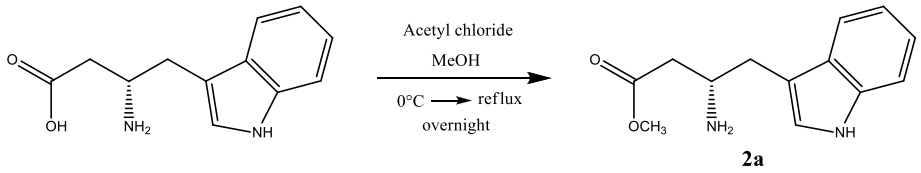
Chemistry

Unless otherwise noted, reagents and solvents were purchased from commercial suppliers (Aldrich and Fluka) and were used without purification. The progress of the reaction was monitored by thin-layer chromatography with F_{254} silica-gel precoated sheets (Merck Darmstadt, Germany). UV light, ninhydrin ethanolic solution (0.3% w/v) and potassium permanganate solution (10% w/v) were used for detection. Flash chromatography was performed using Merck silica-gel 60 (Si 60, 40-63 μm, 230-400 mesh ASTM). Dichloromethane (DCM) was dried by distillation over calcium hydride. All reactions were carried out using flame-dried glassware under atmosphere of nitrogen. Melting points were determined on a Gallenkamp melting point apparatus and were not corrected. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance 400 spectrometer (400MHz); chemical shifts (δ scale) are reported in parts per million (ppm). ^{1}H-NMR spectra are reported in the following order: multiplicity, approximate coupling constants (*J val*ue) in Hertz (Hz) and number of protons; signals were characterized as s (singlet), d (doublet), t (triplet), m (multiplet), b (broad). Mass spectra were recorded on an Applied Biosystem API-150 EX system spectrometer with ESI interface. The final compound was analyzed on a ThermoQuest (Italia) FlashEA 1112 Elemental Analyzer for C, H and N. The percentages found were within ±0.4% of the theoretical values.

Methyl 3(*S*)-amino-4-(1*H*-indol-3-yl)butanoate (2a)

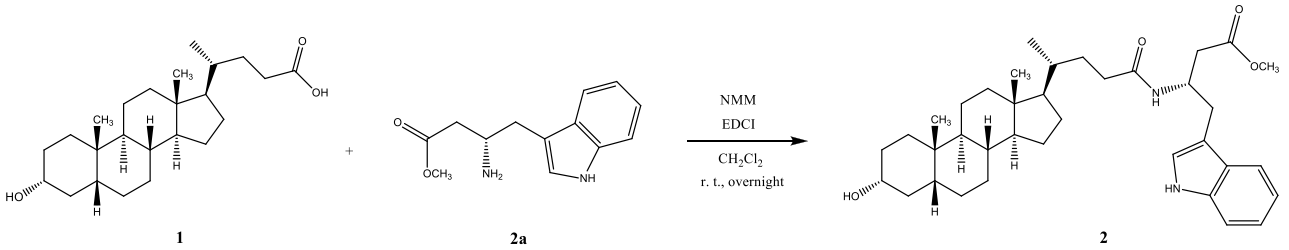


2a

Compound **2a** was synthesized following a modification of a described procedure[1] in which acetyl chloride (1.767 mmol) was added dropwise to dry methanol (10 ml) at 0°C. The mixture was stirred for 15 min and 3(S)-amino-4-(1*H*-indol-3-yl)butanoic acid (0.589 mmol) was then added portionwise to the solution. The resulting mixture was heated to reflux overnight. The solvent was evaporated under reduced pressure afforded the methyl 3(S)-amino-4-(1*H*-indol-3-yl)butanoate **2a** as a brown oil that was immediately used in the next step without further purification. Yield (98%). ^{1}H-NMR (400 MHz, CD_3OD) δ = 2.66 (dd, *J* = 17.2, 7.6 Hz, 1H, CHCH*H*), 2.78 (dd, *J* = 17.2, 4.8 Hz, 1H, CHC*H*H), 3.12 (dd, *J* = 14.8, 7.2 Hz, 1H, CHCH*H*), 3.17 (dd, *J* = 14.8, 6.8 Hz, 1H, CHC*H*H), 3.67 (s, 3H, OCH_3),

3.86-3.93 (m, 1H, C*H*CH_2), 7.07 (td, *J* = 7.2, 1.2 Hz, 1H, Ar), 7.14 (td, *J* = 7.2, 1.2 Hz, 1H, Ar), 7.20 (s, 1H, Ar), 7.39 (d, *J* = 8.0 Hz, 1H, Ar), 7.59 (d, *J* = 7.6 Hz, 1H, Ar). ^{13}C-NMR (100 MHz, CD_3OD) δ = 28.22, 35.47, 48.78, 51.20, 107.49, 111.24, 117.58, 118.87, 121.49, 123.91, 126.96, 136.95, 170.97(C=O).

