Supporting Information

Design and Synthesis of Potent Bivalent Peptide Agonists Targeting the EphA2 Receptor

Srinivas Duggineni[a], Sayantan Mitra[b#] Ilaria Lamberto[b#], Xiaofeng Han[a], Yan Xu[a], Jing An[a], Elena B. Pasquale[b,c*] and Ziwei Huang[a*]

Experimental Procedures

Peptide synthesis. SWL and derivative peptides used for alanine scanning were purchased from GeneScript (Piscataway, NJ) and had a purity of >91. The identity of the peptides and peptide purity were verified by HPLC and mass spectrometry. SWL, SWL-C10, SWL dimer (C6 linker) and SWL dimer (C12 linker) peptides were synthesized on a Symphony peptide synthesizer (Protein Technology) by using Fmoc [N-(9-fluorenyl) methoxycarbonyl] chemistry. Low loading Tenta Gel S RAM amide resin (loading, 0.24 mmol/g; Fluka) was used for the in-house synthesis. 0.5 M 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and N-hydroxybenzo-triazole (HOBt) in N,N-dimethylformamide (DMF) were used as coupling and activating reagents in the presence of 2 M diisopropyl-ethylamine (DIEA). Fmoc group deprotection at each step was conducted using piperidine in DMF (20%). The N-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl group of on the lysine side chain was deprotected by 3 treatments with 2% hydrazine in DMF for 2 min. Final peptides were cleaved from the peptidyl resin as previously reported[1].

For disulfide bond formation to dimerize the purified SWL-C10 peptide, the peptide was dissolved in folding buffer (1 M guanidinium hydrochloride and 0.1 M Trizma base at pH 8.5; 1 mg peptide/ml folding buffer) and dimerization was monitored by analytical RP-HPLC using a Xbridge^{TM} BEH130 C-18 (4.6 X 250 mm) column with a flow rate of 1 ml/min. The following solvents were used: A, water with 0.1% TFA; and B, 10% water in CH_3CN with 0.1% TFA in a linear gradient of 0% to 100% B over 25 min. All peptides were purified by using Xbridge^{TM} BEH130 prep C-18 (10 X 250 mm) column with a flow rate of 5 ml/min and characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Molecular modeling. The binding model of the SWL dimer (C6 linker) peptide was constructed based on the crystal structure of two EphA2 ligand-binding domains from crystals including most of the EphA2 extracellular domains bound to two ephrin-A5 ligands (PDB:3MX0; see main text of the manuscript). The structure of the SWL dimeric peptide was built using Sybyl-X and subjected to energy minimization using the Tripos force field and Gasteiger-Huckel charges implemented in Sybyl-X1.3. The SWL dimer (C6 linker) peptide was manually placed into two neighboring EphA2 ephrin-binding pockets and the resulting complex was subjected to further energy minimization. Molecular dynamics (MD) simulations were then performed using Sybyl x1.3 and Tripos force field for 200 ps while the temperature was maintained at 300 K. During the MD simulations, only residues of the EphA2 binding site and all residues of the SWL dimer (C6 linker) peptide were allowed to move, while the remaining residues were frozen at their positions in the crystal structure.

ELISA assays. To measure inhibition of EphA2-ephrin-A5 binding by the peptides, 1 μg/ml EphA2 Fc (R&D System, Minneapolis MN) diluted in Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) with 0.01% Tween 20 was used to coat Protein A coated 96 well plates (Pierce Thermo Scientific, Rockford, IL) for 1 hour at room temperature. The plates were washed three times with TBS, 0.01% Tween 20 and different peptide concentrations were added to the wells together with ephrin-A5 AP for 3 hours. Unbound ephrin-A5 AP was removed by washing the plates three times with TBS, 0.01% Tween 20. The amount of bound ephrin-A5 AP was measured by using 1 mg/ml p-nitrophenyl phosphate (Pierce Thermo Scientific) dissolved in SEAP buffer (105 mM diethanolamine, 0.5 mM Mg Cl_2, pH 9.8) as the substrate and reading the optical density at 405 nm. The optical density in wells coated with Fc was substracted as the background.

The selectivity of the SWL dimer (C6 linker) peptide for different Eph receptors was measured in similar ELISA using different Eph receptor Fc fusion proteins (R&D System, Minneapolis MN). Ephrin-A5 AP was incubated with EphA receptors and ephrin-B2 AP with EphB receptors in the presence and in the absence of 10 μM SWL dimer (C6 linker) peptide.

