Targeting Eph receptors with chemical compounds and peptides

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CONTENTS

Chapter 1

Introduction

Eph receptors and ephrins

Eph receptors represent the largest family of receptor tyrosine kinases. The first member of this family was cloned in 1987 from an erythropoietin-producing human epatocellular carcinoma cell line, from which the Eph receptors earned their name (Hirai et al., 1987). To date 16 vertebrate Eph receptors have been described, divided into two sub-groups based on sequences similarities and binding preferences for their ligands, the ephrins.

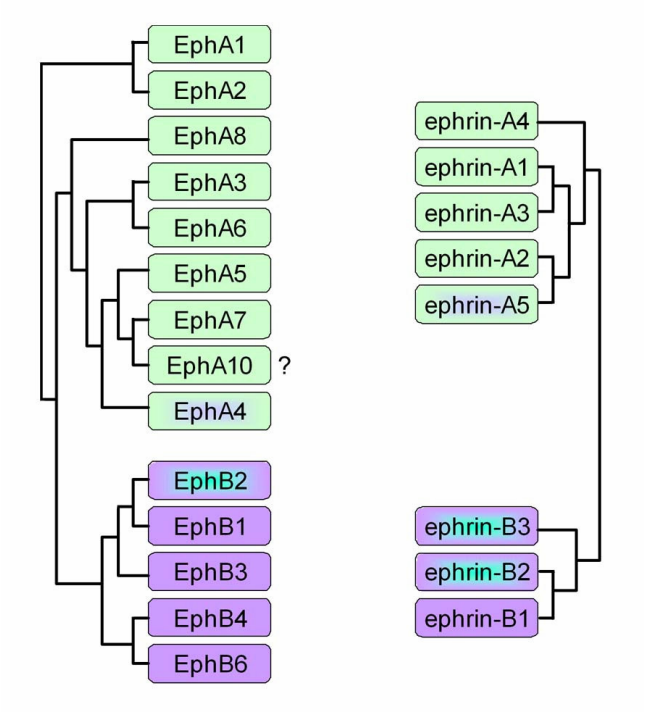

Figure 1.1. **Binding interactions and sequence relationships of Eph receptors and ephrins.** Green and purple indicate the high binding affinity to partners of the A or B class, respectively. Green with a purple center indicates high binding affinity to partners of the A class and low binding affinity to partners of the B class; purple with a green center indicates high binding affinity to partners of the B class and low binding affinity to partners of the A class. The question mark indicates that the binding preference of EphA10 receptor has not yet been determined.

There are 10 EphA (A1 through A10) and 6 EphB (B1 through B6) receptors, which preferentially bind to ephrins of the A or B class, respectively. Receptor-ligand interactions are promiscuous within each class, although binding affinities vary. Despite the initial classification, there are also some examples of inter-class binding: indeed, EphA4 can bind to all the ephrin B ligands, and EphB2 can bind to ephrin-A5 (Himanen et al., 2004; Pasquale, 2004) (**Fig. 1.1**).

Both the Eph receptors and the ephrins are membrane-bound molecules (**Fig. 1.2**). The Eph receptors are transmembrane proteins and they consist of an extracellular region responsible for ligand binding, a single transmembrane domain and a cytoplasmic region, where the catalytic activity resides (**Fig. 1.2**).

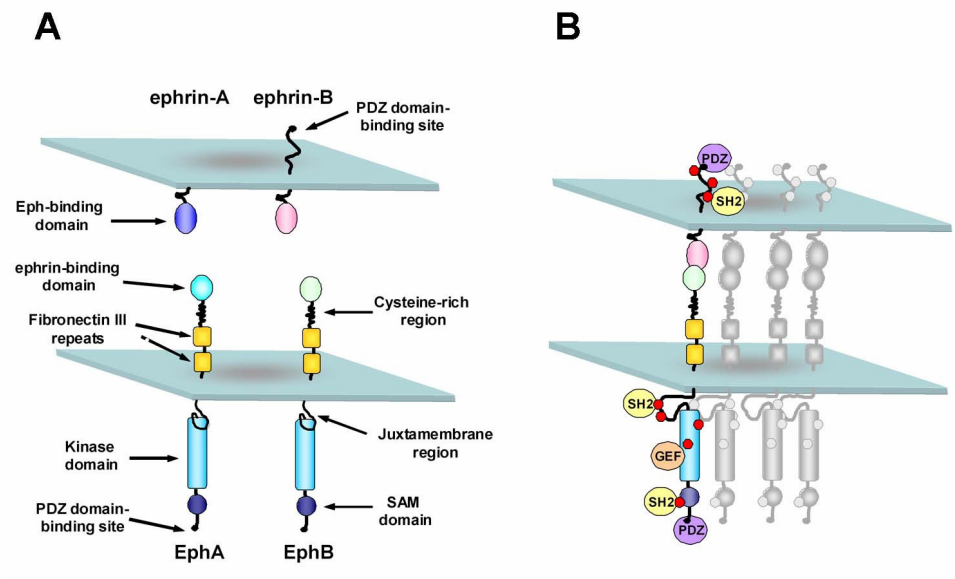

Figure 1.2. **(A)** Domain structure of Eph receptors and ephrins. The ephrin-binding domains of Eph receptors are in different colors to indicate their different specificity for ephrin-A and ephrin–B ligands, respectively. **(B)** Eph receptors-ephrin complex and associated signaling molecules. Eph receptor-ephrin binding results in the lateral aggregation of multiple Eph-ephrin complexes and the transphosphorylation of tyrosine residues (indicated in red) in the cytoplasmic region. This allows conformational changes in the receptor and ephrin structures and the association with intracellular signaling molecules, such as SH2 domain-containing proteins (SH2), PDZ domain-containing proteins (PDZ) and guanine nucleotide exchange factors for Rho GTPases (GEF).

The extracellular region includes a conserved N-terminal ephrin-binding domain, a cysteine-rich linker, and two fibronectin type-III repeats, which seem to be involved in receptor dimerization (Lackmann et al., 1998). The ephrin-binding domains of EphB2 (Himanen et al., 1998) and EphB4 (Chrencik et al., 2006) have been crystallized, showing a conserved jellyroll folding topology formed by 13 antiparallel beta-sheets. The cytoplasmic part of the Eph receptors consists of a juxtamembrane segment, a tyrosine kinase domain, a sterile-alpha-motif (SAM) domain, which is involved in receptor dimerization and signal transduction (Schultz et al., 1997; Stapleton et al., 1999), and a binding motif for post-synaptic density protein zona occludens (PDZ) domains. The juxtamembrane region contains 2 highly conserved tyrosine residues, which are the major autophosphorylation sites involved in receptor signaling (Bruckner and Klein, 1998; Holland et al., 1998).

