Supporting information

Multiplexed Targeting of Barrett's Neoplasia with a Heterobivalent Ligand: Imaging Study on Mouse Xenograft In Vivo and Human Specimens Ex Vivo

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Materials and Methods

Chemicals

Peptide synthesis reagents (N^{α}-Fmoc protected amino acids, HBTU and HOBt) were purchased from either Anaspec or AAPPTec. The side-chain protecting groups used the following amino acids: Arg(N^{ω}-Pbf), Asn(N^{γ}-Trt), Glu(O-*t*Bu), His(N^{im}-Trt), Ser(*t*Bu), Lys(N^{ε}-Alloc), Lys(N^{ε}-Ivdde). Fmoc-AEA, Fmoc-AEEA, Fmoc-AE3A, Fmoc-AE6A Fmoc-AE10A and 6-aminohexanoic acid were purchased from AAPPTec. These reagents are analytical grade with >99% purity, and were used without further purification. Rink amide MBHA resin with initial loading of 0.2 mmol/g was acquired from Protein Technologies, Inc. Cy5.5-NHS ester was acquired from Lumiprobe. Analytical grade solvents for peptide synthesis and HPLC were purchased from Fisher Scientific, and were used without further purification unless otherwise stated.

Peptide monomers and heterodimer

Peptide monomers and heterodimers were synthesized and labeled with Cy5.5 using standard Fmoc solid-phase chemical synthesis with rink amide MBHA resin in a PS3 automatic synthesizer (Protein Technologies Inc). Fmoc protected L-amino acids were applied with standard HBTU/HOBt activation. The C-terminus lysine was incorporated as Fmoc-Lys(Alloc)-OH for fluorophore labeling. Upon completion of peptide assembly, the resin was transferred to a reaction vessel for manual labeling with dye. The Alloc side chain protecting group was removed with a palladium catalyst, as described previously.[35] The resin was washed with dimethylformamide (DMF) and dichloromethane (DCM) for 1 min 3X. The protected resin-bound peptide was reacted with Cy5.5-NHS ester and (N,N-Diisopropylethylamine) DIPEA for

12 hours. The completion of the reaction was monitored with a qualitative ninhydrin test. A cleavage cocktail reagent TFA: TIS: H_2O (95:2.5:2.5 v/v/v) was mixed with the resin, and stirred for 2 hours in dark conditions at 25^oC. The crude peptides were isolated from the resin by filtration and evaporated with N_2 gas followed by precipitation with chilled diethyl ether and stored at -20^oC for 12 hours. The precipitated peptides were centrifuged and washed 2X with ether, dried, dissolved in water, and lyophilized to produce a dark-green powder. The crude peptides were purified by prep-HPLC with a C18 column (Waters Inc) using a water (0.1% TFA)-acetonitrile (0.1% TFA) gradient. Peptide purity was tested using an analytical C18-column. Further characterization was confirmed by either ESI (Waters Inc.) or Q-TOF (Agilent Technologies) mass spectrometry.

The absorbance spectra of the Cy5.5-labeled peptides were characterized with a spectrophotometer (NanoDrop 2000, Thermo Scientific). Fluorescence excitation and emission from a 1 μM peptide solution diluted in PBS was collected with a fiber coupled spectrophotometer (Ocean Optics) using a diode-pumped solid-state laser (Technica Laser Inc) with λ_{ex} = 671nm. The spectra were plotted with Origin 8.5 software (OriginLab Corp).

Peptide heterodimer validation with confocal microscopy

Cells were cultured to ~70% confluence on glass coverslips, washed with PBS, and incubated with 2% BSA in PBS for 30 min to block non-specific binding. 1 μM of Cy5.5-labeled peptides were added and incubated for 30 min at 4°C. The cells were then washed 3X with PBS, fixed with ice cold 4% paraformaldehyde (PFA) for 10 min, washed with PBS 1X, and then mounted on glass slides with ProLong Gold reagent containing DAPI (Invitrogen). For antibody staining, cells were incubated with either anti-EGFR (1:500, #2232S) or anti-ErbB2 (1:500,

#29D8) primary antibody from Cell Signaling Inc overnight at 4°C after fixation, and then washed with PBS 3X and processed with secondary antibody staining. Either goat anti-rabbit IgG (H+L) labeled with Alexa-Fluor 488 (1:1000, #A-11008, Invitrogen) or goat anti-mouse IgG (H&L) labeled with Alexa-Fluor 568 (1:1000, #ab175473, Abcam) was added and incubated for 1 hour at room temperature (RT). The cells were washed with PBS 3X, and mounted onto glass coverslips. Confocal fluorescence images were collected using DAPI, AF488, AF568 and Cy5.5 filter sets. Fluorescence intensities from 3 independent images were quantified using custom Matlab (Mathworks) software.

