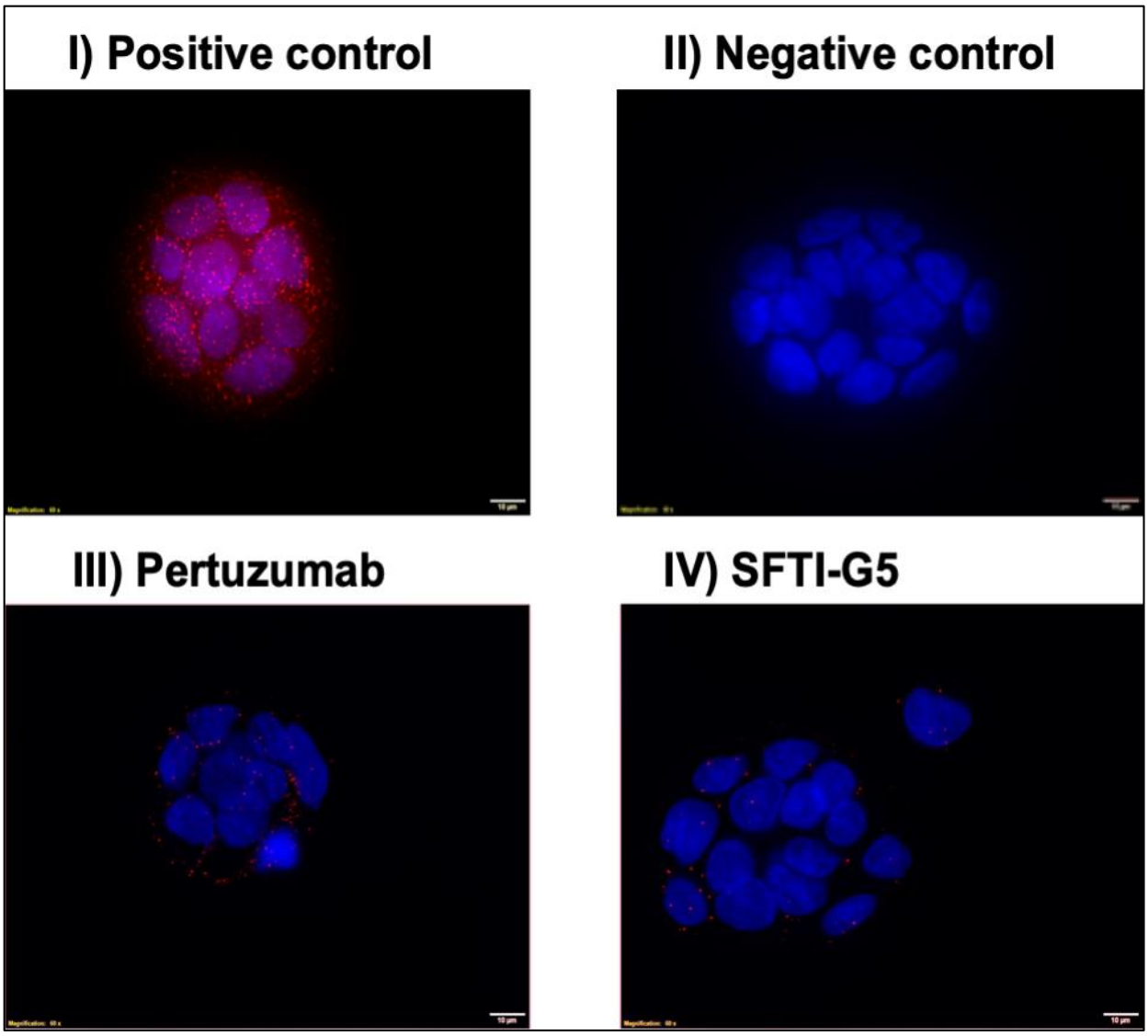

Figure S15. Result of Proximity ligation assay: Inhibition of heterodimerization of EGFR: HER2 in Calu-3 cells. Each red dot represents a heterodimer (I); cells without primary antibodies (II); cells in presence of Pertuzumab (200 nM), a HER2 dimerization inhibitor (III); and cells in presence of SFTI-G5 (1 μM). The nucleus was stained with DAPI. Magnification 60 ×, Scale bar 10 μm.

