Center Probe Report

Project Title: EphA4 Receptor Antagonists for Nervous System Repair
Grant Number: 1-X01-MH077609-01

Screening Center Name: San Diego Center for Chemical Genomics
Principal Investigator of Screening Center: John C. Reed

Assay Provider & Institution: Professor Elena Pasquale; Burnham Institute for Medical Research

Assay or Pathway Target: The EphA4 receptor tyrosine kinase
Probe PubChem Compound Identifier (SID/CID): CID-668744; SID-17465980; BIM-0001471 (pyrrole series).

Assay provider information

Specific Aim:
The aim of this project is to identify small molecule antagonists that inhibit the binding of EphA4 to the KYL peptide which interacts with high affinity to the ephrin-binding site of EphA4. This new approach will lead to the development of potent, specific inhibitors with the potential for a number of therapeutic applications.

Significance:
Damage in the adult central nervous system (CNS) due to injury or degeneration is usually irreparable because neuronal connections cannot regenerate. For example, many spinal cord injuries result in permanent paralysis due to the inability of motor neurons to re-establish connections with muscles. This is due at least in part to the physical barrier formed by glial cells that respond by proliferating and forming a scar and to the presence of inhibitory molecules in the CNS environment [1,2,3].
Recent work has shown that the EphA4 receptor tyrosine kinase plays a critical role in the inhibition of axon regeneration that occurs after spinal cord injury [3]. Axons in EphA4 knockout mice can re-generate past the site of injury and re-establish severed connections resulting in functional recovery. Furthermore, recent data show that the KYL pepide promotes axon sprouting and functional recovery after spinal cord injury in the rat [4] Other evidence suggests that in various regions of the central nervous system EphA4 inhibits axonal and dendritic growth.
Furthermore, EphA4 also plays a role in the maintenance of platelet aggregation during thrombus formation and in prostate and pancreatic cancer [5,6,7]. Thus, inhibiting the interaction between EphA4 and ephrin ligands could be useful, providing potential therapies that promote regeneration after nervous system injury, ameliorate certain neurodegenerative diseases, inhibit blood clot formation, and inhibit progression of certain types of cancer [8].

Rationale:
EphA4 is a member of the large Eph family of receptor tyrosine kinases [8]. All Eph receptors have an extracellular portion, which contains the ligand-binding domain at the amino terminus, and a cytoplasmic portion containing the tyrosine kinase domain. The signaling ability of EphA4 and the other nine closely related EphA receptors is activated by binding the six GPI-linked ephrin-A ligands. In addition, EphA4 also binds the three transmembrane ephrin-B ligands, which are the ligands for the other class of Eph receptors, the EphB receptors (EphB1-EphB6). Eph receptor-ephrin interaction requires cell-cell contact because both the receptor and the ligand are membrane-bound. The interaction leads to clustering, tyrosine phosphorylation of Eph receptors and ephrin-B ligands, and

association with cytoplasmic signaling proteins. Importantly, signals are generated both through the Eph receptor kinase domain (forward signals) and through signaling molecules associated with the ephrins (reverse signals). The goal is to find small molecules that inhibit the interaction of EphA4 with ephrin ligands.

Screening center information

Assay Implementation and Screening

PubChem Bioassay Name: Colorimetric assay for HTS discovery of chemical inhibitors of EphA4 receptor antagonists

List of PubChem bioassay identifiers generated for this screening project (AIDS): 689.

List of relevant AIDs that may be used as counterscreen information: 779 (AP detection system), 720 and 835 (EphB4).

Primary Assay Description as defined in PubChem:

Protocol

EphA4 assay materials:

1) EphA4-AlkalinePhosphatase conditioned media (from EphA4-AP secreting stable cell-line) (cell line: NIH3T3 stably transfected with EphA4-AP), as well as control Alkaline Phosphatase conditioned media, was provided by Dr. Elena Pasquale (Burnham Institute for Medical Research, San Diego, CA).
2) KYL-biotinylated peptide (KYLPYWPVLSSL), re-constituted in PBS, was provided by Dr. Elena Pasquale.
3) Streptavidin (Pierce Cat # PI21125) 1mg/ml in 0.1M Boric Acid pH 8.7
4) Blocking buffer: 5mg/ml BSA in 20mM Tris pH 7.5, 150mM NaCl
5) Assay/Washing buffer: 20mM Tris pH 7.5, 150mM NaCl, 1mM CaCl2, 0.01% Tween-20
6) Detection reagent: 1mg/ml pNPP in 1.1M Diethanolamine, 0.5mM MgCl2 pH 9.8
7) Stop Reagent: 1N NaOH

EphA4 binding assay protocol:

Day 1

1) Add 25ul of streptavidin (1ug/ml) to each well of a microtiter plate
2) Incubate, at least overnight (18hrs), at room temp (keep plates covered and in the dark)
*after the overnight incubation, plates can be place in the cold room for 3-4 weeks if kept tightly covered and in the dark.

Day 2

1) Wash plates once with Assay/Washing buffer using a Titertek MAP C2 plate washer (75ul/well)
2) Bang out plates
3) Block plates by adding 50ul/well of 5mg/ml BSA in TBS for at least 1 hour at room temperature
4) Wash plates once with Assay/Washing buffer using MAP C2 plate washer (75ul/well)
5) Bang out plates
6) Add 25ul/well of fresh KYL-biotinylated peptide solution (0.10 uM)
7) Incubate plates for 15 min at room temperature
8) Wash plates 4 times with Assay/Washing buffer (75ul/well)
9) Bang out plates
10) Add 11 uL of Assay/Washing buffer to columns 1-24.
11) Compound/ 10% DMSO addition

a. Add 2.5ul of compounds (0.1mM in 10% DMSO) to appropriate columns using a Beckman Coulter Biomek FX(columns 3-24)
b. Add 2.5ul of 10% DMSO to columns 1-2

12) Add 12 ul of diluted EphA4-AP (15.6ul of conditioned media per ml of Assay/wash buffer) to columns 2-24 (note that the amount of receptor added is determined empirically for each pool of culture supernatant, this dilution will change).
13) Add 12 ul of diluted AP control (3.13ul of conditioned media per ml of Assay/wash buffer) to column 1.
14) Spin down plates
15) Incubate for 2 hours at room temperature, while shaking.
16) Wash plates 4 times with Assay/Washing buffer (75ul/well)
17) Bang out plates
18) Add 25ul pNPP solution to each well
19) Wait an hour for signal to develop.
20) Add 25 uL of 1 N NaOH to columns 1-24.
21) Read plates at A405 using a Perkin Elmer Envision plate reader

Center Summary of the Primary Screen and Confirmatory Dose-response:
Assay optimization:
The original assay was provided in a 96 well plate format. This assay did not perform well in a 384-well format, therefore, extensive assay optimization was carried out. The assay conditions were optimized to provide better S/N and signal stability. The detailed protocol for the assay after optimization is above and the detailed assay optimization will be published elsewhere.