Western blot

Western blot was performed by lysing cells in Pierce® RIPA lysis buffer containing Halt™ protease inhibitor cocktail (#87786, Thermo Scientific) for 30 min on ice. The lysates were centrifuged at 10,000 rpm for 10 min at 4°C. A BSA protein assay kit (#23227, Thermo Scientific) was used to quantify protein concentration in the supernatant. Aliquots of protein (10 μL) were used for electrophoresis on NuPAGE™ 4-12% Bis-Tris gels (#17031471, Invitrogen) followed by electrophoretic transfer onto membranes (#ISEQ00010, Merck Millipore Ltd). The membranes were incubated with primary rabbit anti-EGFR monoclonal antibody (1:1000, #2232S) and rabbit anti-ErbB2 monoclonal antibody (1:1000, #2165S) from Cell Signaling Inc. Mouse anti-β-tubulin monoclonal antibody (1:500, #32-2600, Invitrogen) was used for loading control. For visualization, horseradish peroxiase (HRP)-conjugated secondary antibodies consisting of goat anti-rabbit IgG (H+L) HRP (1:5000, #65-6120) and goat anti-mouse IgG (H+L) HRP (1:5000, #62-6520) from Thermo Scientific were used, and followed by the ECL kit (#RPN2106, GE Healthcare) per manufacturer instructions.

Statistical Analysis

We fit the fluorescence intensities from the human esophageal specimens according to 4 histological classifications, including squamous (SQ), Barrett's esophagus (BE), high-grade dysplasia (HGD), and esophageal adenocarcinoma (EAC), and used a unpaired *t* test and one-way ANOVA. Co-localization of peptide and antibody binding was evaluated using Pearson's correlation coefficient.