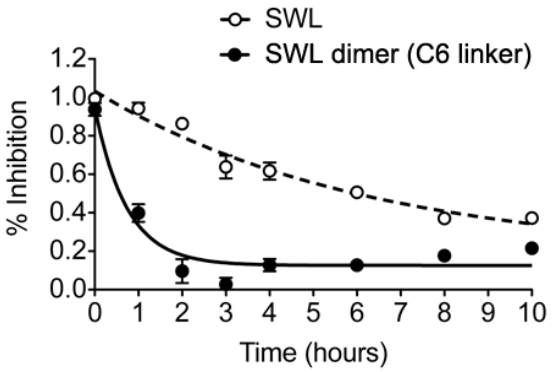
To measure peptide stability, SWL and SWL dimer (C6 linker) peptides were incubated in mouse serum *in vitro* at 37°C. Aliquots were collected and tested in ELISAs at 25 μM for SWL and 0.5 μM for SWL dimer (C6 linker) peptides, which are the concentrations at which the two intact peptides inhibited ephrin-A5 AP binding to EphA2 Fc by ~80%. For these assays, EphA2 Fc or Fc were coated on ELISA wells at 1 μg/ml for 1 hour at room temperature. The wells were washed with TBS, 0.01% Tween 20 and

peptides pre-incubated in serum for various periods of time were added to the wells together with ephrin-A5 AP and incubated for 30 minutes at 4°C. The wells were washed again to remove unbound ephrin-A5 AP and substrate absorbance in wells coated with only Fc was subtracted as background. The absorbance from wells incubated with diluted serum without peptide (vehicle) was used to determine 0% inhibition while 100% inhibition was determined by using peptides that were not incubated in serum.

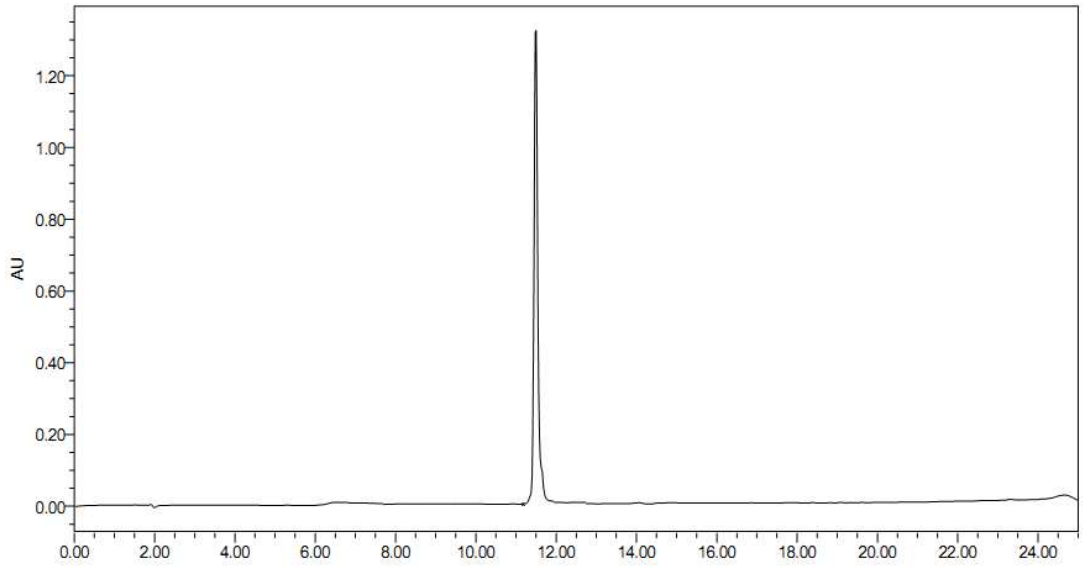
Immunoblotting. To measure EphA2 tyrosine phosphorylation, PC3 prostate cancer cells were grown in F-12K medium (ATCC) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% sodium pyruvate and starved for 3 hours in medium without FBS. The cells were then treated for 20 min with 0.1 μg/ml human Fc (as negative control), 0.1 μg/ml ephrin-A1 Fc (as positive control) or different concentrations of SWL or SWL dimer (C6 linker) peptide. The cells where washed in PBS once, lysed in modified RIPA buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors and centrifuged at 16,000 g for 10 minutes. Twenty μl GammaBind sepharose beads (GE Health Care Life Science, Piscataway, NJ) rinsed in modified RIPA buffer were incubated with 200 μg of PC3 cell lysate for 1 hour at 4 ºC for pre-clearing. To immunoprecipitate EphA2, beads that had been pre-incubated with 3 μg anti-EphA2 antibody (Millipore Upstate Inc., Temecula, CA) for 1 hour at 4 ºC were incubated with precleared cell lysate for 1 hour at 4 ºC. The immunoprecipitates were then rinsed twice in modified RIPA buffer and twice with PBS, probed by immunoblotting with anti-phosphotyrosine antibody (4G10; Millipore Upstate Inc.,

Temecula, CA), and reprobed with anti-EphA2 antibody (Invitrogen/Life Technologies, Carlsbad, CA).