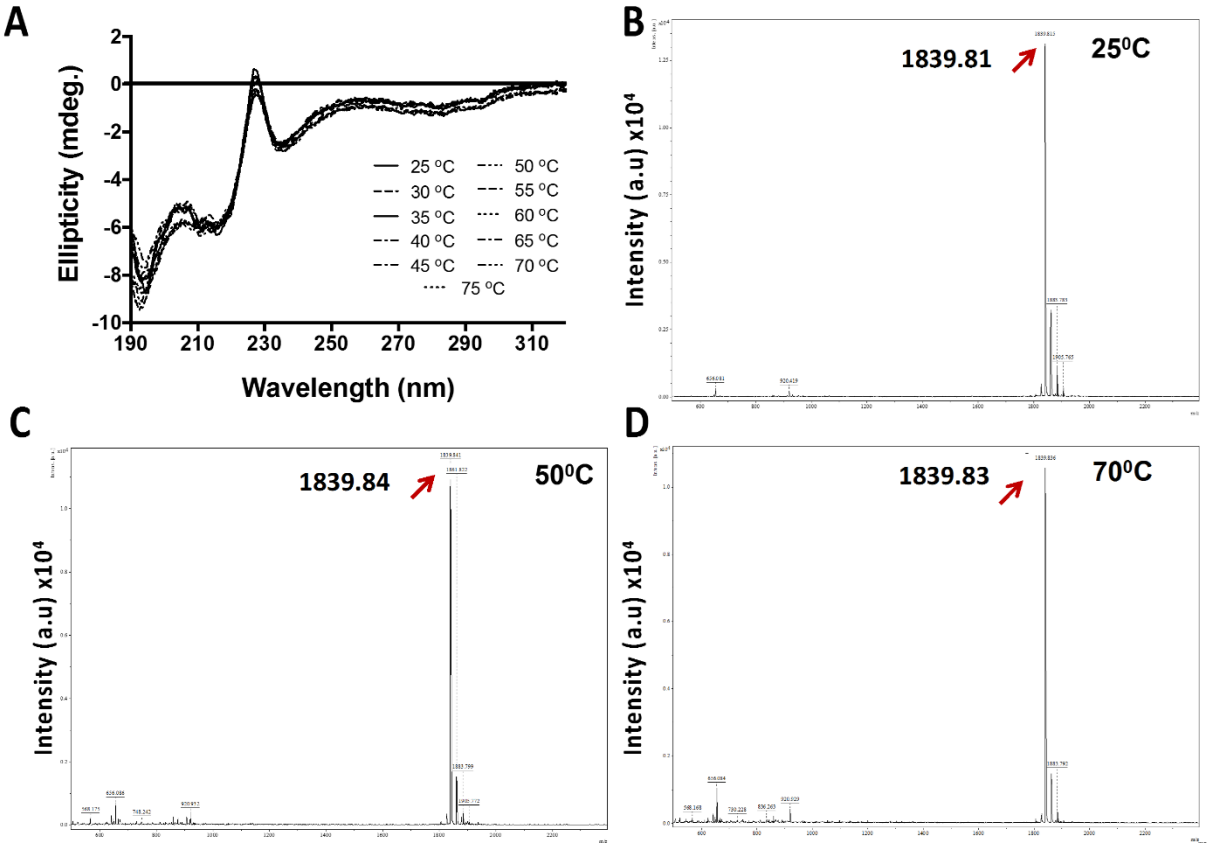

Figure S16: A) Thermal stability of SFTI-G5 evaluated by CD spectroscopy. Note that there was no significant difference in the CD spectra when temperature of the sample was changed from 25 ℃ to 75 ℃. The overall conformation of the peptide remained the same at all temperatures studied. B, C, & D) To verify that the peptide was intact at high temperature (70 ℃), the samples were analyzed by mass spectrometry. Mass spectrometry analysis suggested that the peptide was stable up to 70 ℃ as shown by intact peptide molecular ion (m/z 1839 ± 0.013 at temp 25, 50 and 70 ℃).