The ephrin-B ligands are transmembrane proteins and contain conserved cytoplasmic regions, while the ephrin-A ligands are attached to the membrane through a glycosyl-phosphatidylinositol (GPI)-anchor at their C terminus. The receptor-binding domain of the ephrins of both the A and B class is conserved and has a Greek key beta-barrel folding topology (Nikolov et al., 2007; Nikolov et al., 2005).

Eph-ephrin complex formation

Binding of Eph receptors to their ephrin ligands leads to clustering, autophosphorylation and binding to intracellular signaling molecules, creating what is commonly called “forward signaling”. In addition, ephrin clustering and downstream signaling also occurs, which is referred to as “reverse signaling”.

The crystal structures of the ligand binding domain of EphB2 in the *apo* form or in complex with ephrin-B2 or ephrin-A5 have elucidated how complex formation between Eph receptors and ephrins occurs (Himanen et al., 2001). The ephrin binding domain of EphB2 consists of two interfaces: a high affinity, hydrophobic cavity in the Eph receptor

extracellular domain, which accommodates the protruding G-H loop of the ephrin, and a low affinity interface on the opposite side. Two interfaces, one high affinity and the other low affinity, are also present on the surface of the Eph binding domain of the ephrin. The high affinity interfaces mediate the dimerization of the Eph receptors and ephrins, while the low affinity interfaces facilitate the formation of a heterotetramer, consisting of two Eph/ephrin heterodimers. Additional residues in both proteins also contribute to the binding surfaces, and both ligand and receptor undergo conformational changes, which in the case of EphB2 are different when the receptor is bound to different ligands.

A third molecular surface, located outside the crystallized domain, seems to help higher order oligomerization. Random mutagenesis of the EphA3 receptor revealed that a region located within the cysteine-rich linker also plays a role in EphA3 activation (Smith et al., 2004). A random mutagenesis approach was also used to identify the EphA3-interacting residues on ephrin-A5 surfaces, showing that a third interface is also present on the ephrin and it is located between the dimerization and the tetramerization sites (Day et al., 2005). Despite the presence of at least three binding interfaces, molecules that target the high affinity interface are sufficient to inhibit ligand-receptor binding (Chrencik et al., 2006; Koolpe et al., 2005; Koolpe et al., 2002; Murai et al., 2003b).

Eph activation and signaling

In the unphosphorylated form the Eph receptors are kept in an inactive state by the association of a helix from the juxtamembrane domain with the kinase domain (Wybenga-Groot et al., 2001). As a consequence of ephrin binding, the Eph receptors cluster and the kinase domains come in close proximity, favoring trans-phoshorylation of the cytoplasmic domains, which in turn releases the inhibitory interactions with the juxtamembrane domain and promotes kinase activity.

Tyrosine phosphorylation creates binding sites for Src-homology 2 (SH2) domain-containing proteins, while the interaction with other

signaling proteins, such as PDZ-domain-containing proteins and guanine nucleotide exchange factors for Ras and Rho family proteins, are independent of the kinase activity of the receptor (Noren and Pasquale, 2004). EphB6 and EphA10, which lack an active catalytic domain, can signal through kinase-independent pathways or can be phosphorylated by other receptors and bind cytoplasmic proteins.

The reverse signaling, mediated by the activation of the ephrin, is better understood for the B-class ephrins, which can recruit intracellular signaling or adaptor proteins through their PDZ-binding domain or through phosphorylated tyrosines in their cytoplasmic region. The activation of the A-ephrins is poorly understood and may require the interaction with transmembrane proteins (Pasquale, 2005).

Eph signaling can mediate both cell attraction and cell repulsion. Given that both Eph receptors and ephrins are membrane bound molecules, their interaction occurs at sites of cell contact and mediates cell adhesion. However, Eph signaling causes contraction of the actin cytoskeleton and retraction of the cell periphery. Separation of the two adhering cell surfaces is enabled by endocytosis of Eph-ephrin complexes at sites of cell contact or by proteolitic cleavage of the ephrin or the receptor (Pasquale, 2005).

In many tissues, Eph receptors and ephrins are co-expressed in the same cells. Two different mechanisms have been reported to explain the role of Eph-ephrin coexistence. Eph receptors and ephrins can be segregated in different plasma membrane domains, where they can interact *in trans* with their partners on other cells, independently generating signals (Marquardt et al., 2005; Konstantinova et al., 2007). Alternatively, Eph receptors and ephrins could be intermixed in the same areas of the plasma membrane and interact with each other *in cis*, thus silencing forward and reverse signaling by preventing interaction with partners on other cells (Carvalho et al., 2006).

Eph functions

Eph receptors and ephrins were originally identified as neuronal axon guidance molecules active during development. However, further studies showed how this family of receptors is involved in many other different activities, both during development and in the adult.

In the developing nervous system Eph-ephrin interaction is required to guide growing axons towards their targets through repulsive effects (Wilkinson, 2001). For example, axons with high Eph receptor expression avoid regions with high ephrin expression. Gradients of Eph receptors and ephrins help establish proper neuronal connections in many areas of the brain, including the olfactory and visual systems. Eph receptors and ephrins are also involved in patterning the vertebrate neuraxis (Lumsden and Krumlauf, 1996) and controlling the migration of neural crest cells (Wang and Anderson, 1997) and axon branching and fasciculation (Chen et al., 2004; Yates et al., 2001). Eph receptors and ephrins are also expressed in different cell types in the adult brain, although usually at lower levels compared to the developing stages. The functions of Eph receptors and ephrins in the adult brain remain partly unclear, but it appears that they may play an important role in the formation and plasticity of dendritic spines and synapses in the hippocampus (Murai et al., 2003a; Murai and Pasquale, 2002), which are processes involved in learning and memory.