During assay development it was observed that binding of EphA4 and the peptide does not follow the standard equilibrium rules with continuous change in the bound material. Rather, it resembled an "all-or-nothing" process. The likely explanation is that behavior of the construct of EphA4 was somewhat influenced by alkaline phosphatase fused with it and which is necessary for detection. It is known from the literature that alkaline phosphatase is only active in dimeric form and inactive as a monomer. It is likely that EphA4-AP exists as a dimer of chimeric proteins in solution due to an interaction between monomers of alkaline phosphatase. After binding to KYL peptide and extensive washing, the dimeric form is more likely to be bound (due to the dentate effect and the expected higher affinity of a dimer) and is in fact the only detected form. In addition, streptavidin is a tetrameric protein capable of binding four biotins per tetramer. Therefore, depending on the quality of the plate coating, i.e. the orientation of streptavidin molecules and the density of streptavidin per surface area, the quantity of retained EphA4 and therefore, alkaline phosphatase could vary significantly. The scheme of interactions involved in the assay is depicted in Figure 1.

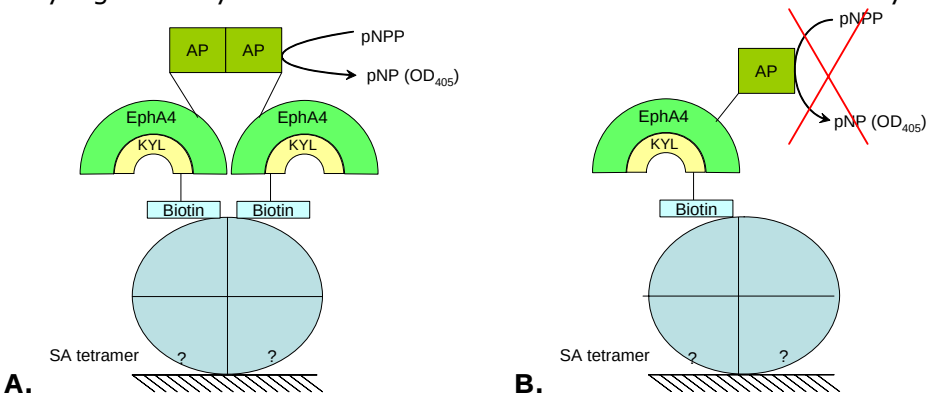


Figure 1: Schematic presentation of binding and detection processes involved in the EphA4 assay. A. EphA4-AP retained as a dimer. B. EphA4-AP retained as a monomer

Summary of HTS

The original assay in 96-well plate format was tested on a total of 12,000 compounds by screening two of Burnham's compound collections. The first library tested was the Spectrum collection from Microsource Discovery Systems Inc. The second was a partial screen of 10,000 compounds from the ChemBridge DiverSet of 50,000 compounds. It was from this collection that the original pyrrole hits were discovered.

Some modifications were made to the original assay (as discussed above) to increase its reliability in 384-well format. Once these modifications were finalized, the assay was screened through 52,404 compounds from the NIH small molecule library. From the screen 140 compounds demonstrated >50% activity in the inhibition of EphA4 binding to the KYL peptide. Of these 140 compounds, 82 generated IC_{50}s in dose response experiments.

The readout of this assay is based on alkaline phosphatase activity and therefore, it was important to determine if the compounds inhibited this enzyme. A separate assay was developed to measure the effect of the compound on alkaline phosphatase. None of the hits inhibited alkaline phosphates in this assay.

Comparative data on probe, similar compound structures and information on existing probes available to the public There are no known small molecule probes for EphA4.

Probe Optimization

Chemical name of probe compound: 4-(2,5-dimethyl-1H-pyrrol-1-yl)-2-hydroxybenzoic acid.

Draw probe chemical structure and show stereochemistry if known: see Figure 2.

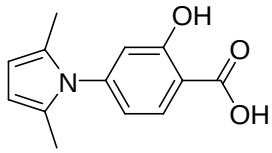


Figure 2. Structure of CID-668744

Describe mode of action for biological activity of probe: The probe reversibly and competitively inhibits ephrin ligand binding to EphA4. Therefore, it blocks the biological activities of EphA4 in cells.

Has this compound been provided to the MLSMR: Yes.

Commercial vendor information if available for probe or analogs that have been purchased: The probe compound is commercially available from Chembridge (cat# 5987008) and Asinex (cat# BAS 06735656)

Description of secondary screens used to optimize probe structure:

A counterscreening assay was developed to assess alkaline phosphatase inhibition using the EphA4-alkaline phosphatase construct as a source of enzyme. The activity of alkaline phosphatase was measured in a colorimetric assay using pNPP as the substrate.

The original assay that measures binding of EphA4 AP to immobilized KYL was used to compare the potency of different compounds related to compound CID-668744. A second screen involves measuring inhibition of EphA4 AP binding to ephrin-A5 Fc immobilized on protein-A-coated plates (ephrin-A5 is one of the natural ligands for EphA4). However, so far only compound CID-668744 and its 1,2 isomer were found to inhibit EphA4-ephrin binding. See Appendix for more detailed information on the assays.