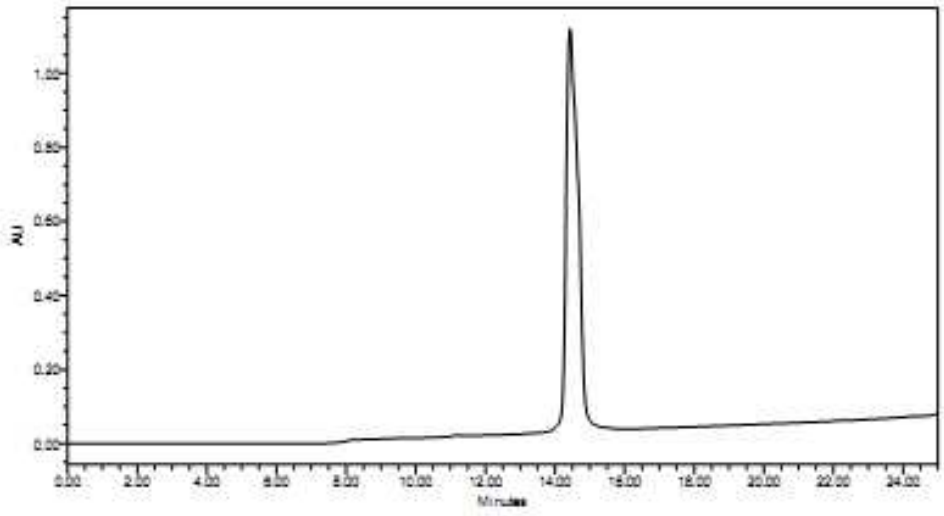
Figure S1. The SWL dimer (C6 linker) peptide has a shorter half-life than the SWL monomer peptide in mouse serum. Peptides were incubated for the indicated times in mouse serum *in vitro* at 37°C at concentrations of 25 μM for SWL and 0.5 μM for SWL dimer (C6 linker) peptide. These concentration of the intact peptides yield 80% inhibition of ephrin-A5 AP binding to EphA2 Fc in ELISA assays. The curves show relative loss of inhibitory activity due to incubation in serum for the indicated times ($t_{1/2}$ = 0.5 hours for SWL dimer (C6 linker) peptide and $t_{1/2}$ = 4.3 hours for SWL monomer peptide).



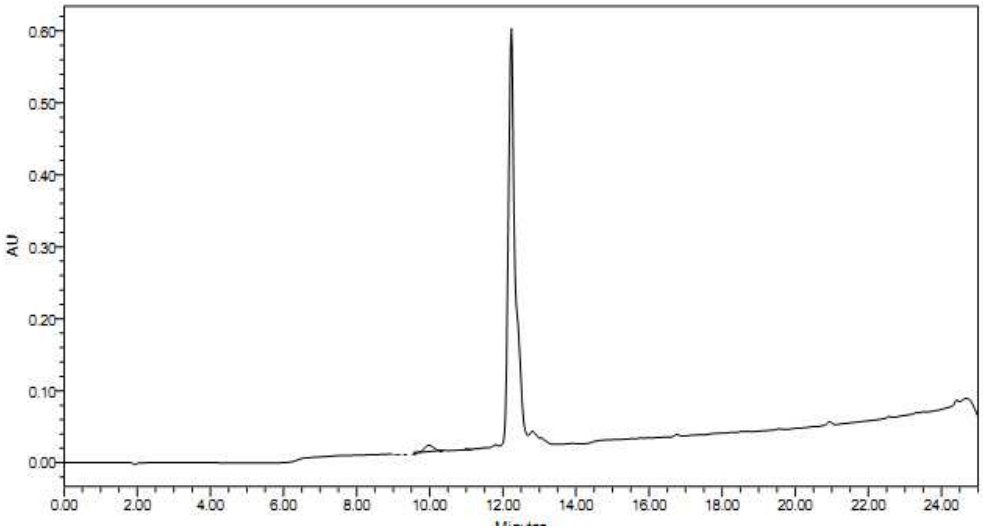
HPLC Spectrum for the SWL-C10 dimer peptide