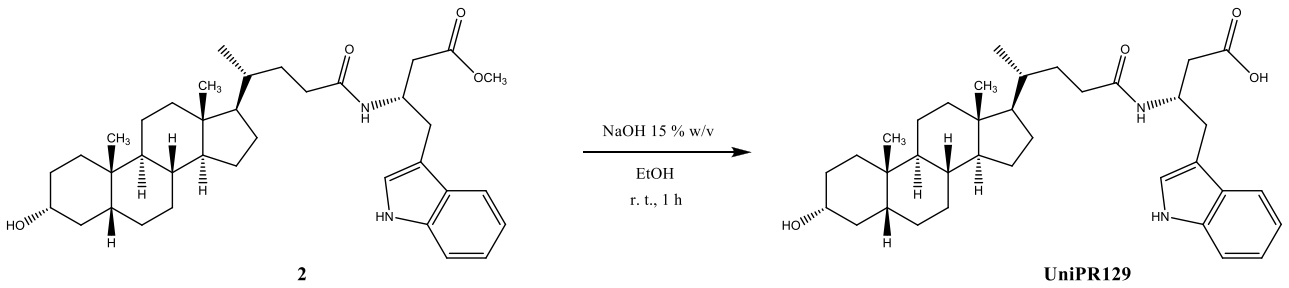
Methyl 3(*S*)-[*N*-(3α-hydroxy-5β-cholan-24-oyl)amino]-4-(1*H*-indol-3-yl)butanoate (2)



1 **2a** **2**

To a stirred solution of lithocholic acid **1** (0.490 mmol), methyl 3(S)-amino-4-(1*H*-indol-3-yl)butanoate (0.588 mmol) and N-methyl morpholine (NMM) (1.225 mmol) in dry CH_2Cl_2 (15 ml) under nitrogen was added N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydroclhoride (EDCI) (0.505 mmol). The reaction mixture was stirred at room temperature overnight and then was diluted with 30 ml of CH_2Cl_2, washed with an aqueous solution of HCl 2N, brine and dried over anhydrous Na_2SO_4. Evaporation of solvent under reduced pressure yielded a solid that was purified by flash chromatography [SiO_2, CH_2Cl_2:EtOH 98:2]. Yield: 57%. Mp: 193-197°C. ^{1}H-NMR (400 MHz, DMSO-d6) δ = 0.59 (s, 3H, CH_3), 0.83-0.91 (m, 7H), 1.00-1.22 (m, 10H), 1.28-1.35 (m, 7H), 1.47-1.50 (m, 2H), 1.55-1.68 (m, 3H), 1.73-1.79 (m, 2H), 1.89-1.95 (m, 2H), 1.98-2.07 (m, 1H), 2.36-2.46 (m, 2H, CHC*H*$_2$), 2.75 (dd, *J* = 14.4, 7.2 Hz, 1H, CHCH*H*), 2.85 (dd, *J* = 14.4, 6.4 Hz, 1H, CHC*H*H), 3.30-3.36 (m, 1H), 3.50 (s, 3H, OCH_3), 4.26-4.31 (m, 1H, C*H*CH_2), 4.43 (d, *J* = 4.4 Hz, 1H, CH*OH*), 6.96 (td, *J* = 7.6, 1.2 Hz, 1H, Ar), 7.05 (td, *J* = 8.0, 1.2 Hz, 1H, Ar), 7.09 (d, *J* = 2.4 Hz, 1H, Ar), 7.31 (d, *J* = 8.0 Hz, 1H, Ar), 7.56 (d, *J* = 8.0 Hz, 1H, Ar), 7.78 (d, *J* = 8.0 Hz, 1H, NH). ^{13}C-NMR (100 MHz, DMSO-d6) δ = 12.33, 18.73, 20.87, 23.73, 24.31, 26.63, 27.36, 28.17, 30.44, 30.86, 31.98, 32.95, 34.67, 35.30, 35.62, 35.85, 36.77, 38.82, 42.00, 42.73, 47.28, 51.65, 56.09, 56.56, 70.33, 111.24, 111.79, 118.71, 118.84, 121.32, 123.91, 127.90, 136.63, 171.90 (C=O), 172.48 (C=O). MS (ESI) calc for $C_{37}H_{54}N_2O_4$: 590.41; found: 591.5 $[M+H]^+$, 613.4 $[M+Na]^+$.