In addition to their roles in the neural system, the Eph receptors are involved in many other physiological processes. One of the most investigated is their activity in the development of the cardiovascular system (Kuijper et al., 2007; Zhang and Hughes, 2006). The cell shaping, migration and compartmentalization required during angiogenesis and remodeling of blood and lymphatic vasculature are regulated by repulsive Eph signals. Eph receptors and ephrins are also involved in the immune system, where they regulate T cell development and immune responses (Hjorthaug and Aasheim, 2007) and dendridic cell trafficking and function (de Saint-Vis et al., 2003; Munthe et al., 2004).

Recent evidence suggests a role for Eph-ephrin signaling in the response of pancreatic β-cells to glucose. In the absence of glucose, Eph forward signaling, which inhibits insulin secretion, is predominant. In the presence of glucose, the cells shift to ephrin reverse signaling to enhance insulin secretion (Konstantinova et al., 2007).

Eph-ephrin bidirectional signaling is also involved in the regulation of bone homeostasis (Zhao et al., 2006) and in platelet aggregation (Prevost et al., 2002; Prevost et al., 2005).

Finally, Eph receptors and ephrins are expressed in different types of stem cells, including skin, haematopoietic, neural, intestinal and embryonic stem cells, where they can influence self-renewal and differentiation (Pasquale, 2005).

Targeting Eph receptors

Given the wide distribution and multiple functions of the Eph receptors and their ligands, it is not surprising that they are involved in many diseases. The Eph receptors are upregulated after central nervous system injury, and they may inhibit axon regeneration by stimulating growth cone collapse, astrocytic glyosis and the formation of the glial scar (Goldshmit et al., 2006). Furthermore, many Eph receptors are overexpressed or dysregulated in different types of cancer, where they can affect tumor growth, tumor vascularization and metastatic dissemination. Therefore, targeting Eph receptors may be beneficial for central nervous system regeneration after injury and for the treatment of different types of cancer. Furthermore, given the role of Eph-ephrin interaction in promoting platelet aggregation and thrombus stability, blocking the Eph-ephrin signaling could be useful for the prevention of blood clotting under pathological conditions, such as stroke and coronary artery occlusion. Drugs targeting Eph signaling could also be used in diabetes and osteoporosis treatment, and to control stem cell migration and fate determination.

Aim of the work

The goal of this work is the development and characterization of chemical compounds and peptides that inhibit Eph receptor-ephrin binding (**Fig. 1.3**).

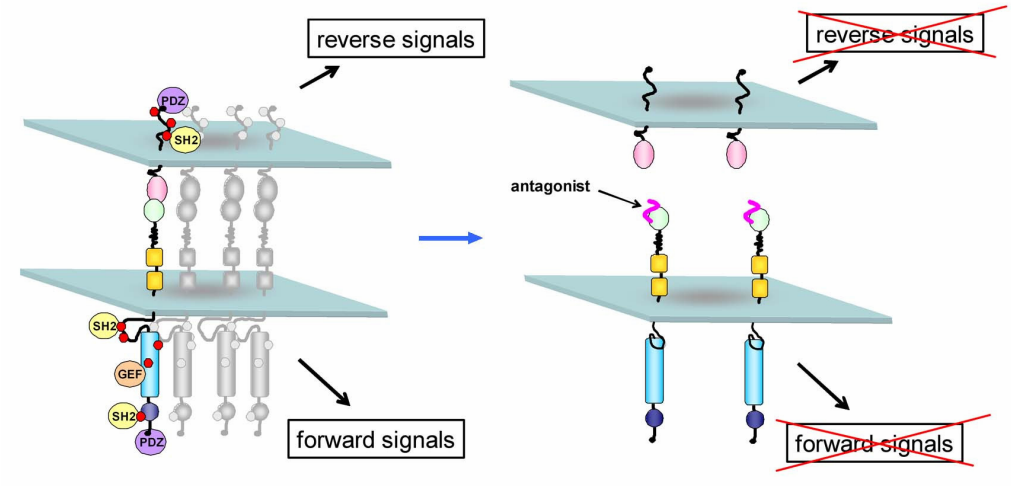

Figure 1.3. Molecules targeting the Eph-ephrin binding pocket can inhibit bidirectional signals, by disrupting the interaction between Eph receptors and ephrins.

The identification of such molecules will help in understanding the mechanism of recognition between the receptors and their ligands and will be useful for the treatment of diseases where Eph-ephrin interaction is involved. Some molecules that modulate Eph-ephrin signaling have already been identified, including agonistic and antagonistic peptides, antibodies and soluble portions of Eph receptors and ephrins (Carles-Kinch et al., 2002; Conover et al., 2000; Koolpe et al., 2005; Koolpe et al., 2002; Murai et al., 2003b; Noblitt et al., 2004). However none of the molecules identified so far have ideal pharmacological properties: they are difficult to make, not sufficiently stable or do not show adequate specificity.

Although the interaction surface involved in Eph receptor-ephrin binding is relatively large, a survey analyzing the suitability of binding

pockets in terms of size, polarity and geometry of protein-protein interaction surfaces showed that the high affinity ephrin binding pocket of the Eph receptors is amenable to tight binding of a druglike compound (Fry and Vassilev, 2005).

In this study we focused on the identification of compound inhibitors targeting the Eph receptors EphA4 and EphA2 using high throughput screening or combinatorial approaches. We also modified TNYL-RAW, a potent peptide antagonist for EphB4, in order to improve its stability and delay its *in vivo* clearance from the blood circulation by coupling it with poly ethylene glycol (PEG) or the Fc portion of human IgG.

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Chapter 2

Small molecules that target the EphA4 receptor

ABSTRACT

The EphA4 receptor is a well-known regulator of axon guidance and tissue patterning during nervous system development. Recent evidence also links the expression and activity of this receptor to different pathological conditions, such as inhibition of spinal cord regeneration after injury, improper platelet aggregation and cancer. Thus, EphA4 is a promising novel target for drug development. Here we report the identification by high throughput screening of two 2,5-dimethylpyrrolyl benzoic acid derivatives that inhibit EphA4-ephrin binding and, among the large family of Eph receptors, are specific for EphA4 and EphA2 only. These compounds inhibit ephrin-dependent phosphorylation of EphA4 and EphA2 in cells, EphA4-mediated growth cone collapse in retinal explants and EphA2-dependent retraction of the cell periphery in prostate cancer cells. The inhibitory activities of these compounds in cell biological assays suggest that they are promising lead compounds for the development of therapies to treat diseases in which EphA4 and EphA2 are involved. We also report some examples of artifacts that could be encountered when screening small molecule in a high throughput format.