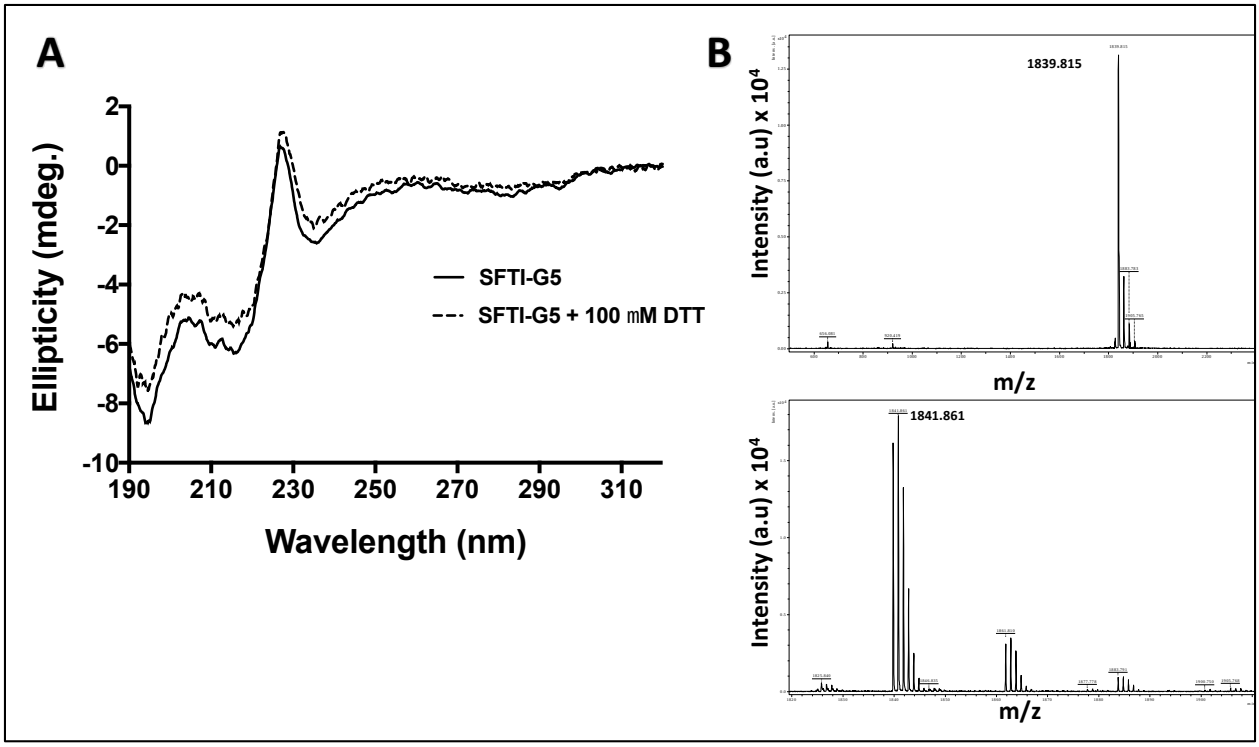

Figure S17. A) Overlay CD spectra of SFTI-G5 incubated with a reducing agent, DTT. SFTI-G5 was mixed with 100 µM concentration of DTT and analyzed in CD. B) Mass spectra of SFTI-G5 before and after the addition of different concentrations of DTT. Samples used for CD studies above were analyzed by MALDI-TOF mass spectrometry. SFTI-G5 without DTT showing molecular ion at 1839.81 showing intact disulfide bond and the addition of DTT to SFTI-G5. Molecular ion corresponding to 1841.86 indicates the reduction of disulfide bond.