Center comments on chemistry strategy leading to probe identification:

In order identify EphA4 hits that might be suitable to optimize into chemical probes a screening dataset of 10K compounds from the Burnham collection was analyzed. *N*-Arylpyrroles were identified as selective inhibitors of EphA4 displaying micromolar activity with both the natural ephrin ligands and the synthetic KYL peptide. In order to establish SAR around the primary screening hits, chemistry efforts were initiated that generated 60 analogues of this class of compounds. It was observed that the active compounds contained carboxylic acid functionality; therefore efforts were made to include this group in the derivative compounds. Two key reactions were developed to furnish diverse *N*-aryl pyrroles (see Scheme 1 below). The first method utilized a copper-catalyzed *N*-arylation of pyrroles (Eq. 1). In this Ullmann-type coupling reaction, the aryl halide was mixed with excess pyrrole, CuI, and potassium carbonate in DMSO. It was found that the addition of an amino acid such as *N*-methylglycine accelerated the reaction; most likely by chelating copper and making it more reactive to oxidative addition. This route furnished a variety of *N*-aryl pyrroles in moderate to good yields. The second route utilized a Paal-Knorr pyrrole synthesis (Eq. 2). Functionalized anilines were combined with one equivalent of 2,5-hexanedione and catalytic p-toluenesulfonic acid in refluxing toluene. The resulting pyrroles were collected by filtration to furnish products with purities greater than 90%. By utilizing both of these routes for analogue synthesis, along with acquisition of derivatives from commercial sources, a variety of functionalized pyrroles were produced that displayed EphA4 activity (Table 1). Based on these efforts CID-668744 (BIM-0001471) was selected as a small molecule probe for EphA4.

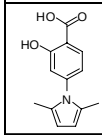
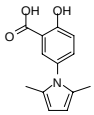
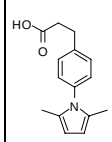
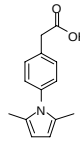
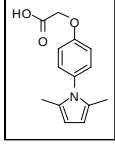
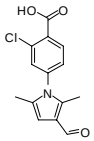
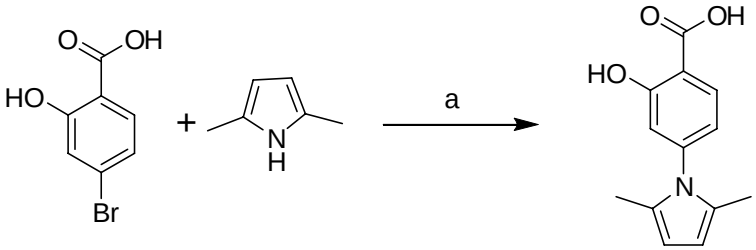
Structure	EphA4-KYL IC_{50} (μM)	Structure	EphA4-KYL IC_{50} (μM)
	2.1μM **PROBE**		2.7μM
	7.7μM		14μM
	25μM		32μM

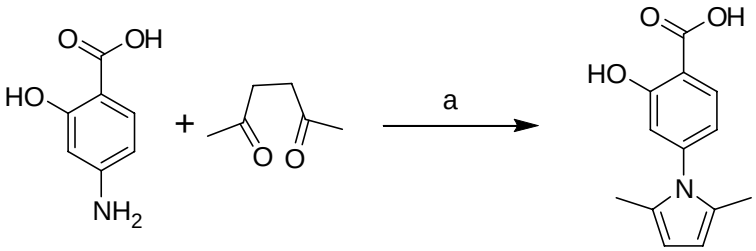
Table 1. Potency data for selected pyrrole derivatives.

Reduced to practice detailed synthetic pathway for making probe:

Eq. 1.



Eq. 2.



Scheme 1. Synthesis of *N*-aryl pyrroles. Reagents and conditions: (a) CuI, *N*-methylglycine, K_2CO_3, DMSO, 90°C, 36h; 36%; (b) 5 mol % pTsOH, toluene, reflux, 6h, 47%..

Known probe properties: The probe is a reversible and competitive inhibitor for the natural ligand ephrin-A5, it has a Ki value of ~9 uM, and it is not toxic to cultured cells at the concentrations tested (up to 400uM). See Appendix for more detailed information.

Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool:
The probe has favorable calculated properties, low molecular weight (231.2) and structural features that are similar to known drugs, and can thus be considered a "drug-like" molecule. It has a log P value of 2.76, with 1-3 being the range of most oral drugs. The total polar surface area (TPSA) is 62, also indicative of good oral bioavailability. It is also a rigid molecule with low H-bond donor and acceptor count. Thus, this probe's physicochemical properties make it useful for continued study, including *in vivo* models of efficacy and tolerance.
The probe can be used to block cell responses to ephrin stimulation of EphA4 and also EphA2, the only other Eph receptor that is inhibited by it. As shown in the Appendix, the probe successfully prevents EphA4 and EphA2 activation by ephrin ligands in cultured cells and cellular responses such as EphA4-dependent growth cone collapse in neuronal cells and EphA2-dependent changes in the shape of cancer cells. Since each ephrin ligand can bind many Eph receptors, the probe can also be used to discriminate responses that are mediated by EphA4 or EphA2 rather than other Eph receptors that may be present and activated by the same ligands. Future *in vivo* work may be possible, although *in vivo* properties of the probe in animal models remain to be characterized. See Appendix for more detailed information.

EphA4 pyrrole series Appendices

Comparative data showing probe specificity for target or for cell-based/phenotypic assays data should be shown in orthogonal cell-based assay systems that address the pathway of interest:

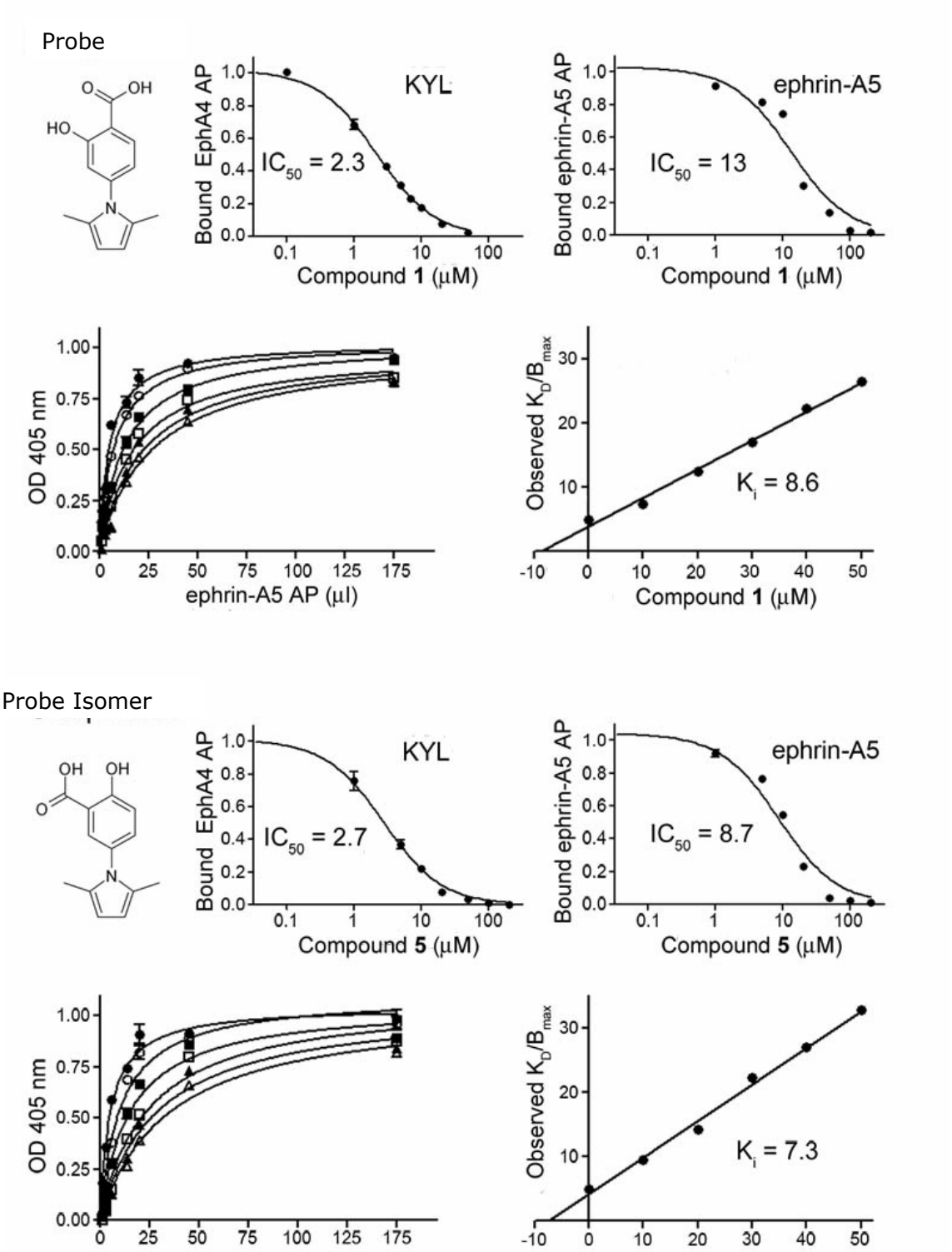


Fig. 1. Small molecules identified by high throughput screening inhibit ephrin-A5 binding to EphA4 in a competitive manner. The probe and its 1,2 isomer inhibit EphA4 AP binding to immobilized biotinylated KYL peptide and ephrin-A5 AP binding to immobilized EphA4 ectodomain Fc in a concentration-dependent manner, as shown in the two top panels for each compound. The bottom-left panels show the binding of ephrin-A5 AP to immobilized EphA4 Fc in the presence of different concentration of each compound.(●) 0 μM (O) 10 μM (■) 20 μ M (□) 30 μ M (solid black triangle) 40 μ M (Δ) 50 μ M. In bottom-right panels the dissociation constants, obtained using non-linear regression, were plotted against the compound concentrations in order to determine K_i values. Error bars indicate standard error from triplicate measurements.