3(*S*)-[*N*-(3α-hydroxy-5β-cholan-24-oyl)amino]-4-(1*H*-indol-3-yl)butanoic acid (UniPR129)



2 **UniPR129**

To a solution of compound **2** (0.169 mmol) in ethanol (15 ml) was added a solution of sodium hydroxide 15% w/v (10 ml) and the mixture was stirred at room temperature for 1 hour. Ethanol was removed under reduced pressure and the solution was acidified with concentrated hydrochloric acid until a precipitate was formed. The resulting suspension was filtered under vacuum and the white residue washed with water. The crude product was crystallized from ethanol-water to give the title compound **UniPR129** as a white solid. Yield: 95%. Mp: 185-189°C. ^{1}H-NMR (400 MHz, DMSO-d6) δ = 0.59 (s, 3H, CH_3), 0.84-0.91 (m, 7H), 1.00-1.07 (m, 4H), 1.12-1.22 (m, 6H), 1.28-1.35 (m, 7H), 1.47-1.50 (m, 2H), 1.56-1.68 (m, 3H), 1.74-1.77 (m, 2H), 1.87-1.94 (m, 2H), 1.99-2.07 (m, 1H), 2.33 (d, *J* = 6.8 Hz, 2H, CHC*H*$_2$), 2.76 (dd, *J* = 14.0, 6.8 Hz, 1H, CHCH*H*), 2.84 (dd, *J* = 14.0, 6.4 Hz, 1H, CHC*H*H), 3.33-3.38 (m, 1H), 4.21-4.28 (m, 1H, C*H*CH$_2$), 6.95 (t, *J* = 8.0 Hz, 1H, Ar), 7.04 (t, *J* = 8.0 Hz, 1H, Ar), 7.08 (d, *J* = 2.4 Hz, 1H, Ar), 7.31 (d, *J* = 8.4 Hz, 1H, Ar), 7.58 (d, *J* = 7.6 Hz, 1H, Ar), 7.76 (d, *J* = 8.4 Hz, 1H, NH). ^{13}C-NMR (100 MHz, DMSO-d6) δ = 12.34, 18.78, 20.87, 23.73, 24.32, 26.64, 27.36, 28.17, 30.32, 30.85, 32.01, 33.02, 34.68, 35.37, 35.62, 35.86, 36.77, 39.07, 42.01, 42.73, 47.32, 56.07, 56.56, 70.34, 111.49, 111.76, 118.65, 118.92, 121.28, 123.80, 128.01, 136.63, 172.44 (C=O), 173.26 (C=O). MS (ESI) calc for $C_{36}H_{52}N_2O_4$: 576.39; found: 575.6 $[M-H]^-$. Anal. calc for $C_{26}H_{52}N_2O_4$: C, 74.96; H, 9.09; N, 4.86; found: C, 74.99; H, 9.10; N, 4.83.

[1] A. N. Hulme, K. S. Curley, *J. Chem. Soc. Perkin Trans.* **2002**, 1, 1083-1091.