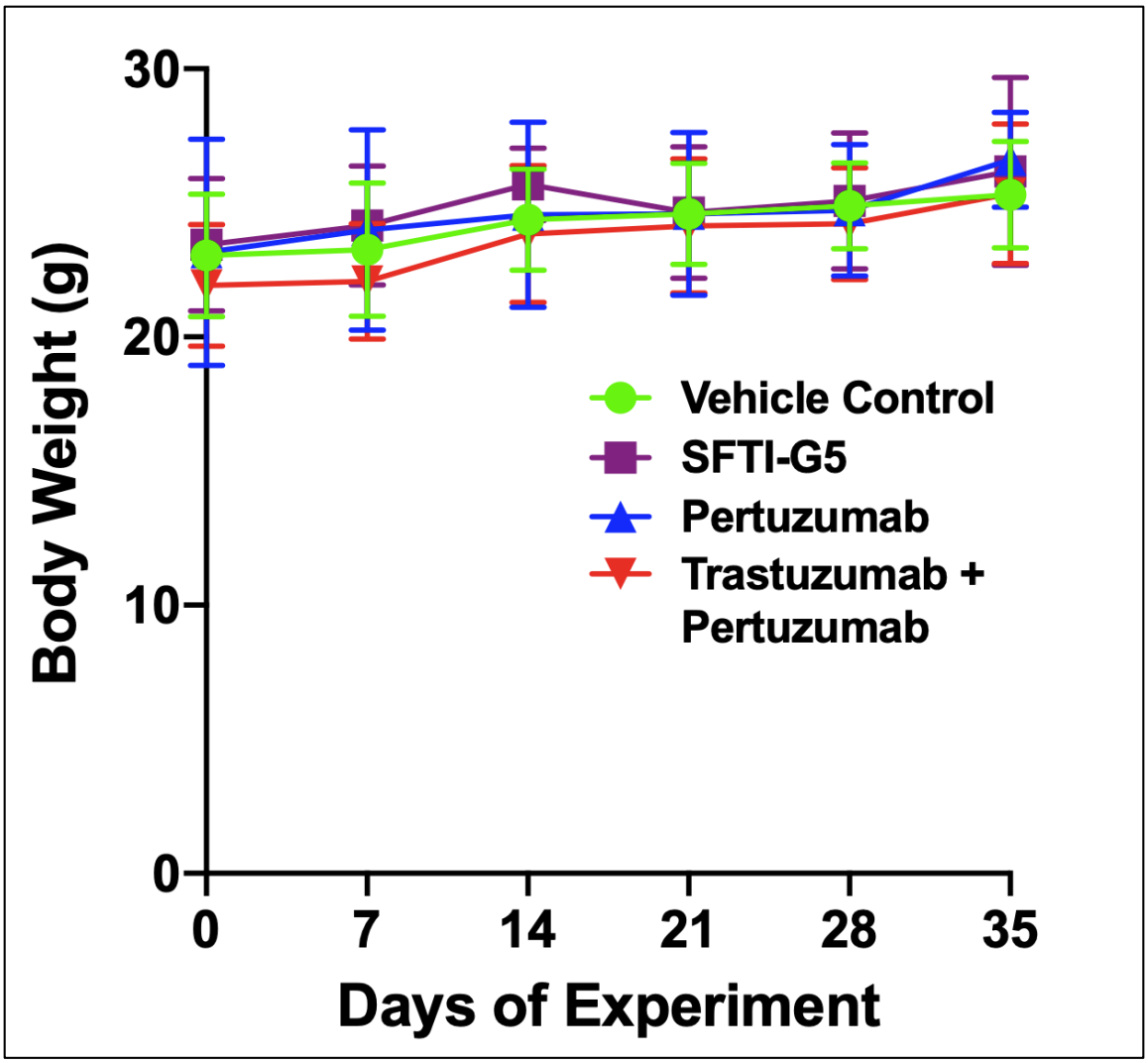

Figure S18. The body weight changes of Foxn1 nude mice treated with SFTI-G5, Pertuzumab, and trastuzumab and pertuzumab in combination in comparison with vehicle control group. Error bar represents standard error of the mean, n=6 for each group.