Supplementary Figures

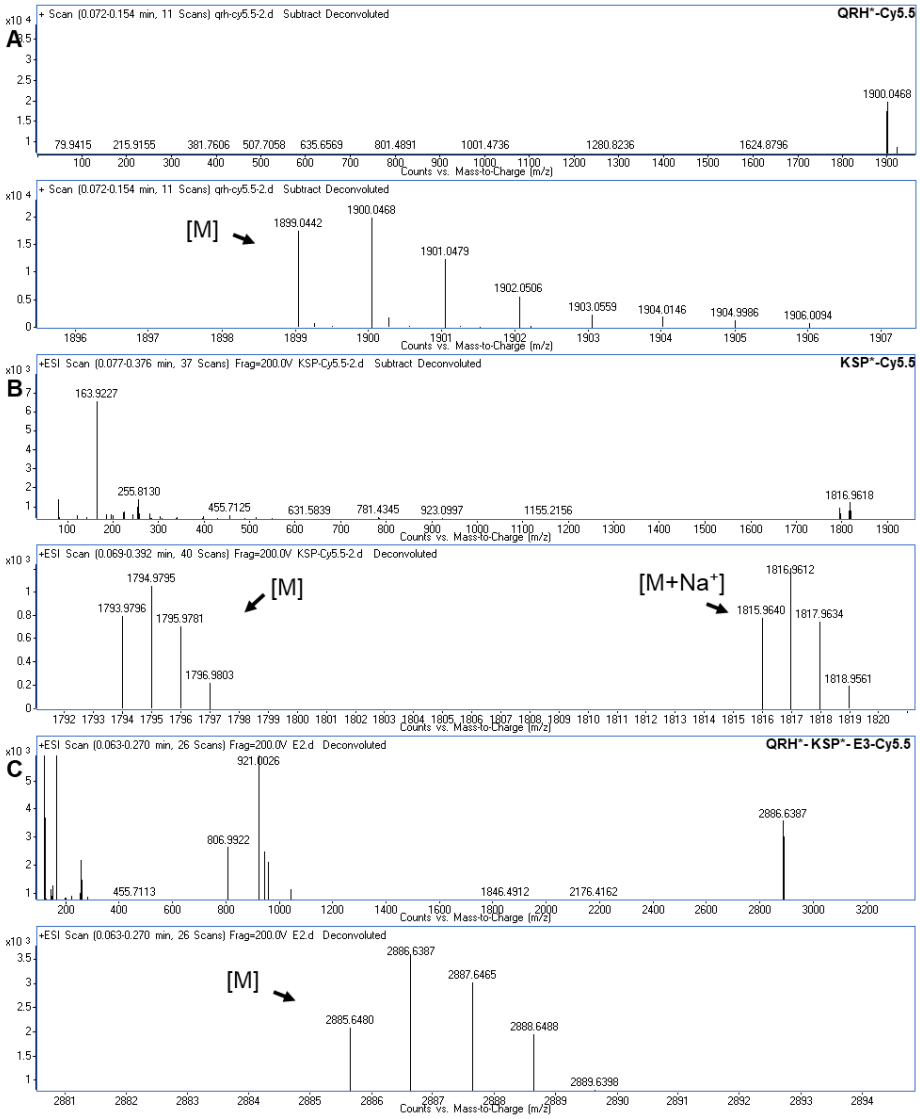


Figure S1 – **Mass spectrometry analysis**. The experimental mass-to-charge (m/z) ratios for the Cy5.5-labeled peptides were found to be 1794.98, 1900.04, and 2974.69 for **A**) QRH*-Cy5.5, **B**) KSP*-Cy5.5, and **C**) QRH*-KSP*-E3-Cy5.5, respectively. These results agree with expected values. Key: M - molecular weight, Na^+ - sodium.

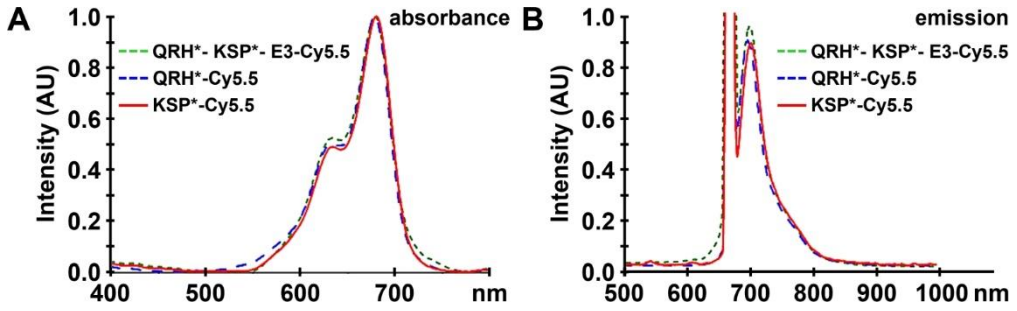


Figure S2 – **Spectral properties**. QRH*-Cy5.5, KSP*-Cy5.5 and QRH*-KSP*-E3-Cy5.5 show **A**) maximum absorbance at λ = 680 nm and **B**) peak emission at λ = 708 nm.

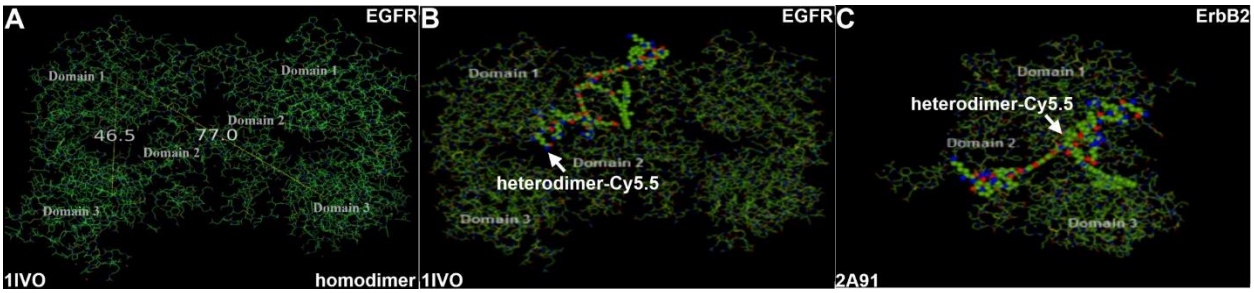


Figure S3 – **Peptide binding on structural models**. **A**) EGFR (1IVO) forms a homodimer prior to binding. The extracellular domain (ECD) has a head-to-head and head-to-tail dimensions of ~46.5 and ~77.0 Å, respectively. The triethyleneglycol (PEG3) linker (E3) provides the best spacing for the heterodimer QRH*-KSP*-E3-Cy5.5 to bind to **B**) domain 2 of EGFR with energy E_t = -656.47 and **C**) domain 3 of ErbB2 with E_t = -632.83.