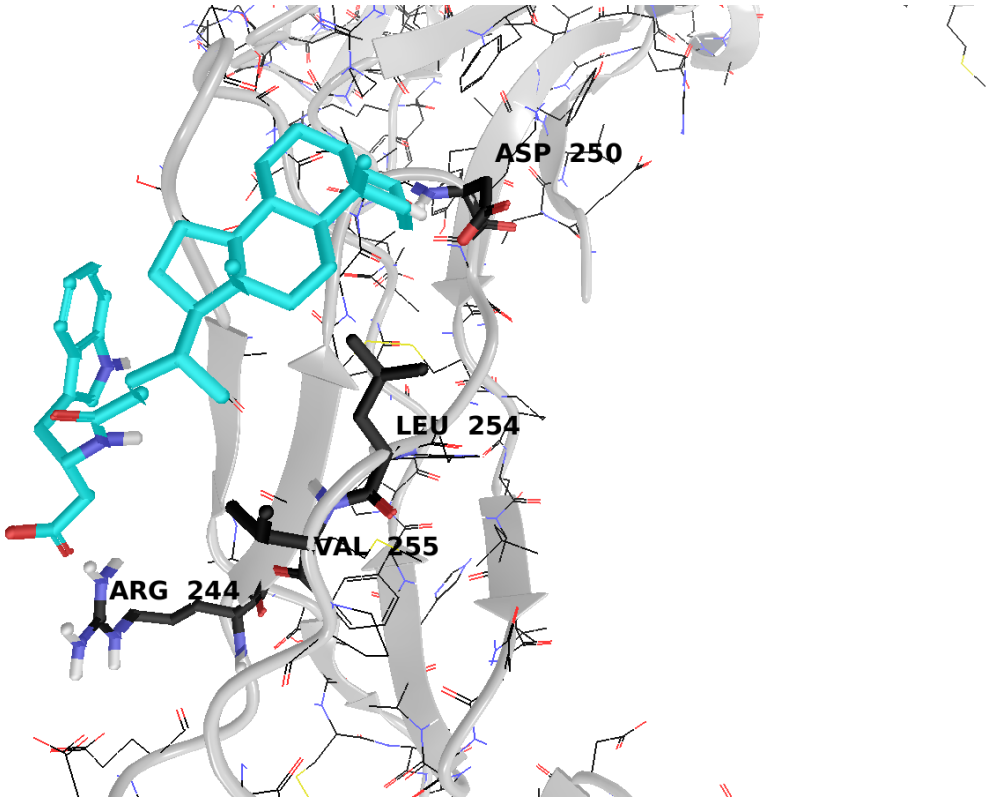


Figure S1.

Docking of UniPR129 (cyan carbon atoms) in the CRD domain of EphA2 receptor (white ribbons with black carbon atoms). In evidence, Leu254 and Val255 of the leucine zipper region, as well as polar residues (i.e. Arg244 and Asp250).

To quantitatively compare this new binding mode to that at the LBD, MM-GBSA calculations were performed on docking complexes (Table S1). However, these calculations indicate EphA2-UniPR129 complex is significantly less stable (by nearly 26 kcal/mol) when the compound binds CRD domain, thus indicating (at least from a theoretical standpoint and in absence of other experimental evidences) that UniPR129 likely binds the LBD of EphA2.

Table S1. MM-GBSA calculations for UniPR129 in the LBD and in CRD domains of EphA2.

