

## **Development and Structural Analysis of a Nanomolar Cyclic Peptide Antagonist for the EphA4 Receptor**

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## SUPPORTING METHODS

**Peptide Synthesis.** The APY peptide with a free amine at the N terminus and a carboxylic acid at the C terminus and >95% pure was purchased from GenScript. Peptide amides were synthesized using manual synthetic cycles for 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis. Typically syntheses were performed on a 0.2 mmol scale using Rink amide resin (0.69 mmol/g, Novabiochem). Couplings were performed for 20 min using Fmoc protected amino acids (1.1 mmol) dissolved in 2.5 mL 0.4 M 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU; 1.0 mmol) and diisopropylethylamine (DIEA; 261  $\mu$ L, 1.5 mmol). Fmoc deprotection was facilitated by treating with an excess of 20% 4-Me piperidine for a total of 7 min. Peptides were deprotected and cleaved from resin using trifluoroacetic acid (TFA):triisopropylsilane (TIS):1,2 ethanedithiol (EDT):H<sub>2</sub>O (92.5:2.5:2.5:2.5) while agitating for 2 hours at room temperature. The TFA was 80% evaporated under N<sub>2</sub>, precipitated using ice-cold diethyl ether, filtered and further washed with cold ether. The crude peptides were dissolved in 45% acetonitrile/water, 0.05% TFA and lyophilized. Samples were solubilized with 20% acetic acid prior to analysis by reversed-phase high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry. If the sample was sufficiently homogeneous, it was oxidatively folded in 0.1 M NH<sub>4</sub>HCO<sub>3</sub>, pH 8 at a peptide concentration of 0.1 mg/mL followed by HPLC purification. If significant synthetic byproducts were present, the peptide was purified by HPLC prior to oxidation. The identity and purity of the peptides (>95%) were verified by reversed-phase HPLC and electrospray ionization mass spectrometry. For further analysis, peptides were dissolved in DMSO or H<sub>2</sub>O at a concentration of ~10 mM and the concentration was verified by measuring the optical density at 280 nm.

**Secondary Phage Display Screens.** The secondary phage display libraries were generated by using PAGE-purified oligonucleotides and the M13KE gIII cloning vector according to the recommendations of the manufacturer (New England BioLabs). The following primers were used to generate the libraries: library 1 (APXCVXRGSWSC, where X indicates any amino acid residue) 5'-CATGTTTCGGCCGATCCTCCTCCACACGACCAAGAACCCTMNNCACACAMNNCGGAG CAGAGTGAGAATAGAAAGGTACCCGGG-3'; library 2 (APY/FCXY/F/W/L/CXGXXC) 5'-CATGTTTC GGCCGATCCTCCTCCACAMNNMNNMNNACCMNNMHAMNNACA AWACGGAGCAGAGTGAGAATAGAA AGGTACCCGGG-3'; library 3 (APYCVYXGXWXC) 5'-CATGTTTCGGCCGATCCTCCTCCACAMNN CCAMNNACCMNNATACACACAATACGGAGCAGAGTGAGAATAGAAAGGTACCCGGG-3'; library 4 (XXXCVYRGSWSC) 5'-CATGTTTCGGCCGATCCTCCTCCACACGACCAAGAACCCTATACACACA MNNMNNMNNAGAGTGAGAATAGAAAGGTACCCGGG-3'. The size of the libraries generated was ~4x10<sup>3</sup> plaque forming units for library 1 (complexity of ~1x10<sup>3</sup> for the DNA sequences and 4x10<sup>2</sup> for the amino acid sequences), ~1x10<sup>5</sup> plaque forming units for library 2 (complexity of ~4x10<sup>8</sup> for the DNA sequences and ~4x10<sup>7</sup> for the amino acid sequences) and ~4x10<sup>4</sup> for libraries 3 and 4 (complexity of ~3x10<sup>4</sup> for the DNA sequences and 8x10<sup>3</sup> for the amino acid sequences).

The libraries were amplified and one round of panning was performed using mouse EphA4 ectodomain fused to Fc (EphA4 Fc; R&D Systems) immobilized at 10  $\mu$ g/mL in Tris-buffered saline (TBS; 50 mM TrisHCl, 150 mM NaCl, pH 7.5) in a protein G-coated well.<sup>28</sup> A well without EphA4 Fc was used as a negative control. Wells were blocked for 1 hour at room temperature with 0.5% milk in TBS and washed in washing buffer (TBS containing 0.5% Tween-20) prior to incubation with phage libraries (10<sup>7</sup>-10<sup>8</sup> plaque forming units) diluted in 100  $\mu$ l TBS containing 0.1% Tween-20 for 1 hour at room temperature. Unbound phage were washed away with washing buffer and bound phage were eluted with 0.2 M glycine HCl, pH 2.2 for 10 min. The eluates were immediately neutralized with 1 M TrisHCl, pH 9.0.

To measure the titer of the eluted phage, *E.coli* ER2738 cells grown to ~0.6 OD<sub>600</sub> in LB containing 10  $\mu$ g/mL tetracycline were infected for 5 min with different dilutions of the phage

eluted from wells coated with EphA4 Fc or from control wells, according to the manufacturer's recommendations (New England BioLabs, <https://www.neb.com/protocols/2014/05/08/m13-titer-protocol>). Plaques from plates enriched in EphA4-binding phage were amplified according to the manufacturer's recommendations (<https://www.neb.com/protocols/1/01/01/m13-amplification>) and tested in ELISAs for EphA4 binding. Phage DNA was purified according to the recommendations of the manufacturer (New England BioLabs) and sequenced.