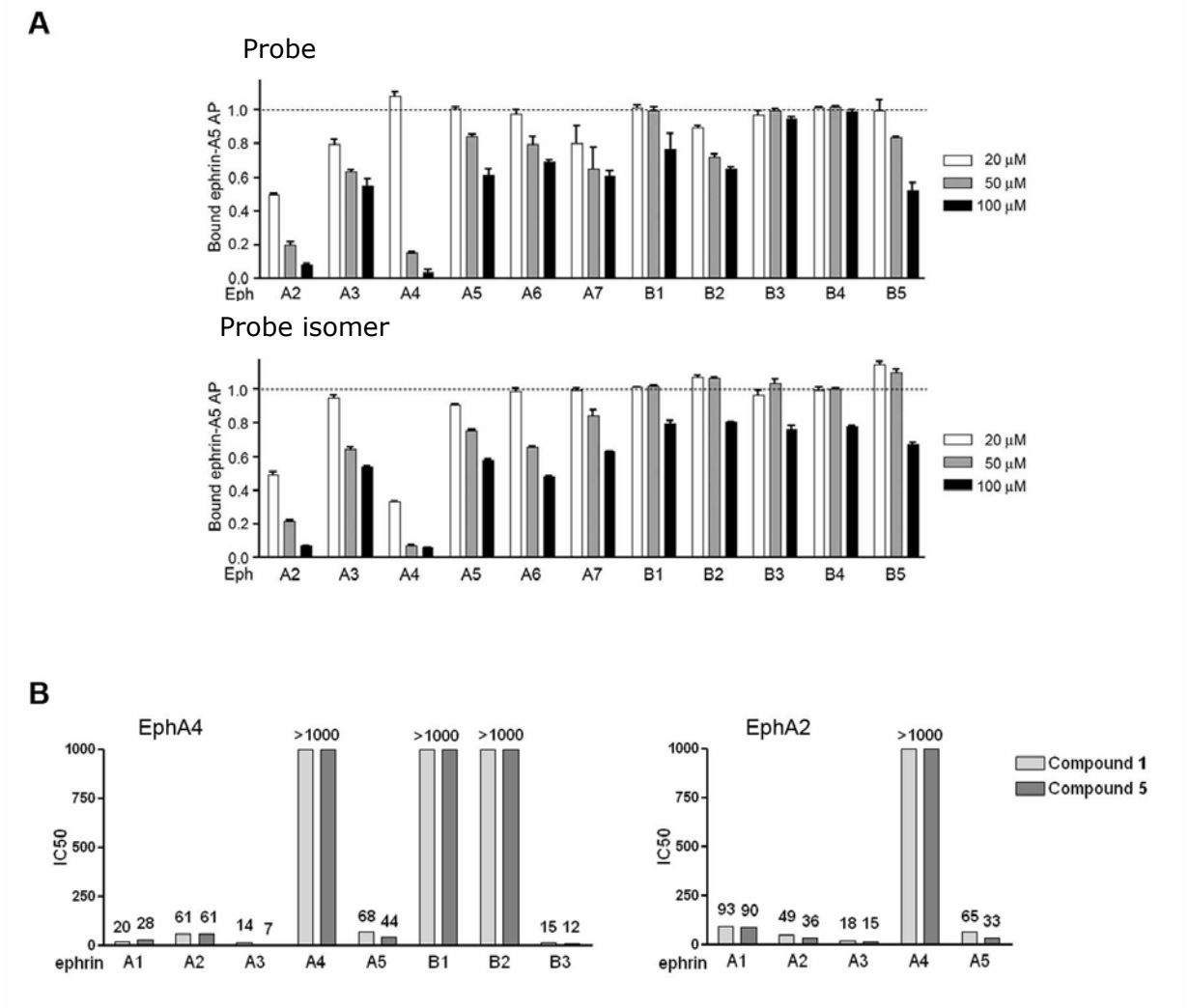


Fig. 2. The probe compound and its isomer show selectivity in their inhibition of different Eph receptors or ephrins. (A) Ephrin-A5 AP or ephrin-B2 AP binding to immobilized EphA or EphB receptor Fc fusion proteins, was measured in the presence of the indicated compound concentrations. The ratio of ephrin AP bound in the presence and in the absence of compounds is shown. Bars indicate standard error from triplicate measurements. **(B)** IC_{50} values for inhibition of EphA4 AP or EphA2 AP binding to the indicated ephrins immobilized on protein A-coated plates.