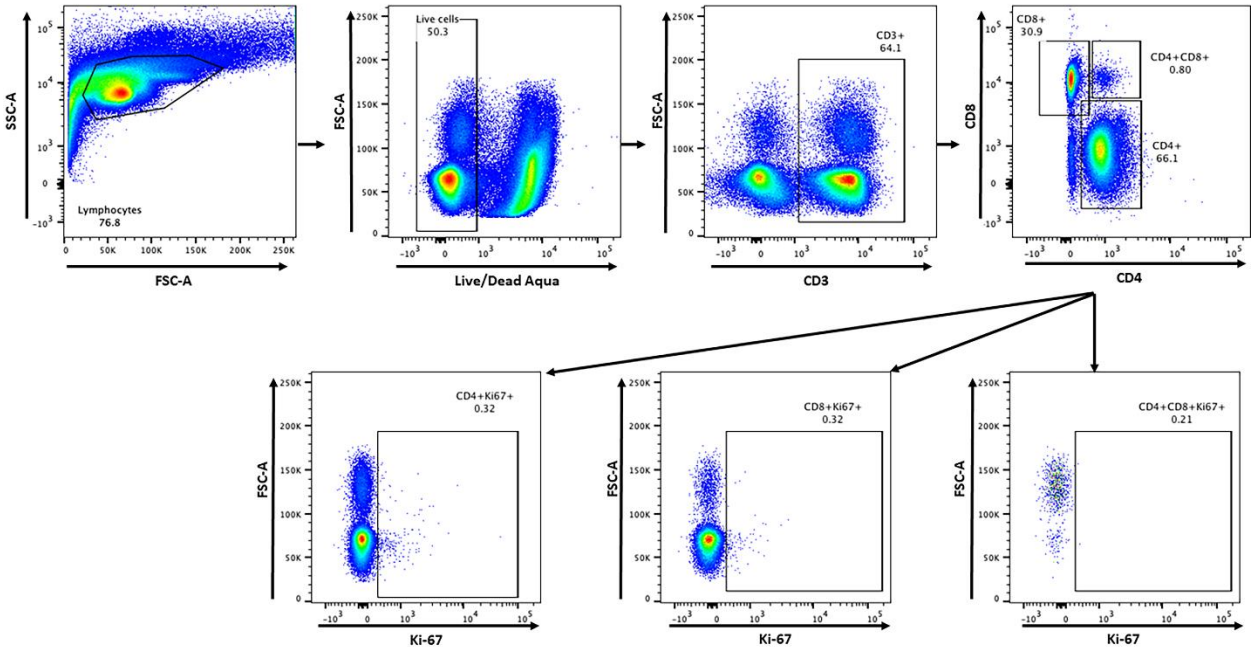

Figure S19. Gating strategy for the detection of ki-67 expression in CD3+ populations (CD4+, CD8+, and in CD4+8+ populations) in control group.