INTRODUCTION

The Eph family of tyrosine kinase receptors and their ligands, the ephrins, are critical regulators of nervous system development. Eph-ephrin signaling mediates axon pathfinding and helps organizing topographical maps during neural development by mediating repulsive cues (O'Leary and Wilkinson, 1999; Wilkinson, 2001). EphA4 is a member of the A class of Eph receptors (see Introduction) and modulates diverse processes by interacting with both ephrin A and B ligands. Similar to other members of the Eph family, EphA4 is involved in the development of the nervous system, where it helps organize and compartmentalize developing structures, usually by mediating repulsive signals in response to the binding to its ephrin ligands. For instance, during early development of the hindbrain and somites, EphA4 signaling helps segregate anatomical compartment expressing the receptor from compartment expressing ephrin ligands (Mellitzer et al., 1999; Xu et al., 1995; Xu et al., 1999). Later in development, EphA4 is required in the corticospinal tract, where by interacting with ephrin-B3 expressed at the spinal cord midline it prevents contralateral corticospinal projections from recrossing the midline (Coonan et al., 2001; Dottori et al., 1998; Kullander et al., 2001; Yokoyama et al., 2001). EphA4 also regulates the trajectory of motor axons from the lateral motor column of the spinal cord to limb muscles (Eberhart et al., 2004; Eberhart et al., 2002). Finally, in the developing visual system EphA4 guides the topographical connections between retinal ganglion cells axons and the visual centers of the brain (Connor et al., 1998; Dutting et al., 1999; Hornberger et al., 1999; Reber et al., 2004).

Despite being expressed at higher levels in the embryo, EphA4 is also present in the adult brain, particularly in synaptic-rich structures that show on-going remodeling, such as the hippocampus and the cortex (Murai et al., 2003a). In hippocampal pyramidal neurons, EphA4 is expressed in dendritic spines, which are the small dendritic protrusions where the excitatory synapses are located, and it regulates their morphological remodeling, which is believed to modulate synaptic plasticity and learning

and memory processes. Activation of EphA4 causes shortening and retraction of spines through mechanisms involving different signaling pathways, such as integrin signaling (Bourgin et al., 2007), phospholipaseγ1 signaling (Zhou et al., 2007) and activation of a guanine nucleotide exchange factor for the RhoA GTPase (Fu et al., 2007).

The EphA4 receptor has also been implicated in different pathological conditions. First, it has been shown to inhibit the ability of spinal cord neurons to re-grow after injury. Damage to the adult central nervous system usually results in very limited regeneration of lesioned axons. The limited capability of central nervous system neuron to regenerate is due to different factors, including the reduced intrinsic activity of mature axons to grow, the presence in the environment of myelin-associated inhibitors and an excessive and ipertrophic response of glial cells, which form a barrier to the growth of axons called glial scar (Stichel and Muller, 1998). EphA4 is overexpressed and activated in glial cells at the site of spinal cord injury, consistent with the observation that inflammatory cytokines upregulate EphA4 expression in cultured astrocytes (Goldshmit et al., 2004). EphA4 knock-out mice show a significantly reduced glial scar and an improved ability to regenerate spinal cord connections after injury. Furthermore, EphA4 null astrocytes in culture lose their ability to respond to inflammatory cytokines (Goldshmit et al., 2004), suggesting that this receptor may play a role in the inhibition of axon regeneration in the spinal cord after injury. Here, EphA4 may act as a negative regulator of axon regeneration by mediating the formation of the glial scar and by stimulating ephrin reverse signaling in the axon. In addition, it has been shown that EphA4 accumulates in the damaged axons after injury and may interact with ephrin-B2 expressed in the surrounding astrocytes and ephrin-B3 expressed in myelin, leading to an inhibitory response (Benson et al., 2005; Fabes et al., 2006). The relative importance of these different effects is not yet known, however it seems likely that inhibiting EphA4 function may be beneficial for the treatment of spinal cord injuries. Although one study showed that blockade of EphA4 expression at the site of spinal cord injury

with antisense oligonucletides does not induce axonal regeneration or return of nerve conduction (Cruz-Orengo et al., 2007), another study showed that infusion with a peptide specifically targeting EphA4, KYL (Murai et al., 2003b), into lesioned spinal cords results in improved axon sprouting, reduced cavity formation and improved behavioral recovery (Fabes et al., 2007).

Besides their application in the treatment of spinal cord injury, EphA4 inhibitors may be beneficial for the treatment of other diseases. Indeed, EphA4 is expressed on the surface of human platelets and its interaction with ephrin-B1 favors thrombus formation and stability by promoting integrin activation (Prevost et al., 2002; Prevost et al., 2005; Prevost et al., 2004). Inhibitors of EphA4-ephrin binding could help preventing blood clotting under pathological conditions, such as stroke and recurrent occlusion of the coronary artery, that are due to excessive or inappropriate platelet aggregation. Furthermore, EphA4 is overexpressed in prostate cancer (Ashida et al., 2004), particularly in the transition to more malignant stages. Prostate cancer cell growth is inhibited by small interfering RNA, showing how targeting EphA4 may be also beneficial in the treatment if this type of cancer. Finally, EphA4 is also overexpressed in pancreatic ductal adenocarcinoma, where its interaction with ephrin-A3 promotes cancer cell growth (Iiizumi et al., 2006).

Compounds identified by targeting EphA4 could serve both as tools to study the physiological roles of this receptor and as leads for drug development. However, no small molecule inhibitors of EphA4 have been identified so far. Small molecules are chemical compounds with a molecular weight lower than 500 Da, and they usually show better pharmacological properties compared to peptide and antibodies. Several small molecule inhibitors that target the kinase domain of tyrosine kinases have been identified. However, given the conservation of the kinase domains, these inhibitors typically target multiple kinase families. In this study we report the identification by high through-put screening of two 2,5-dimethylpyrrolyl benzoic acid derivatives that inhibit EphA4 binding to ephrin and EphA4 function.