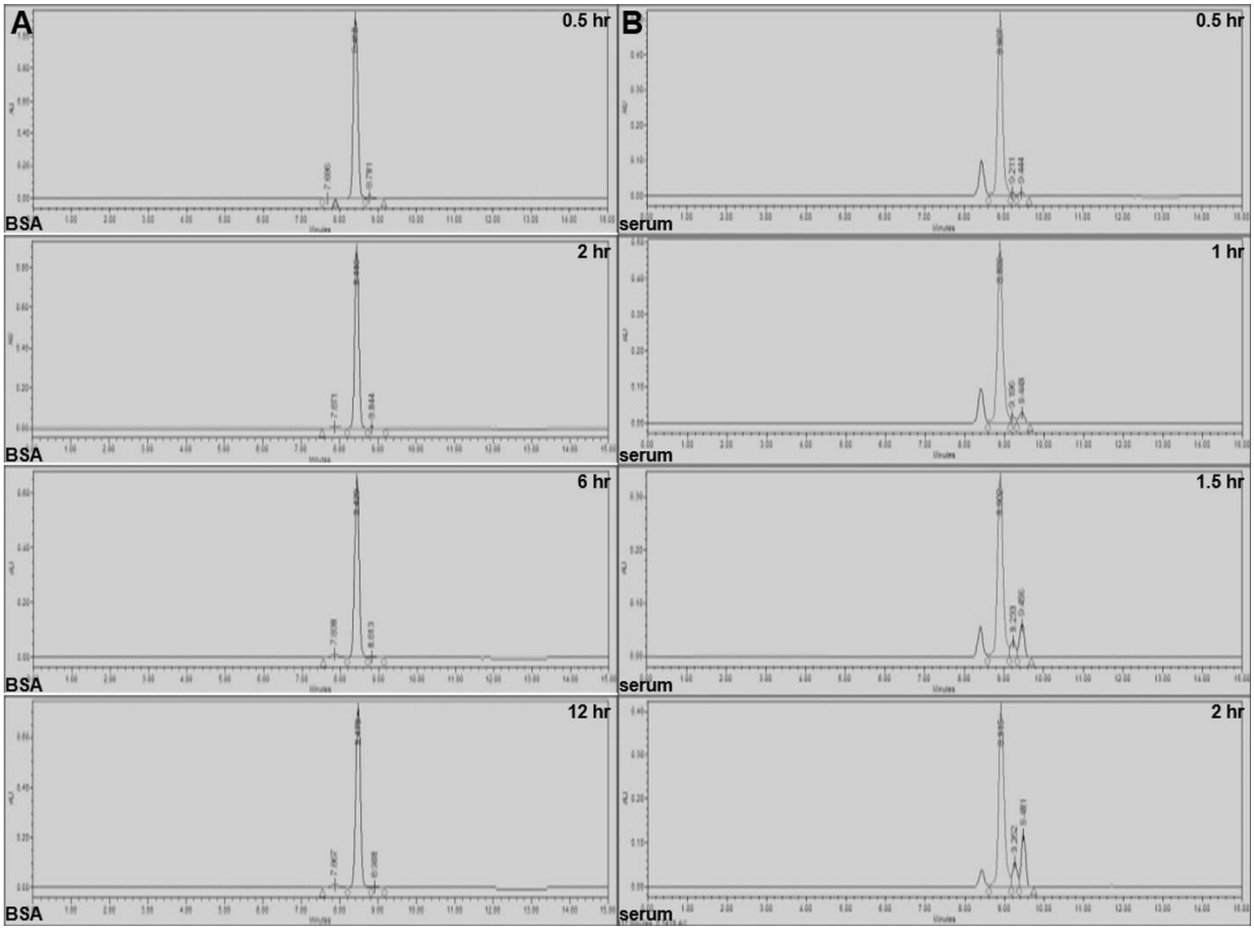


Figure S4 – **Heterodimer stability**. At 37ºC, no noticeable heterodimer degradation was observed by HPLC in either **A)** PBS containing 0.1% BSA at 0.5, 2, 6 and 12 hours or in **B)** mouse serum with 30 μM concentration at 0.5, 1.0, 1.5 and 2 hours. We observed a new peak formed that may arise from degradation and at 2 hour the area of new formed peak is about ~20%.