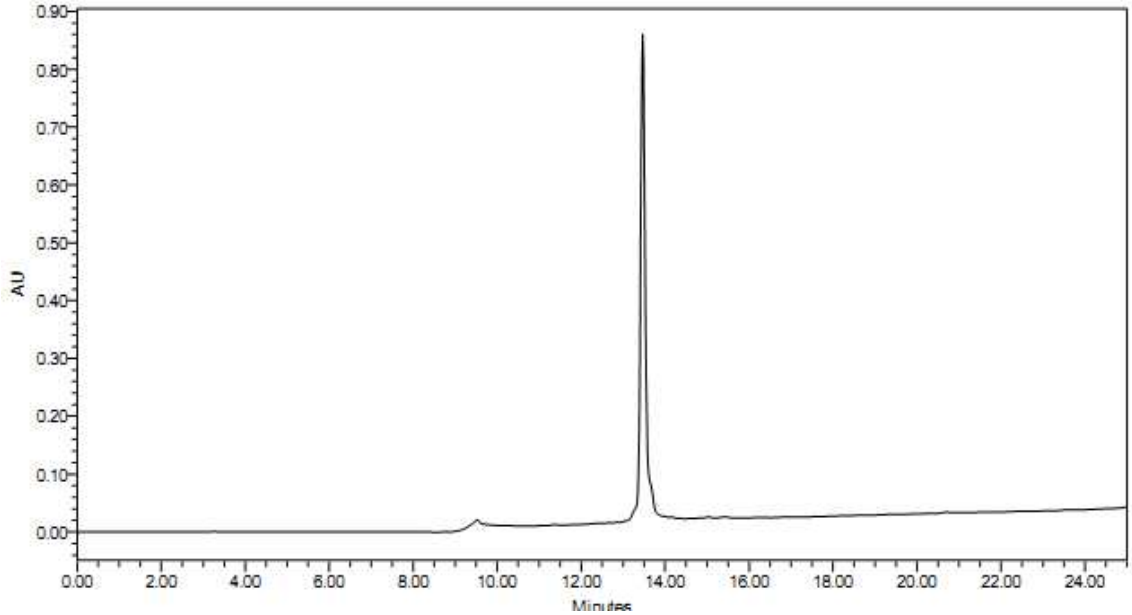
HPLC Spectrum for the SWL dimer (C12 linker) peptide



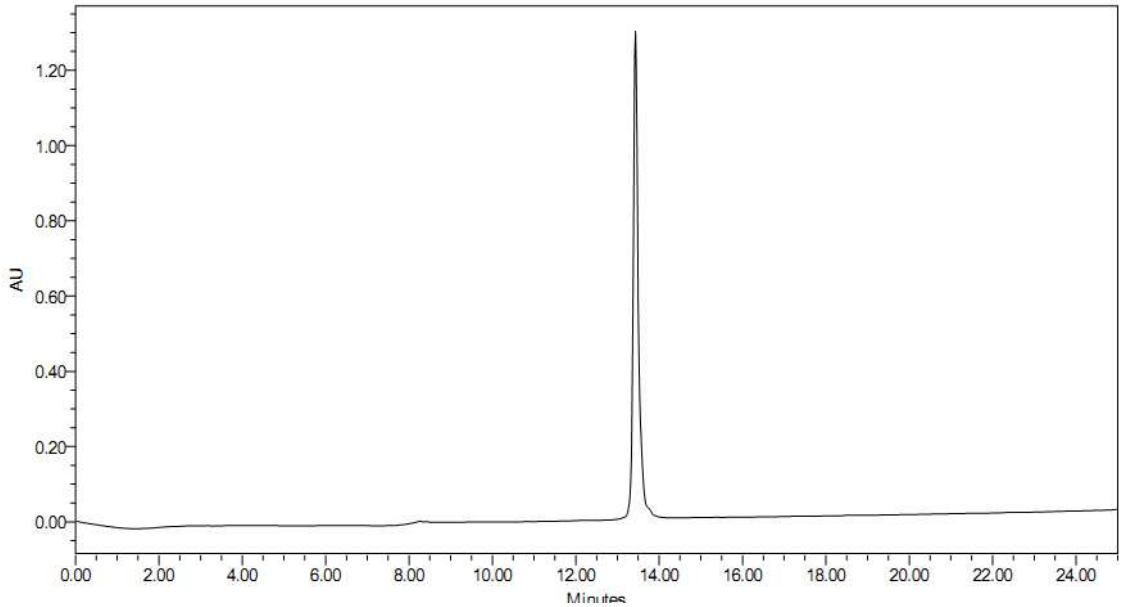
HPLC spectrum for the SWL dimer (C6 linker) peptide



HPLC Spectrum for the SWL Y'5A dimer peptide



HPLC Spectrum for the SWL Y'11A dimer peptide



References

1. Mitra, S.; Duggineni, S.; Koolpe, M.; Zhu, X.; Huang, Z.; Pasquale, E. B. Structure-activity relationship analysis of peptides targeting the EphA2 receptor. *Biochemistry* **2010,** 49, 6687-95.