MATERIALS AND METHODS

Chemical library screening for EphA4 inhibitors

A 96-well or 384-well format *in vitro* assay was used for compound screening. Polystyrene high binding capacity plates (Corning, Corning, NY) were incubated overnight at 4°C with 2 μg/ml streptavidin (Pierce Biotecnology, Rockford, IL) diluted in borate buffer 0.1 M pH 8.7 and then coated by overnight incubation with 0.1 μM of biotinylated KYL peptide in binding buffer (Tris-buffered saline (TBS) (150 mM NaCl; 50 mM Tris-HCl, pH 7.5 plus 1mM $CaCl_2$ and 0.01% Tween 20). Compounds were added to the wells at 10 μg/ml in 100% dimethylsulfoxide (DMSO) together with EphA4 alkaline phosphatase fusion protein (EphA4 AP) produced from transfected cells. Cell culture medium containing the secreted EphA4 AP was diluted 1: 16 in binding buffer. The mixture was incubated for 3 hours at room temperature before adding *p*-nitrophenylphosphate (pNPP) (Pierce Biotecnology, Rockford, IL) as substrate. After 1 hour the reaction was stopped by adding 2N NaOH and the absorbance at 405 nm was measured using an ELISA plate reader. Alkaline phosphatase activity from wells where alkaline phosphatase (AP) was added instead of EphA4 AP was subtracted as background. The inhibitory activity of the compounds was calculated by dividing the absorbance observed in the presence of each compound and the absorbance from wells where no compound was added. Compounds with inhibitory activity higher than 50% were considered hits. The inhibitory activity of the hits was confirmed by repeating the assay.

ELISA assays and K_i determination

Protein A-coated wells (Pierce Biotecnology, Rockford, IL) were used to immobilize ephrin Fc fusion proteins (R&D Systems, Minneapolis, MN). Compounds at different concentrations were incubated with EphA4, EphA2 or EphB4 AP for 3 hours. Alternatively, Eph receptor Fc fusion proteins were immobilized on protein A-coated wells and ephrin-A5 AP or ephrin-B2

AP was added with the compounds. In order to test if the inhibition observed was reversible, an assay where the compounds were removed and the wells washed for 3 hours before the addition of the AP fusion protein was also performed. The amount of bound AP-fusion protein was quantified using pNPP. Alkaline phosphatase activity from wells with Fc only was subtracted as background.

For estimations of the inhibition constant (K_i) values, the binding of ephrin-A5 AP to EphA4 Fc immobilized on protein A-coated wells was measured in the absence or in the presence of the compounds at different concentrations. Each data set was fitted to the Michaelis-Menten equation (Equation 1) using non linear regression and the program GraphPad (Prism).

$$B = B_{max} [S]/(K_D + [S]) \quad \text{(Equation 1)}$$

where [S] is the concentration of ephrin AP fusion protein and K_D is the dissociation constant observed in the absence or in the presence of the compound.

To evaluate whether the inhibition is competitive, non competitive or uncompetitive the K_D and the B_{max} values were determined at different compound concentrations. The K_i was obtained from the linear regression plot of K_D/B_{max} as a function of the concentration of the inhibitor according to:

$$K_D/B_{max} = (K_D [S])/(K_i \cdot B_{max}) + K_D/B_{max} \quad \text{(Equation 2)}$$

Alternatively, K_i values were obtained from the dose response curves, using the Cheng-Prusoff equation (Cheng and Prusoff, 1973):

$$K_i = IC_{50}/(1 + [S]/K_D) \quad \text{(Equation 3)}$$

Assays to identify nonspecific inhibitors

Goat anti-EphA4 antibody (R&D Systems, Minneapolis, MN) was immobilized on protein G-coated wells (Pierce Biotecnology, Rockford, IL), followed by the addition of EphA4 AP containing medium diluted 1:16 in binding buffer. The receptor immobilized in the wells was incubated with the compounds in 100% DMSO for 3 hours before washing the compounds off the plate and adding pNPP substrate. Absorbance was measured at 405 nm. In some experiments, after removing the solution containing unbound EphA4 AP and compounds, the wells were incubated for an additional 3 hours in binding buffer, to examine whether the inhibitory effects of the compounds were reversible. In other experiments, the wells were not washed before measuring AP activity to discriminate between inhibition of alkaline phosphatase activity and inhibition of binding interactions. Experiments where compounds were: (1) added to protein G-coated wells and washed off before the addition of the anti-EphA4 antibody or (2) added to the immobilized anti EphA4 antibody and washed off before adding EphA4 AP were also performed. The results were normalized to alkaline phosphatase activity from wells containing the same amount of DMSO but without the compounds.

Measurement of receptor tyrosine phosphorylation in cells

HT22 neuronal cells, which endogenously express EphA4, and COS7 cells, which endogenously express EphA2, EphB2 and the EGF receptor (EGFR) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Inc, Herndon, VA) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and Pen/Strep. For EphA4 immunoprecipitations, HT22 cells were serum-starved overnight in 0.5% FBS in DMEM and incubated for 15 min with the compounds or DMSO as a control. The cells were then stimulated with 0.5 μg/ml ephrin-A5 Fc or Fc protein for 20 min in the presence of the compounds. After stimulation the cells were lysed in modified RIPA buffer (1% Triton X-100, 1% Na deoxycholate; 0.1 SDS; 20 mM Tris; 150 mM NaCl; 1 mM EDTA) containing 10 μM NaF, 1 μM sodium pervanadate and

protease inhibitors. The protein concentration was calculated using the BCA protein assay kit (Pierce Biotecnology, Rockford, IL). Cell lysates were immunoprecipitated with 4 μg anti-EphA4 antibody. For EphA2 and EphB2 immunoprecipitations, serum-starved COS7 cells were stimulated with 0.1 μg/ml ephrin-A1 Fc or 0.5 μg/ml ephrin-B2 Fc, respectively. The cells were then lysed and incubated with 2 μg of anti-EphA2 antibody (Millipore, Inc, Temecula, CA) or 7 μg anti-EphB2 antibody.

To assess EGFR phosphorylation, COS7 cells were serum-starved overnight in 0.2% FBS in DMEM. The cells were preincubated with the compounds as described above and then stimulated for 15 min with 0.1 μM EGF. Immunoprecipitates and lysates were probed by immunoblotting with anti-phosphotyrosine antibody (Millipore, Inc, Temecula, CA) and reprobed with anti-Eph receptor antibodies followed by a secondary anti-IgG peroxidase-conjugated antibody (GE Healthcare, UK).