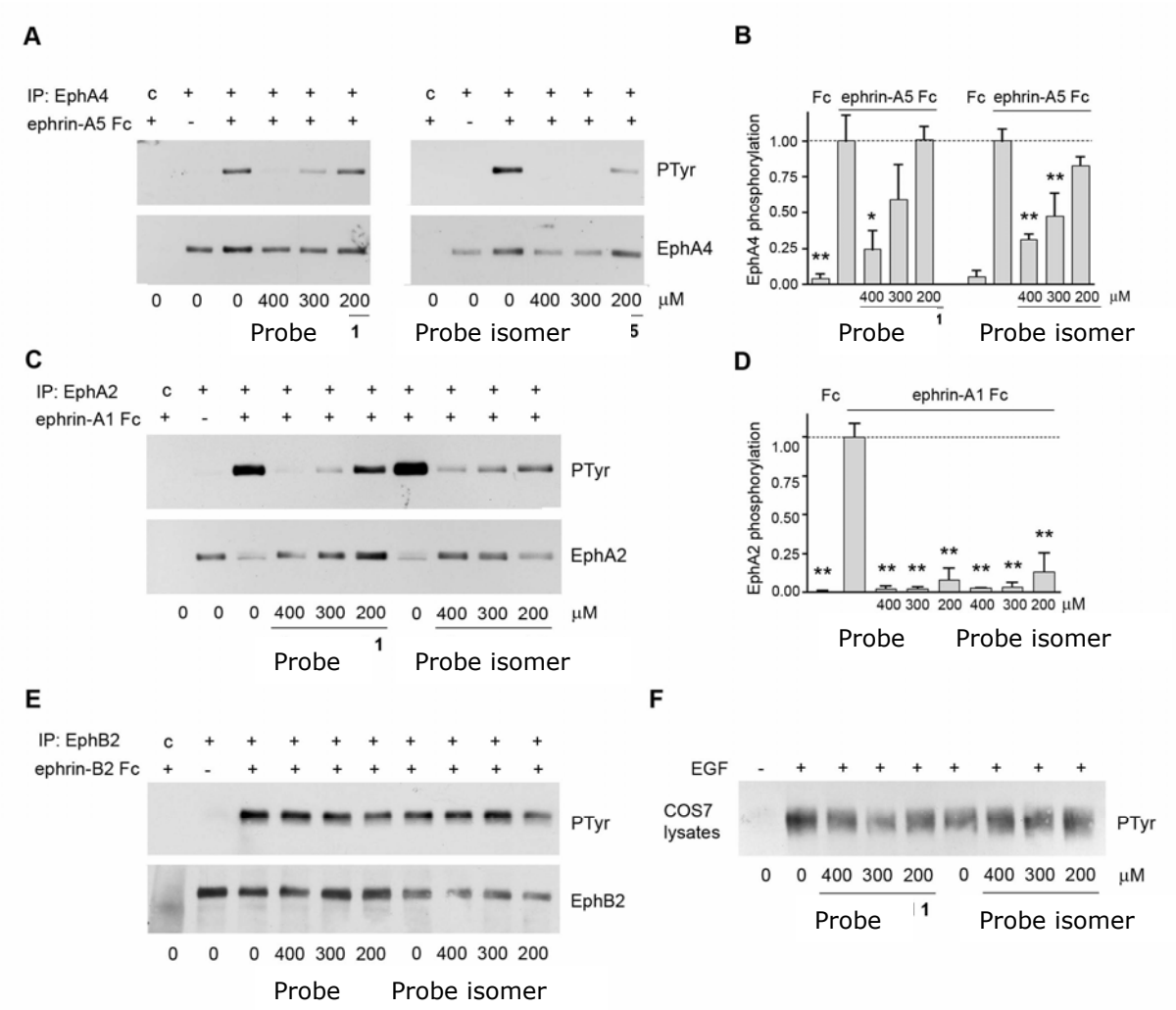


Fig. 3. The probe compound and its isomer inhibit the ligand-induced phosphorylation of EphA4 and EphA2 but not that of EphB2 or the EGF receptor in cells. (A) HT22 neuronal cells pretreated with the indicated concentrations of the compounds for 15 min were stimulated with 0.5 μg/ml ephrin-A5 Fc (+) or Fc as a control (-) for 20 min in the continued presence of the compounds. EphA4 immunoprecipitates were probed with anti-phosphotyrosine antibody (PTyr) and reprobed with anti-EphA4 antibody. C indicates immunoprecipitations performed with control IgG. **(B)** The histogram shows the relative levels of EphA4 phosphorylation quantified from the experiment in (A) and normalized to the EphA4 levels in the immunoprecipitates. Error bars indicate the standard error from at least 3 experiments. **(C)** COS7 cells were stimulated with 0.1 μg/ml of ephrin-A1 Fc or Fc as a control in the absence or presence of the indicated compound concentrations. EphA2 immunoprecipitates were probed with anti-phosphotyrosine antibody and reprobed for EphA2. C indicates the immunoprecipitations performed with control IgG. **(D)** The histogram shows the relative levels of EphA2 phosphorylation quantified from the experiment in (B) and normalized to the EphA2 levels in the immunoprecipitates. Bars represent the standard error from 2 experiments **(E)** The same protocol described in (C) was used, except that COS7 cells were stimulated with 0.5 μg/ml of ephrin-B2 Fc and the EphB2 receptor was immunoprecipitated. **(F)** COS7 cells pretreated with the indicated compound concentrations were stimulated with EGF (+) or left unstimulated (–). Lysates were probed with anti-phosphotyrosine antibody. Receptor phoshorylation levels were compared with those in ephrin-stimulated samples in the absence of compounds by one-way ANOVA and Dunnett's post test. * $P<0.05$ and ** $P<0.01$.

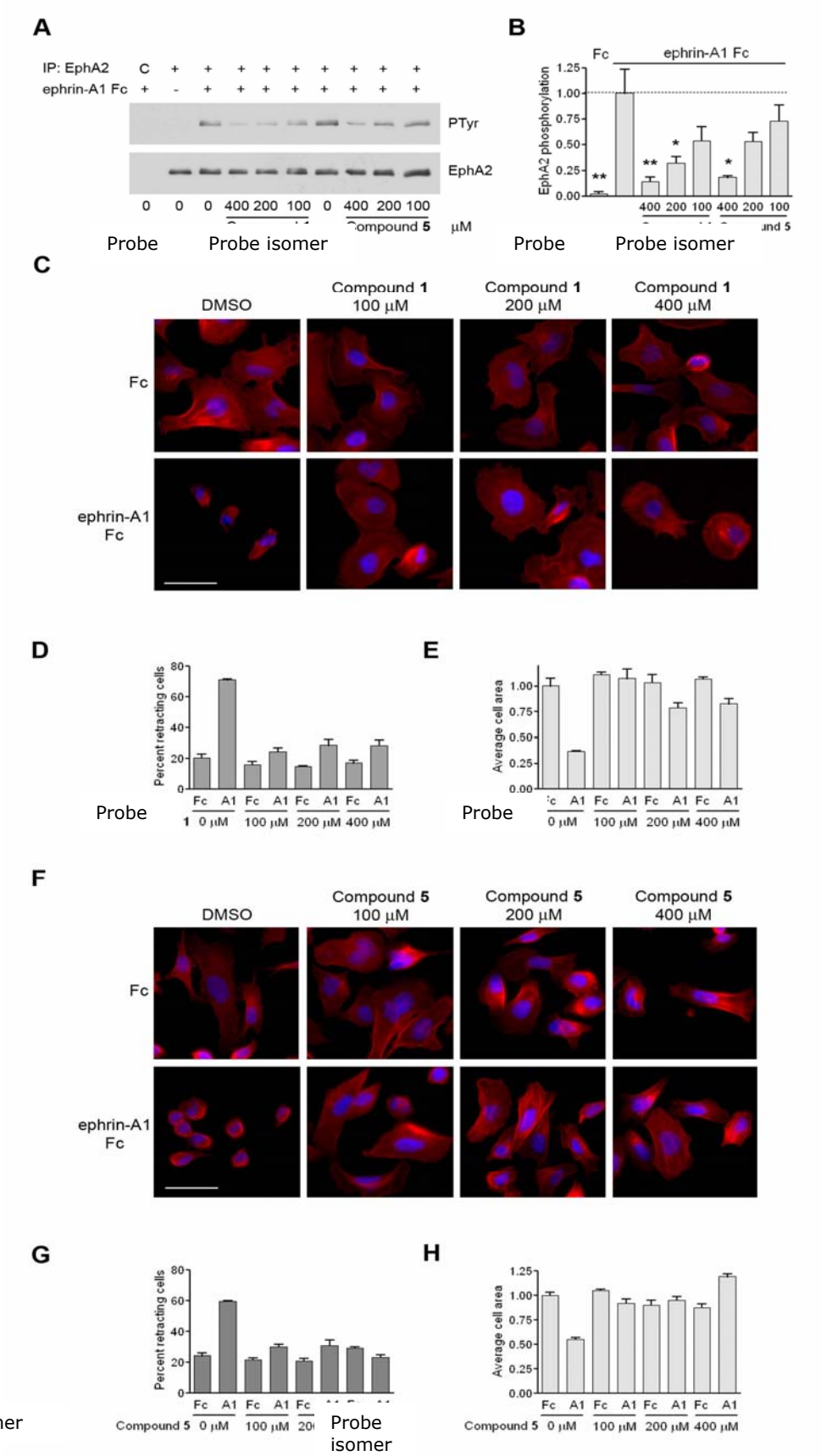

A
IP: EphA2
ephrin-A1 Fc
PTyr
EphA2
Compound 5
μM
Probe
Probe isomer
B
Fc
ephrin-A1 Fc
EphA2 phosphorylation
Probe
Probe isomer
C
DMSO
Compound 1 100 μM
Compound 1 200 μM
Compound 1 400 μM
Fc
ephrin-A1 Fc
D
Percent retracting cells
Probe
E
Average cell area
Probe
F
DMSO
Compound 5 100 μM
Compound 5 200 μM
Compound 5 400 μM
Fc
ephrin-A1 Fc
G
Percent retracting cells
Probe isomer
Probe isomer
H
Average cell area