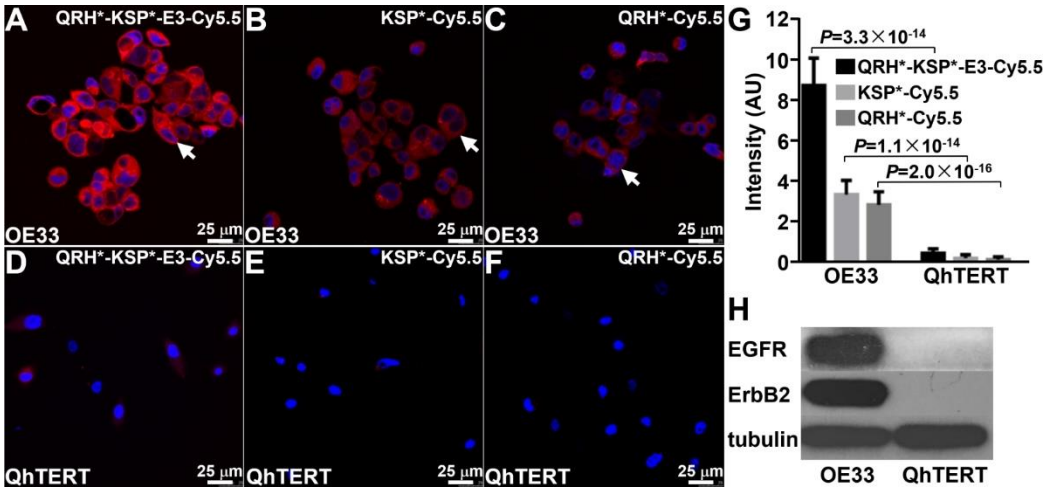


Figure S5 – **Comparison of binding between heterodimer and monomers**. On confocal microscopy, greater fluorescence intensity was observed from binding of the **A**) peptide heterodimer to the surface (arrow) of OE33 (human esophageal adenocarcinoma) cells by comparison with **B**,**C**) monomers. **D-F**) Minimal signal was seen with QhTERT (human non-dysplastic Barrett's esophagus) cells used as control. **G**) Quantified results show a mean fluorescence intensity of 8.83±1.36 for QRH*-KSP*-E3-Cy5.5 versus 3.39±0.72 and 2.87±0.65 for KSP*-Cy5.5 and QRH*-Cy5.5, respectively. Measurements are an average of 10 randomly chosen cells from 3 images collected independently. *P*-values were calculated by unpaired *t* test. **H**) Western blot for OE33 and QhTERT cells is shown.

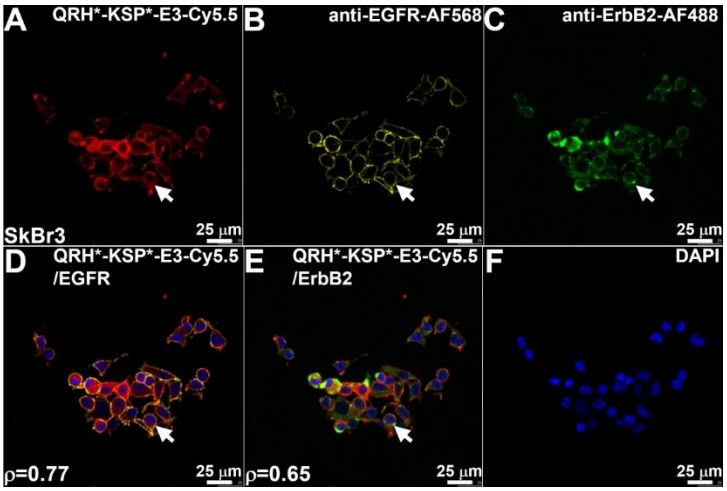


Figure S6 – **Co-localization of peptide and antibody binding**. On confocal microscopy, strong binding is seen by **A**) QRH*-KSP*-E3-Cy5.5 (red), **B**) AF568-labeled anti-EGFR antibody (yellow), and **C**) AF488-labeled anti-ErbB2 antibody (green) to the surface (arrow) of SKBR3 cells. Co-localization of binding between the heterodimer and antibodies was characterized with a Pearson's correlation coefficient of **D**) $\rho = 0.77$ and **E**) $\rho = 0.65$ for EGFR and ErbB2, respectively. **F**) DAPI shows location of cell nuclei. Results are representative of 3 independent experiments.