MTT assay

The cytotoxicity of the compounds was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cells were seeded in a 96 well plate and treated with compounds or DMSO starting 3, 2 or 1 day before they reached 100% confluency. For the assay, MTT (Sigma-Aldrich, Steinheim, Germany) was added at a final concentration of 0.5 μg/ml and incubated with the cells for 3 hours. The resulting formazan crystals were then solubilized by addition of 100% DMSO. The absorbance in each well was measured at 570 nm using an ELISA plate reader. The results were expressed as the ratio of the absorbance of the treated and untreated cells.

PC3 cell retraction

PC3 cells were plated onto glass coverslips and grown in RPMI 1640 medium (Mediatech, Inc, Herndon, VA) with 10% FBS and Pen/Strep. After 17 hours the cells were starved for 3 hours in 0.5% FBS in DMEM and then

incubated for 40 min with the compounds or DMSO, before stimulation for 10 min with 0.5 μg/ml of ephrin-A1 Fc or Fc as a control. The cells were fixed in 4% formaldehyde in PBS, permeabilized in 0.5% Triton X-100 in TBS and stained with rhodamine-conjugated phalloidin (Molecular Probes) and DAPI. Cell area was measured in a blinded manner using ImageJ software and normalized to the average area obtained in the Fc stimulated condition. Cells having rounded shape and area equal or below 20% of the control area were considered as retracting.

Growth cone collapse assay

Nasal retina explants from E6 chicken embryos were cultured on polylysine- (200 μg/ml) and laminin- (20 μg/ml) coated coverslips for 12 to 24 hours in DMEM/F-12 containing 10% FBS and 0.4% methylcellulose. Three hours before adding the Fc fusion proteins, the medium was changed to DMEM/F-12 without methylcellulose. The explants were incubated for 15 min with the KYL peptide or the compounds and then stimulated for 30 min with 1 μg/ml of preclustered ephrin-A5 Fc or Fc as a control. The cultures were fixed for 30 min in 4% paraformaldehyde/4% sucrose, permeabilized in 0.1% Triton X-100 and stained with rhodamine-conjugated phalloidin (Invitrogen, Carlsbad, CA). Growth cones where scored in a blinded manner as collapsed when no lamellipodia or filopodia were present at the tip of the neurite, as not collapsed when lamellipodia or more than 3 filopodia were present and as partially collapsed in the other cases.

RESULTS AND DISCUSSION

High throughput chemical library screening to identify lead compounds that inhibit ligand binding to the EphA4 receptor

To identify small molecule inhibitors of ligand binding to the EphA4 receptor, an assay was designed that takes advantage of a 12 amino acid peptide ligand previously identified by phage display (Murai et al., 2003b). The

peptide – known as KYL – has some sequence similarity with the ephrin-A G-H loop, which mediates high affinity binding to Eph receptors (Himanen et al., 1998). Furthermore, the KYL peptide was shown to competitively inhibit ephrin binding to EphA4, suggesting that it targets the high affinity ligand-binding site of the receptor (Murai et al., 2003b).

The biotinylated KYL peptide was immobilized on streptavidin-coated ELISA wells and binding of the extracellular domain of EphA4 fused to alkaline phosphatase (EphA4 AP) was measured in the presence of chemical compounds. Two screens were performed, where 10,000 compounds from the DIVERSet^{TM} library (ChemBridge, Inc.) and 61,500 compounds from NIH were tested at 10 μg/ml in a 96- and 384- well format, identifying 43 and 80 compounds, respectively, that inhibited EphA4 AP binding by more than 50% in both the original screen and in a rescreen of the hits.

Compound showing a reproducible high inhibition of EphA4-KYL binding were then tested for inhibition of EphA4 interaction with one of its ligands, ephrin-A5.

Some of the hits in the high throughput screen for EphA4 antagonists act by a nonspecific mechanism

Compound **1***, 1-(2-fluorophenyl)-5-(2-furanylmethylene) dihydro-2-thioxo-4,6(1H,5H)-pyrimidinedione, a derivative of barbituric acid, showed a reproducible 97% inhibition of EphA4-KYL binding in the high throughput screen. The IC_{50} for inhibition of EphA4-KYL and EphA4-ephrin-A5 binding were 3 and 40 μM, respectively (**Fig. 2.1A**). Compound **1*** inhibited ephrin-binding to all EphA and EphB receptors except EphA7 and EphB5 (**Fig. 2.1B**). When tested for inhibition of EphA4 binding to different ephrin-A ligands, it showed inhibitory activity towards all ephrins, with the exception of ephrin-A4 (**Fig. 2.1C**). Compound **1*** at 100 μM also inhibited EphA4, EphB4 and EphA2 phosphorylation in cells stimulated with ephrin-A5 Fc, ephrin-B2 Fc and ephrin-A1 Fc, respectively (**Fig. 2.2A-C**).

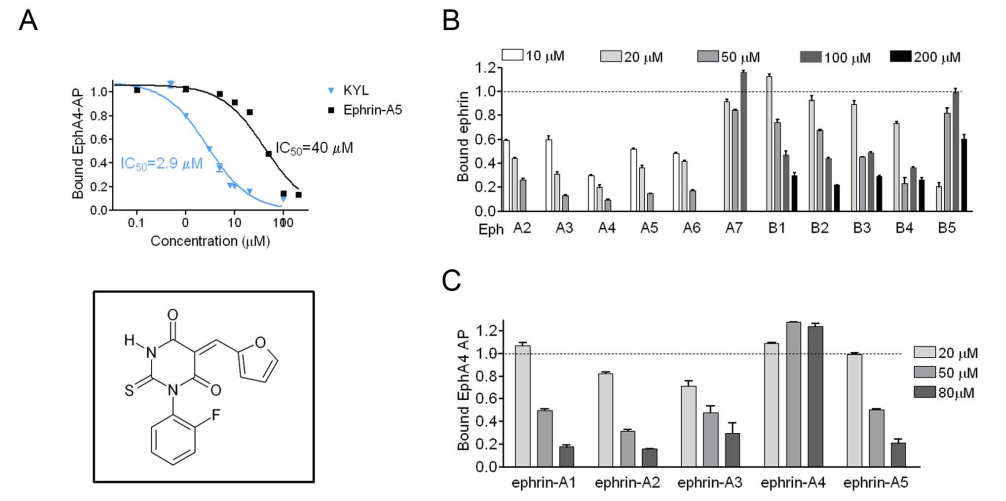


Fig. 2.1. **Compound 1* inhibits ephrin-binding to EphA and EphB receptors**. **(A)** Compound **1*** inhibits EphA4 binding to immobilized KYL peptide (blue) and ephrin-A5 binding to immobilized EphA4 (black) in a concentration-dependent manner. **(B)** Ephrin-A5 AP or ephrin-B2 AP binding to immobilized EphA or EphB receptors, respectively, was measured in the presence of the indicated concentrations of compound **1*** . The ratio of ephrin AP bound in the presence and in the absence of compound is shown. **(C)** EphA4 AP binding to ephrin-A ligands immobilized on protein A-coated plates was detected. The ratios of EphA4 AP bound in the presence and in the absence of the indicated concentrations of compound **1*** are shown. Bars indicate the standard error from triplicate measurements in all the panels.