Fig. 4. **The probe compound and its isomer inhibit ephrin-induced retraction of PC3 prostate cancer cells**. **(A-B)** PC3 cells pretreated for 15 min with the indicated compound concentrations were stimulated with 0.5 μg/ml ephrin-A1 Fc (+) or Fc as a control (–) for 20 min in the continued presence of the compounds. EphA2 immunoprecipitates were probed with anti-phosphotyrosine antibody and reprobed with anti-EphA2 antibody. C indicates the immunoprecipitations performed with control IgG. The histogram in (B) shows the relative levels of EphA2 phosphorylations, which were normalized to the amount of EphA2 in the immunoprecipitates. Error bars indicate standard error from three experiments. Receptor phoshorylation levels were compared with the ephrin-stimulated sample by one-way ANOVA and Dunnett's post test. * $P<0.05$ and ** $P<0.01$. **(C-E)** PC3 cells stimulated as in (A) were stained with DAPI and rhodamine-phalloidin to label filamentous actin. DMSO was used as a control (0 μM). Bar, 50 um. The histogram in (D) shows the mean percentage of retracting cells. Cells having rounded shape and area less than 20% of the mean value obtained for the Fc stimulated cells were scored as retracting. The histogram in (E) shows the mean area of the cells normalized to the value obtained for the Fc stimulated cells. Bars in (D) and (E) indicate standard error from 3 experiments. **(F-H)** The same experiments as in (C-E) were performed using the probe isomer.

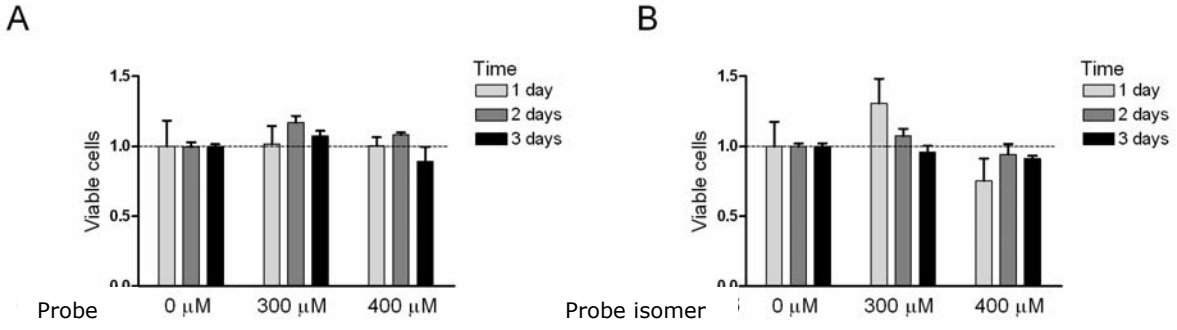


Fig. 5. **The probe compound and its isomer do not have toxic effects in cell culture**. HT22 neuronal cells were grown in the presence of the probe compound **(A)** or its isomer **(B)** at the indicated concentration for 1, 2 or 3 days. DMSO in place of the compounds was used in the "0 μM" sample, as a control. After addition of MTT the absorbance was measured at 570 nm. The histograms show the absorbance obtained for each condition normalized to the absorbance in the absence of the compounds. Bars represent standard error from triplicate measurements.