**ELISAs.** To measure phage binding to EphA4, Ni-NTA HisSorb Strips (Qiagen) were incubated for 1 hour in TBS with 1  $\mu\text{g}/\text{mL}$  EphA4 Fc, which has a hexahistidine C-terminal tag, or only with TBS as a control. Wells were then blocked with 0.5% milk diluted in TBS for 1 hour and washed in washing buffer before incubation for 1 hour at room temperature with a 1:200 dilution of phage amplified from a single plaque. Wells were then washed before addition of horseradish peroxidase (HRP)-conjugated anti-M13 antibody (GE Healthcare; #27-9421-01). Phage binding was detected with the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) substrate dissolved in 50 mM citric acid, pH 4.0.  $\text{OD}_{405}$  was measured and the absorbance from wells without EphA4 Fc was subtracted as background.

To compare the strength of EphA4 binding of different phage clones, Ni-NTA HisSorb Strips were coated with EphA4 Fc or Fc (Fisher Scientific) as the background. The wells were incubated for 1 hour with a phage concentration corresponding to 1  $\text{OD}_{280}$  in 50  $\mu\text{L}$  TBS, 0.1% Tween-20 in the presence of different concentrations of the KYL antagonist peptide or in the absence of KYL. Bound phage was quantified with HRP-conjugated anti-M13 antibody.

To determine peptide  $\text{IC}_{50}$  values for inhibition of EphA4-ephrin-A5 binding, protein A-coated 96-well plates (Pierce/Thermo Scientific) were incubated with 1  $\mu\text{g}/\text{mL}$  EphA4 Fc in 80  $\mu\text{L}/\text{well}$  TBST (50 mM Tris HCl, 150 mM NaCl, pH 7.5 containing 0.01% Tween-20) for 1 hour at room temperature. The wells were washed 3 times with TBST and incubated for 1.5 hours at room temperature with 0.05 nM ephrin-A5 alkaline phosphatase (AP) and different concentrations of peptides in 40  $\mu\text{L}/\text{well}$  TBST. The wells were then washed and bound ephrin-A5 AP was quantified by adding 1 mg/mL *p*-nitrophenylphosphate substrate (Pierce/ThermoScientific) diluted in SEAP buffer (105 mM diethanolamine, 0.5 mM  $\text{MgCl}_2$ , pH 9.8).  $\text{OD}_{405}$  was measured and the absorbance from wells coated with Fc was subtracted as background.

To assess the Eph receptor selectivity of APY- $\beta\text{Ala}$ .am, Eph receptor Fc fusion proteins were immobilized at 1  $\mu\text{g}/\text{mL}$  on protein A-coated wells and incubated with 0.05 nM ephrin-A5 AP (for EphA receptors) or ephrin-B2 AP (for EphB receptors) in the presence or in the absence of the peptide. Ephrin-A5 AP and ephrin-B2 AP for the ELISAs were produced in transiently transfected HEK293T cells as previously described.<sup>32</sup>

**MTT Assay.** Peptide cytotoxicity was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. HT22 mouse hippocampal neurons were grown in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate and antibiotics. Cells were seeded in 96 well plates and incubated for 24 hours with 30  $\mu\text{M}$  APY- $\beta\text{Ala}$ 8.am (diluted from a stock dissolved in water) or with no peptide as a control. The cells were then incubated for 2 hours in 5 mg/mL MTT (Sigma-Aldrich) in PBS at 37°C in a  $\text{CO}_2$  incubator. The resulting formazan crystals were solubilized by addition of 100% DMSO and  $\text{OD}_{570}$  was measured.

## SUPPORTING TABLES

**Supporting Table 1.** Data collection, phasing and refinement statistics for the APY and APY- $\beta\text{Ala}$ 8.am peptides in complex with the EphA4 LBD.

**Supporting Table 2.** Phage display libraries.

**Supporting Table 3.** Sequences of phage clones randomly selected from the secondary phage display libraries.

## SUPPORTING FIGURE LEGENDS

**Supporting Figure 1.** Detailed structure of APY in the EphA4 ephrin-binding pocket. APY is shown in orange stick representation with oxygens in red, nitrogens in blue and disulfide bond in yellow. The EphA4 LBD is shown in gray ribbon representation with the residues within 5 Å of APY residues shown in cyan stick representation and labeled. Residues that when mutated to Ala (or to Ser in the case of Ala193) abolish APY binding are shown in bold and enlarged. Hydrogen bonds are shown as green dotted lines.

**Supporting Figure 2.** Role of EphA4 Thr69 in APY binding and APY stabilization by Trp10 interactions. (A) Thr69 does not directly interact with the bound APY peptide, but forms a hydrogen bond (green dashes) with the side chain of EphA4 Gln71. This favorably positions the side chain of Gln71 for formation of the crucial hydrogen bonds with the backbone of Tyr3 in APY. (B) Tyr6, Trp10 and the disulfide bonded Cys4 and Cys12, which are involved in a network of hydrophobic interactions that internally stabilize the APY peptide, are shown as spheres. (C) Atomic distances relevant to the hydrophobic pattern involving Tyr6, Trp10, Cys4 and Cys12 of APY are shown as grey dashes with the measurements in Å.

**Supporting Figure 3.** Comparison of  $\beta$ -turn and hydrogen bonds in the four APY and APY- $\beta$ Ala8.am molecules (A, B, C and D). The distances of the  $\beta$ -turn (red) and the hydrogen bonds (black) are indicated in the structures and summarized in the table (all distances are in Å). The hydrogen bond distances are less variable in the four APY- $\beta$ Ala8.am molecules than in the four APY molecules.

**Supporting Figure 4.** Isothermal titration calorimetry profiles for peptide binding to EphA4 (upper part of each panel) and plots of the integrated values for the reaction heats (after blank subtraction and normalization to the amount of injected peptide) versus EphA4/peptide molar ratio (lower part of each panel).