Cell viability after 17 hours and EGFR phoshorylation in response to EGF stimulation were not affected by treatment with up to 200 μM of compound **1*** (**Fig. 2.2D** and data not shown), suggesting that the compound is not toxic and that it is not a general inhibitor of receptor tyrosine kinases. However, when the compound was tested in ELISA assays aimed at determining whether the observed inhibition was reversible or irreversible, compound **1*** showed some peculiar properties. EphA4 was immobilized on ELISA wells and incubated with compound **1***, followed by washes and incubation in binding buffer for several hours before adding ephrin-A5 AP. Under these conditions, ephrin-A5 binding was restored even in the presence of the compound (**Fig. 2.3A**). However, when the same experiment was performed by measuring EphA4 AP binding to immobilized ephrin-A5, no reduction in activity was observed several hours after removing the compound, suggesting that the compound binds to ephrin-A5 in an irreversible way (**Fig. 2.3B**).

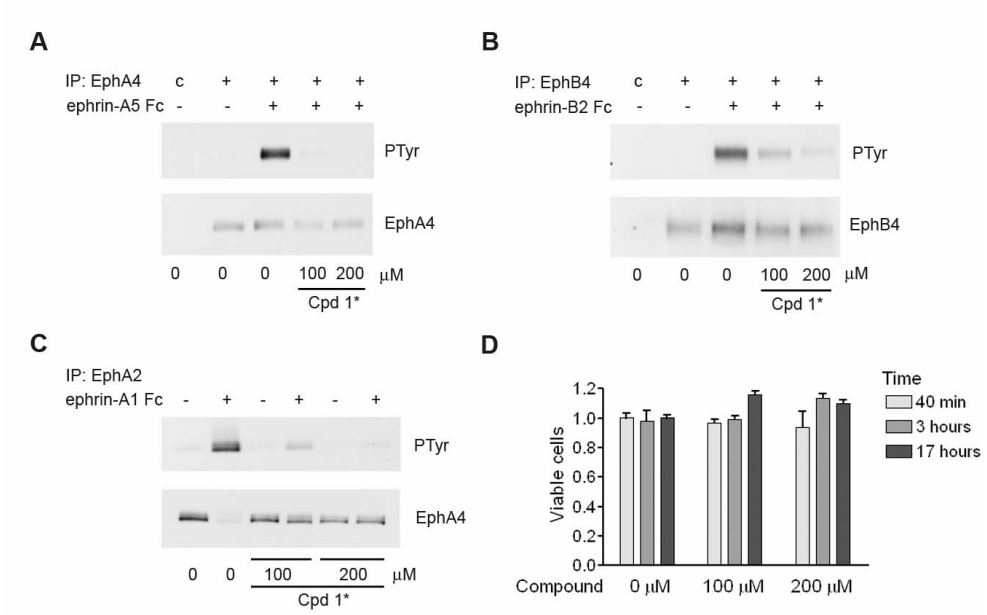


Fig. 2.2. **Compound 1* inhibits EphA4, EphA2 and EphB4 phosphorylation in cells without affecting their viability**. **(A-C)** Cells pretreated with the indicated concentrations of compound **1*** for 15 min were stimulated for 20 min with ephrin Fc (+) or Fc as a control (–) in the continued presence of the compound. Eph receptors immunoprecipitates were probed with anti-phosphotyrosine antibody (PTyr) and reprobed with the indicated anti-Eph antibody. C indicates the immunoprecipitations performed with control IgG. HT22 cells (A), MDA-MB-435 cells (B) and COS7 cells (C) were stimulated with ephrin-A5 Fc, ephrin-B2 Fc or ephrin-A1 Fc, respectively, and EphA4, EphB4 or EphA2 were subsequently immunoprecipitated as indicated. **(D)** HT22 cells were grown in the presence of compound **1*** at the indicated concentration for 40 min, 3 hours or 17 hours. DMSO only was used in the "0 µM" sample. After addition of MTT the absorbance was measured at 570 nm. The histogram shows the absorbance obtained under each condition normalized to the value obtained in the control. Bars represent the standard error from triplicate measurements.

Interestingly, despite binding to ephrin-A5 but not to EphA4, compound **1*** was identified in an assay based on EphA4-KYL binding inhibition, which did not involve the ephrin. An additional ELISA assay with the immobilized KYL peptide was then performed, which demonstrated that compound **1*** also exhibits irreversible activity with the peptide (**Fig. 2.3C**). The hypothesis that compound **1*** covalently binds to the KYL peptide was confirmed by electrospray ionization mass spectrometry, which was performed by the Medicinal Chemistry facility of the Burnham Institute for Medical Research. Indeed, the mass spectrum of the peptide incubated with and without compound **1*** showed a 317 Da shift in the peak corresponding

to the peptide mass, which is the exact molecular weight of compound **1*** (**Fig. 2.3D**).