Rank	Receptor domain	MM-GBSA energy (kcal/mol)
1	LBD	-101.1
2	CRD	-74.2

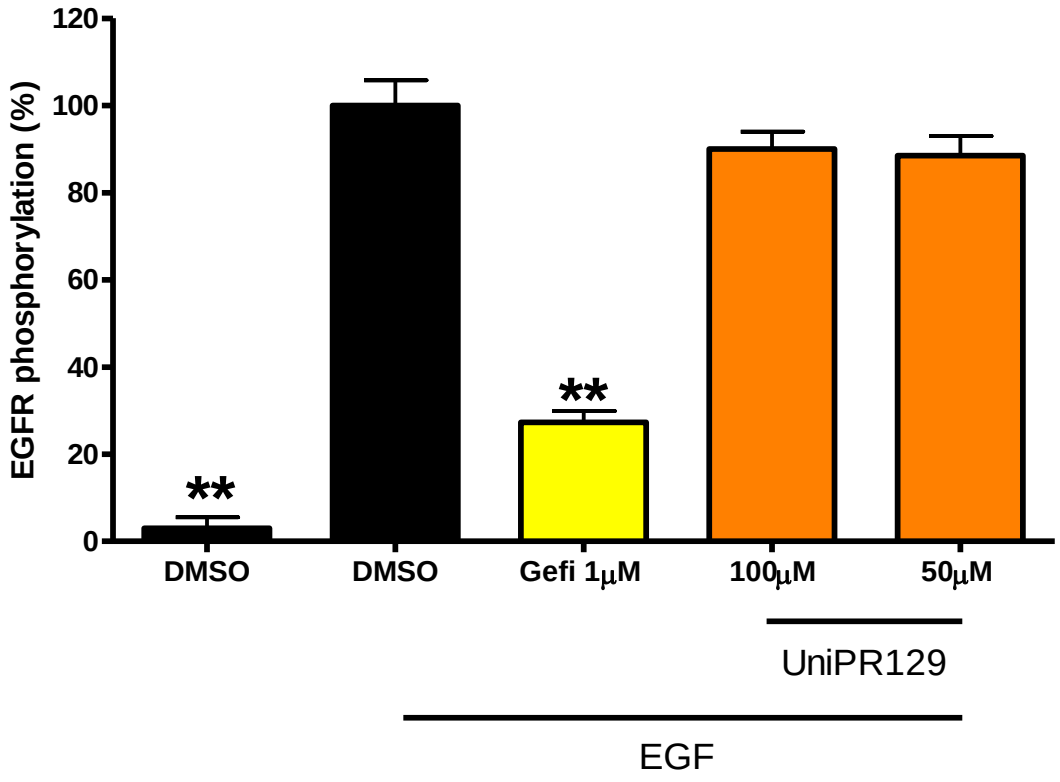


Figure S2. UniPR129 does not affect EGF-induced EGFR-phosphorylation on PC3 cells

*Cells were pretreated for 20 minutes with the indicated concentrations of the compound, or 1% DMSO as a control and then stimulated for 20 minutes with 30 ng mL^{-1} EGF (236-EG-200, R&D Systems). The levels of phosphorylated EGFR were detected with the Human Phospho-EGF-R sandwich ELISA kit (DYC1095, R&D Systems) and normalized to the cells treated with DMSO+EGF in the absence of compound(s). Gefitinib (Gefi) 1μM was used as a control. Data are the means of at least three independent experiments ± st. err. One-way ANOVA followed by Dunnet's post-test was performed to compare EGF+DMSO to all other columns. **, p<0.01.*