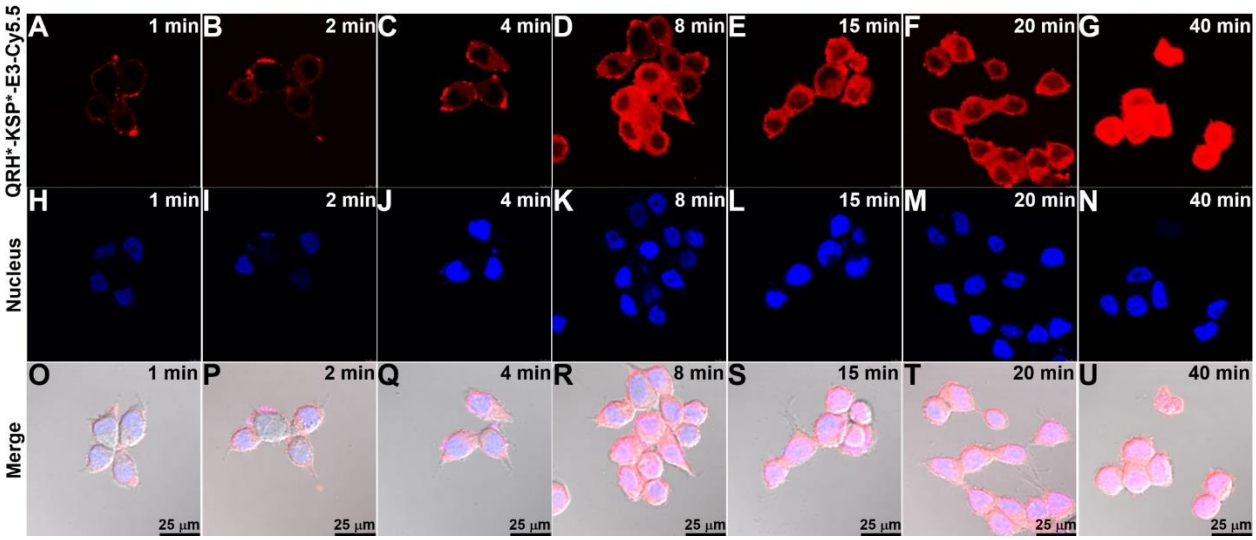


Figure S7 – **Heterodimer internalization**. **A**-**G**) From 1-8 min, QRH*-KSP*-E3-Cy5.5 accumulates on the surface (arrow) of SKBR3 cells. From 8-20 min, the ligand shows signs of internalization, which is complete at 40 min. **H**-**N**) DAPI shows location of cell nuclei. **O**-**U**) Merged images.

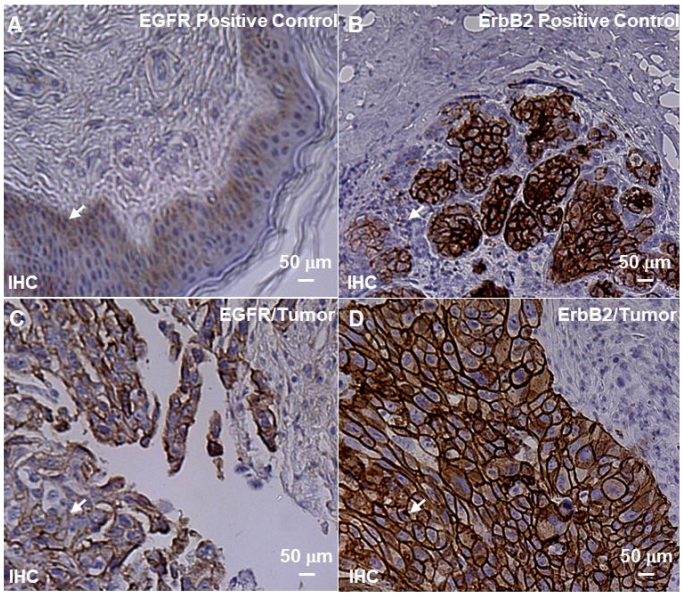


Figure S8 – **Immunohistochemistry (IHC)**. **A**) EGFR positive control and **B**) ErbB2 positive control. Antibody staining validates expression of C) EGFR and D) ErbB2 in OE33 xenograft tumor sections, (scale bar: 50 µm).