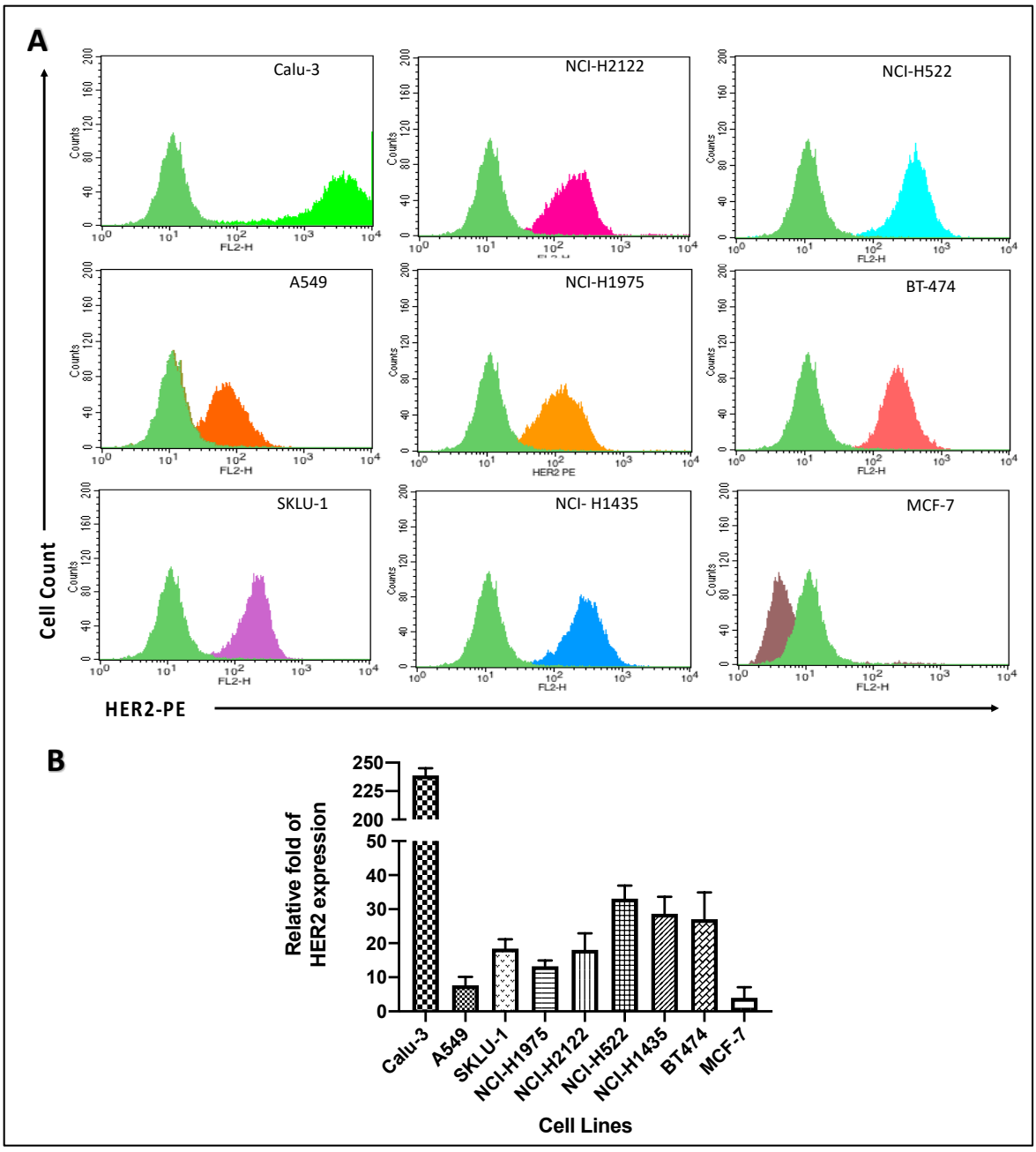

Figure 20. Fluorescence-activated cell sorter analysis of expression of HER2 determined by using PE-HER2 antibody in different cell lines. A) For cells that overexpress HER2, there was a shift in cell population towards the right side after binding of PE-HER2 antibody compared to cells without antibody label. B) Quantification of relative amount of HER2 expression with respect to cells without PE-HER2 antibody.

References

1. R. Sable, T. Durek, V. Taneja, D. J. Craik, S. Pallerla, T. Gauthier and S. Jois, *ACS Chem Biol*, 2016, **11**, 2366-2374.
2. O. Cheneval, C. I. Schroeder, T. Durek, P. Walsh, Y. H. Huang, S. Liras, D. A. Price and D. J. Craik, *J Org Chem*, 2014, **79**, 5538-5544.