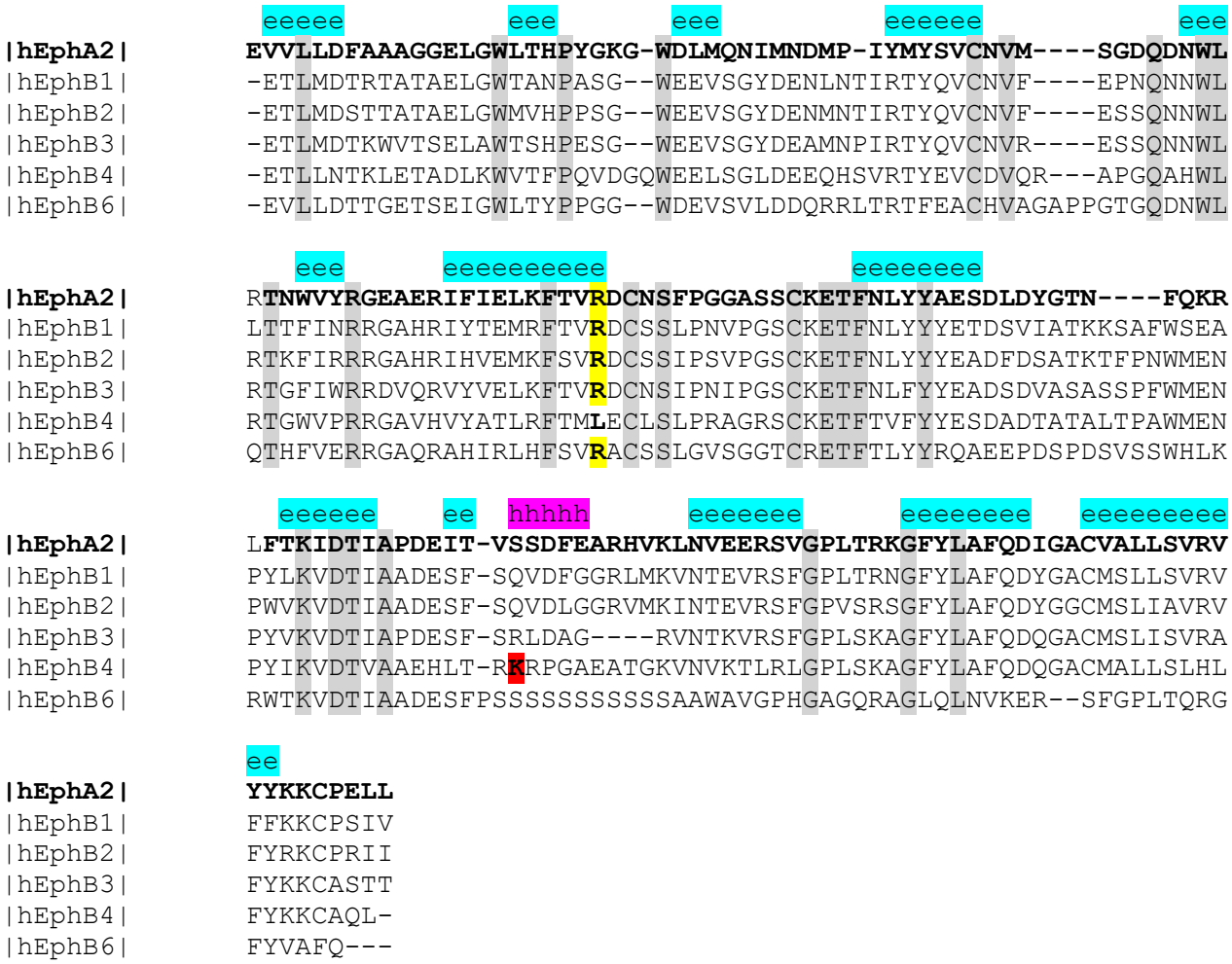


Figure S3.

Multiple sequence alignment of human Eph receptors. Secondary structure elements are shown above the sequences (h: helix; e: sheet) and are referred to the structure of EphA2 as it appears from the X-ray coordinates reported in the 3HEI.pdb complex.

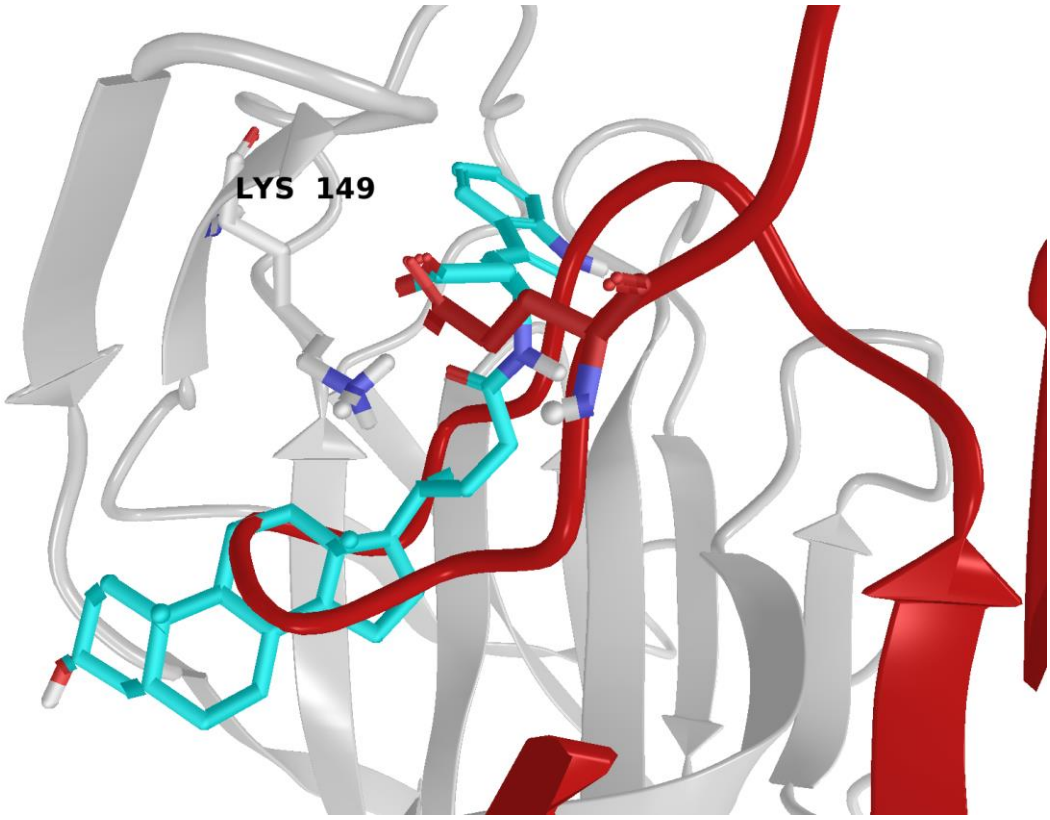


Figure S4*.*

Docking of UniPR129 (cyan carbon atoms) in the high-affinity ephrin binding pocket of the EphB4 receptor (white ribbons with gray side chain carbon atoms). The G–H loop of ephrinB2 is also displayed (red ribbons). In evidence, Lys149 of EphB4 and Glu128 of ephrinB2.