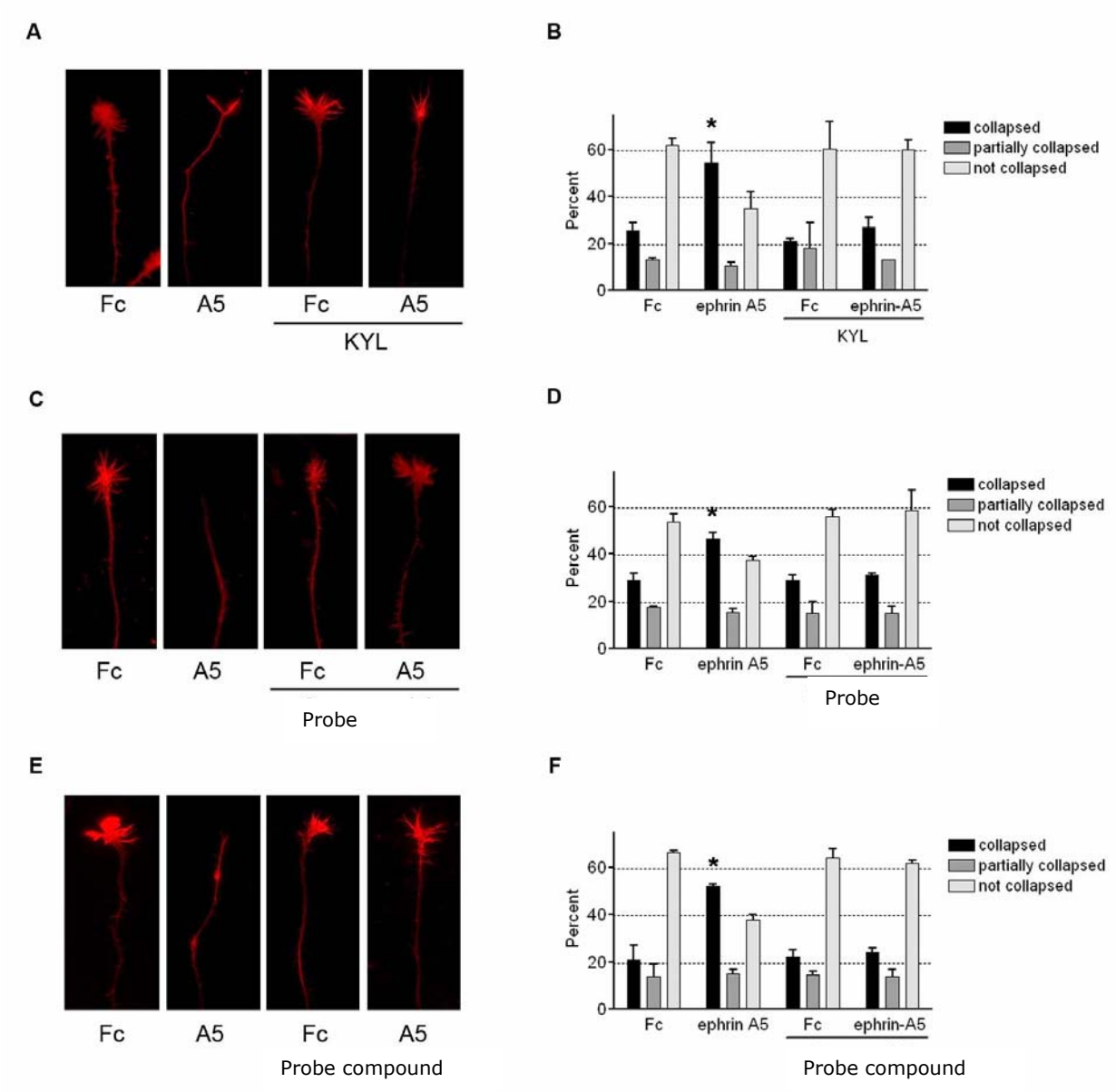


Fig. 6. The KYL peptide, the probe compound and its isomer prevent ephrin-induced growth cone collapse in retinal neurons. **(A,B)** Retina explants from E6 chicken embryos were pre-incubated with 5 μM of KYL peptide for 15 min, stimulated for 30 min with 1 μg/ml ephrin-A5 Fc, or Fc as a control, and stained with rhodamine-phalloidin. Approximately 30-80 growth cones were scored for each condition. The histogram in (B) shows the mean percentages of collapsed, partially collapsed and not collapsed growth cones. Growth cones were scored as "not collapsed" when lamellipodia or more than 3 filopodia where present, as "collapsed" when no lamellipodia or filopodia were visible and as "partially collapsed" in the other cases. Bars indicate standard error from 2 independent experiments. **(C-F)** The same experiment as in (A) was performed, but retina explants were treated with the probe compound (C-D) or its isomer (E-F) at 400 μM. Approximately 80-250 growth cones were scored for each experiment and each condition. Bars in (D) indicate standard errors from measurements in 2 independent experiments and bars in (F) indicate standard errors from duplicate measurements in 1 expt. Collapsed growth cones (%) in the different conditions were compared to Fc control by one-way ANOVA and Newman-Keul's post test. * $P<0.05$

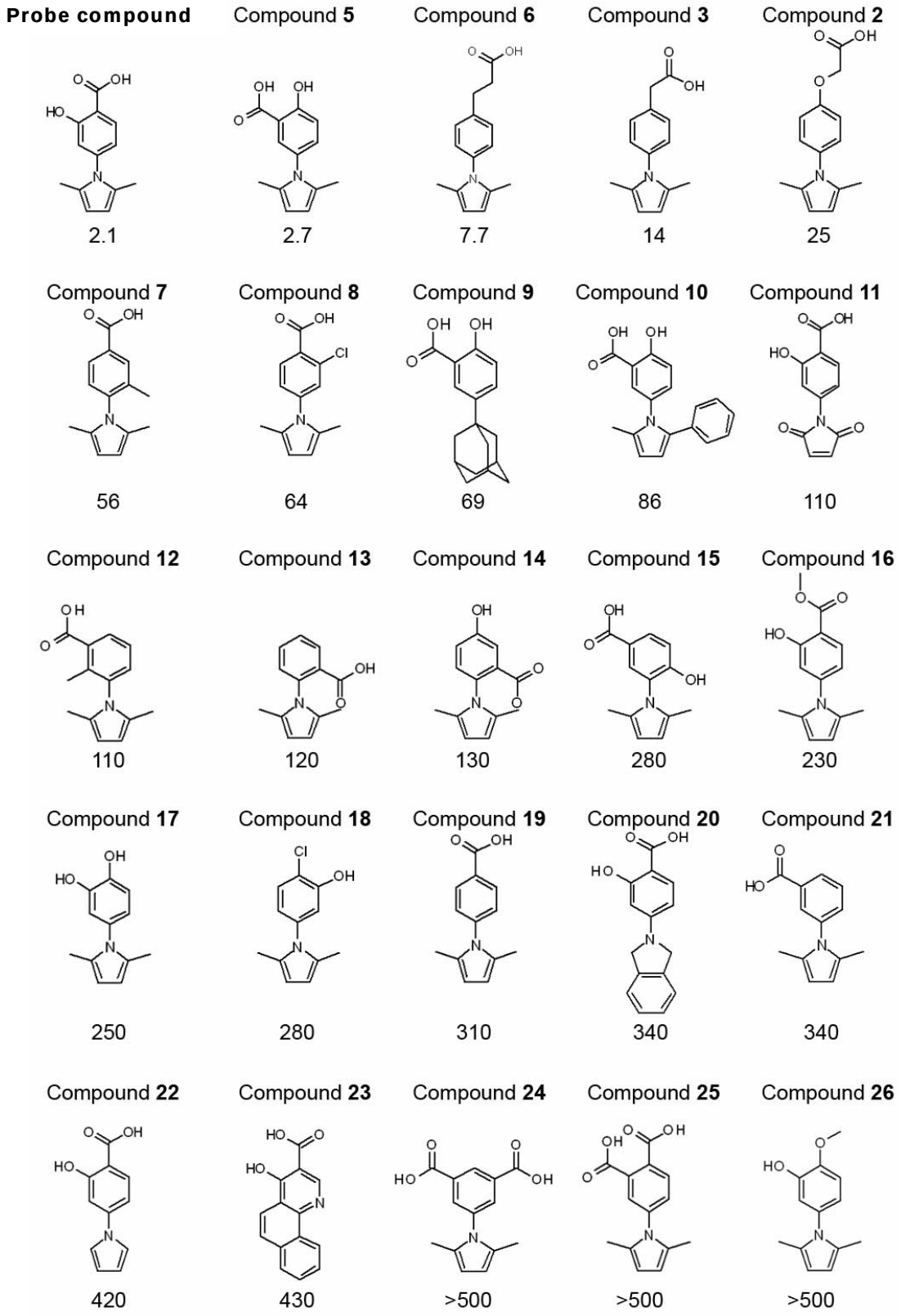


Fig. 7. Structures of 2,5-dimethylpyrrolyl benzoic acid analogs and IC_{50} values in μM for inhibition of EphA4 AP binding to immobilized biotinylated KYL peptide.

Bibliography

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