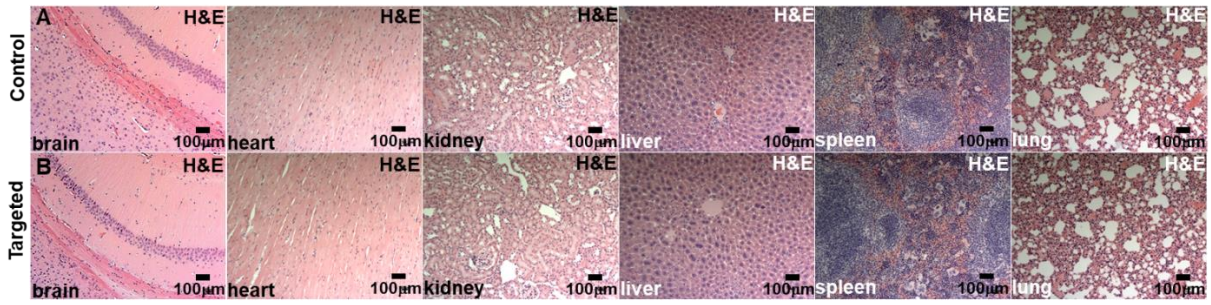
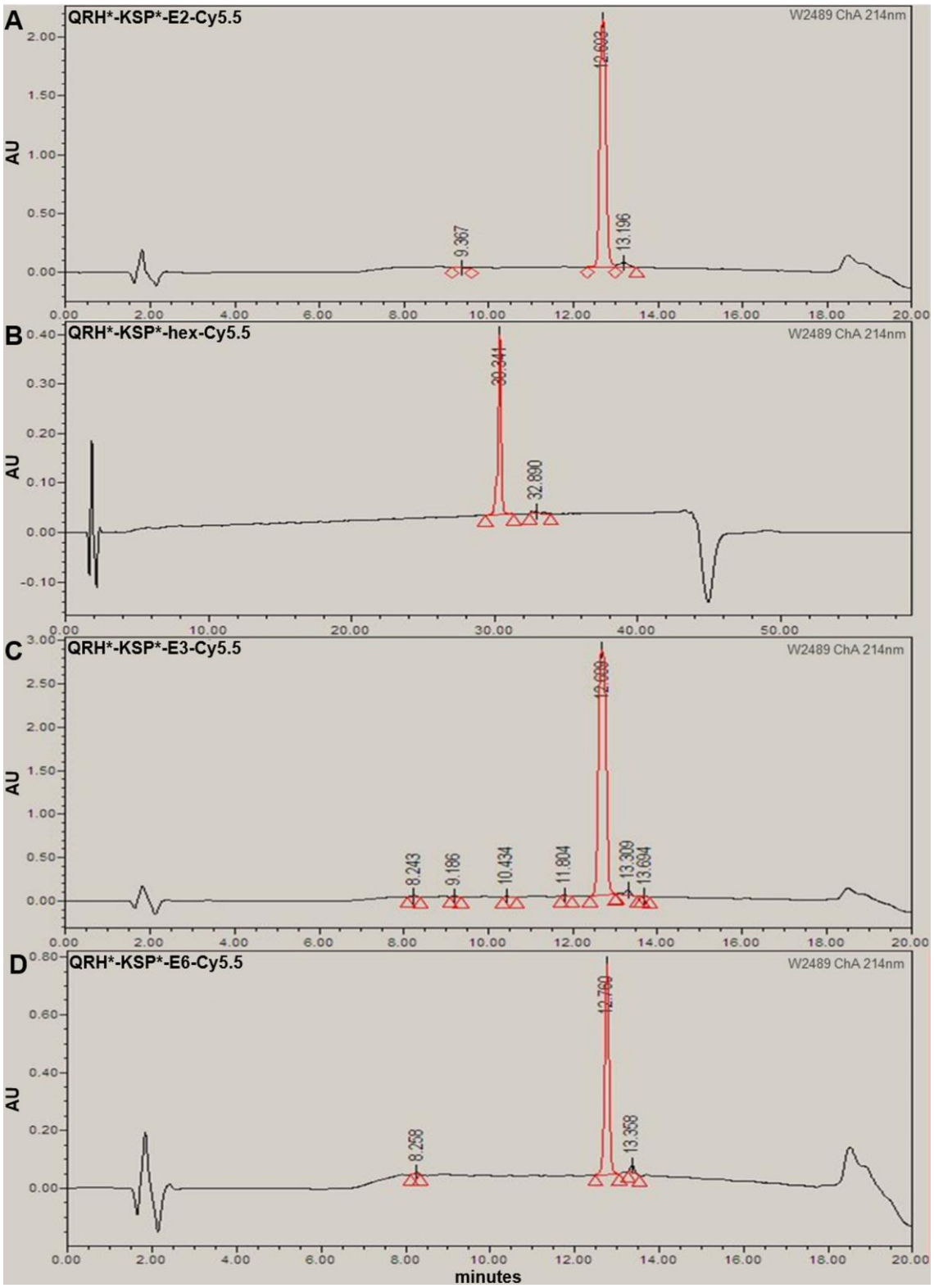


Figure S9 – Histology (H&E). Mice were systemically administered with the **A**) $(GGGAGGG)_2KK$-Cy5.5 control and **B**) QRH*-KSP*-E3-Cy5.5 targeted peptides. Euthanasia was performed after day 15, and sections of major organs including brain, heart, kidney, liver, spleen and lung were evaluated. No sign of acute toxicity was found.


A
QRH*-KSP*-E2-Cy5.5
W2489 ChA 214nm
AU
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12.603
13.196
B
QRH*-KSP*-hex-Cy5.5
W2489 ChA 214nm
AU
30.341
32.890
C
QRH*-KSP*-E3-Cy5.5
W2489 ChA 214nm
AU
8.243
9.186
10.434
11.804
12.609
13.309
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minutes

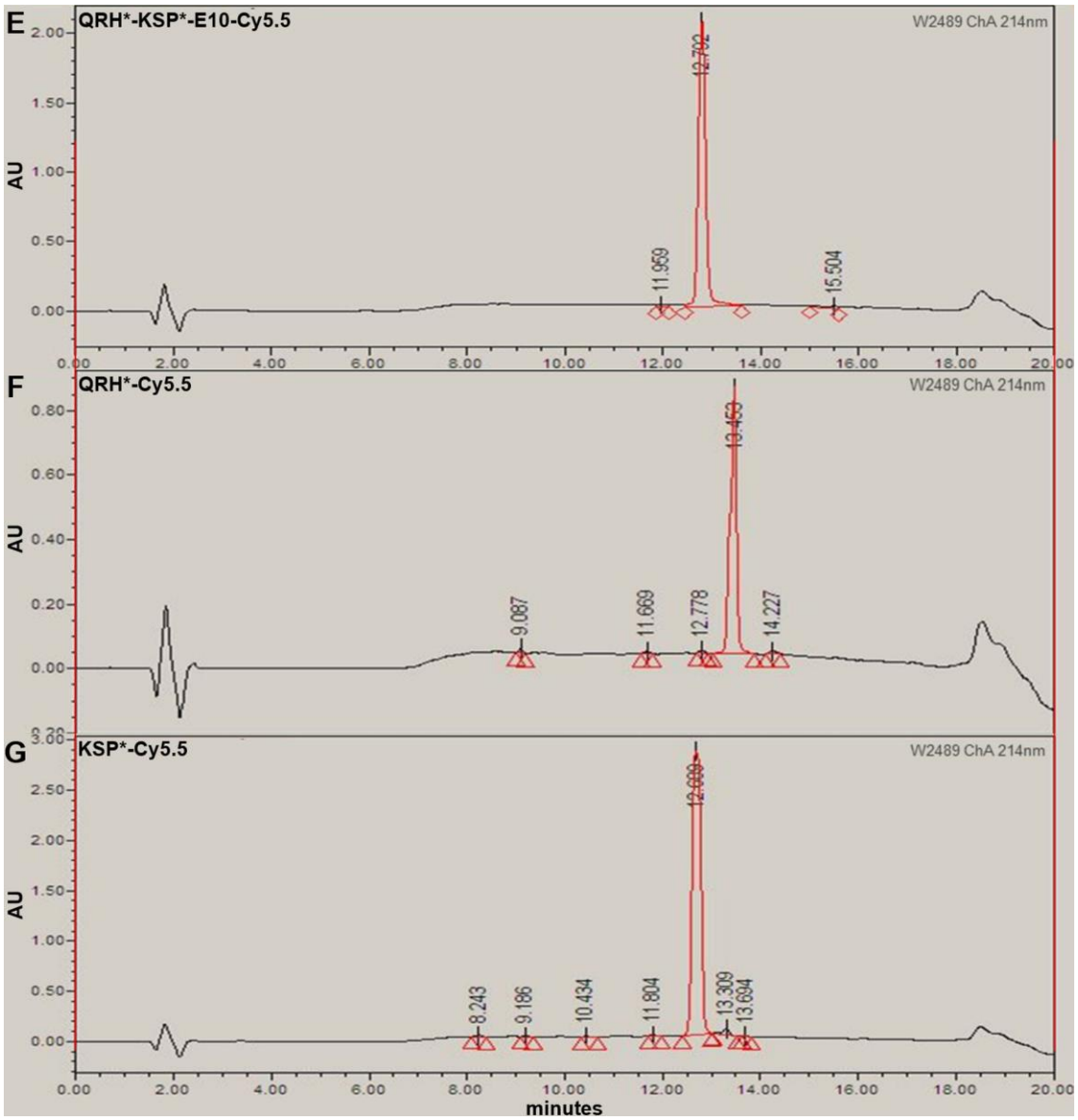

Figure S10 – **HPLC spectra**. The chemical purity of **A**) QRH*-KSP*-E2-Cy5.5, **B**) QRH*-KSP*-Hex-Cy5.5, **C**) QRH*-KSP*-E3-Cy5.5, **D**) QRH*-KSP*-E6-Cy5.5, **E**) QRH*-KSP*-E10-Cy5.5, **F**) QRH*-Cy5.5, and **G**) KSP*-Cy5.5 analyzed at 214 nm is shown.

Table S1 – Biochemical properties of peptide monomers and heterodimers

Compound	Linker (atoms)	Expt Mass[a]	Yield (%)	Purity (%)[b]
QRH*-KSP*-E2-Cy5.5	17	2886.64	55.4	>95%
QRH*-KSP*-Hex-Cy5.5	19	2822.66	38.3	>95%
QRH*-KSP*-E3-Cy5.5	23	2974.69	40.2	>95%
QRH*-KSP*-E6-Cy5.5	49	3266.88	30.1	>95%
QRH*-KSP*-E10-Cy5.5	74	3619.09	35.2	>95%
QRH*-GGGSK-Cy5.5	-	1794.98	61.8	>95%
KSP*-GGGSK-Cy5.5	-	1900.04	50.9	>95%

[a]Exact mass was calculated from most abundant isotope of element, and was characterized by Q-TOF mass spectrometry. [b]Analytical HPLC with C18-column using water (0.1% TFA) as gradient at 280 nm.