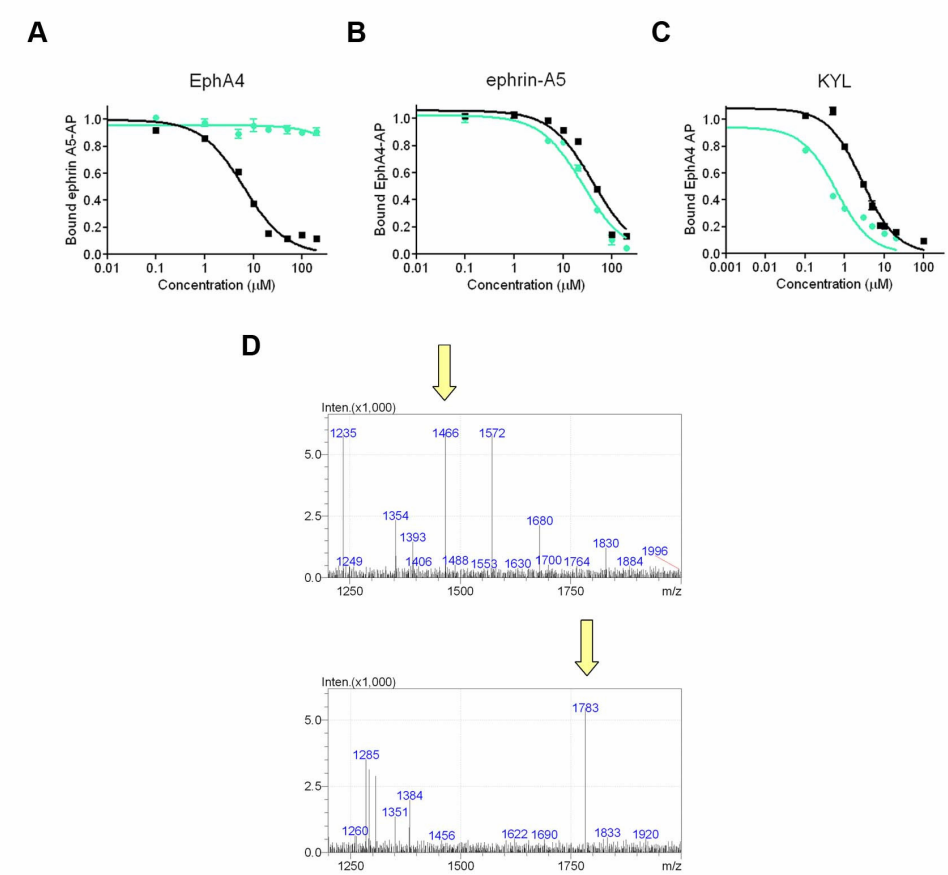


Fig. 2.3. **Compound 1* irreversibly binds to the KYL peptide**. **(A-C)** Ephrin-A5 AP (A) or EphA4 AP (B, C) binding to immobilized EphA4 Fc (A), ephrin-A5 Fc (B) or KYL peptide (C) was measured after incubating compound **1*** at the indicated concentrations together with the AP fusion proteins (black). Alternatively, compound **1*** was removed before addition of the AP fusion proteins, in order to assess whether the compound was irreversibly bound (green). **(D)** The KYL peptide was preincubated for 2 hours with an excess of compound **1*** before performing electrospray ionization mass spectrometry. Peptide MW = 1465, peptide + compound MW = 1782.

The fact that compound **1*** is a reactive molecule which could undergo chemical reactions such as electrophilic addition at the sulfur atom supports the idea that it acts through covalent binding. It would be interesting to identify which amino acid is covalently modified by the compound. Although some covalent-acting drugs identified through biological assays are effective antibiotics or antineoplastic agent, when these types of

compounds are developed using biochemical screens they often have nonspecific activities and are therefore not suited for further development (Rishton, 2003).

Another set of molecules that were identified in the second EphA4 high throughput screen initially showed promising features, having IC_{50} values for inhibition of EphA4-ephrin-A5 binding in the low micromolar range. Three of these molecules, compounds **2***, **3*** and **4***, are shown in **Fig. 2.4A**. When these compounds were tested in a control experiment involving EphA4 AP immobilized through an anti-EphA4 antibody to protein G-coated ELISA plates, however, they showed an inhibition level comparable to the one obtained in the EphA4-ephrin-A5 assay, suggesting that the observed effect was not specific. The inhibition could be due to inhibition of AP activity or to a nonspecific inhibition of one of the protein-protein interactions involved in the assay. None of the compounds inhibited AP activity of EphA4 AP or placental alkaline phosphatase (PLAP) in solution, indicating that they are not AP inhibitors (**Fig. 2.4B**).

In order to better understand the behavior of these compounds, additional ELISA assays were performed (Experiments. I-V; **Fig. 2.4C**). The effects of compounds **2***-**4*** were observed even when the ELISA wells were washed for several hours before measuring AP activity (Exp. II). Compounds **2*** and **4*** showed inhibition when added before EphA4 AP to anti-EphA4 antibody immobilized on the wells (Exp. III), but not when added to the wells before the anti-EphA4 antibody (Exp. IV), suggesting that they inhibit the interaction between the anti-EphA4 antibody and EphA4 AP. In contrast, compound **3*** showed inhibitory activity both when added to protein G-coated plates before the anti-EphA4 antibody and when added after the anti-EphA4 antibody and before EphA4 AP, suggesting that this compound inhibits at least partially the interaction between protein G and the anti-EphA4 antibody. Compound **3*** is a charged reactive molecule, a feature that could explain its promiscuous activity, which is likely due to covalently nonspecific binding through conjugate addition.

In order to test the hypothesis that compounds **2***-**4*** act by nonspecifically disrupting protein-protein interactions, the experiment where the compounds were added to EphA4 AP immobilized through anti-EphA4 antibody to ELISA plates was repeated without washing the compounds off before measuring AP activity (Exp. V). If the hypothesis is correct, no decrease of alkaline phosphatase activity should be observed in this assay. This is what we observed for compound **2***, but not for compounds **3*** and **4***. The behavior of compounds **3*** and **4*** was unexpected, and so far not explained.

Small molecule high throughput screens have become one of the most exploited tools in drug discovery. Although being a powerful tool for the identification of new drugs, high throughput screening results can sometimes be ambiguous and lead to artifacts. Such artifacts can be due to a variety of factors, including chemical reactivity, oxidation, optical opacity, perturbation of the assay or the detection method and nonspecific inhibition due to formation of aggregates (Rishton, 2003; Shoichet, 2006). In the high throughput screens for small molecule inhibitors of EphA4 receptor we encountered some of these artifacts, including chemical reactivity and nonspecific inhibition of protein-protein interactions, in addition to nonspecific inhibition of unknown nature. The prompt identification of such nonspecific inhibitors through the use of appropriate control experiments is indispensable in order to eliminate them as leads for drug development and focus on compounds with better pharmacological properties.

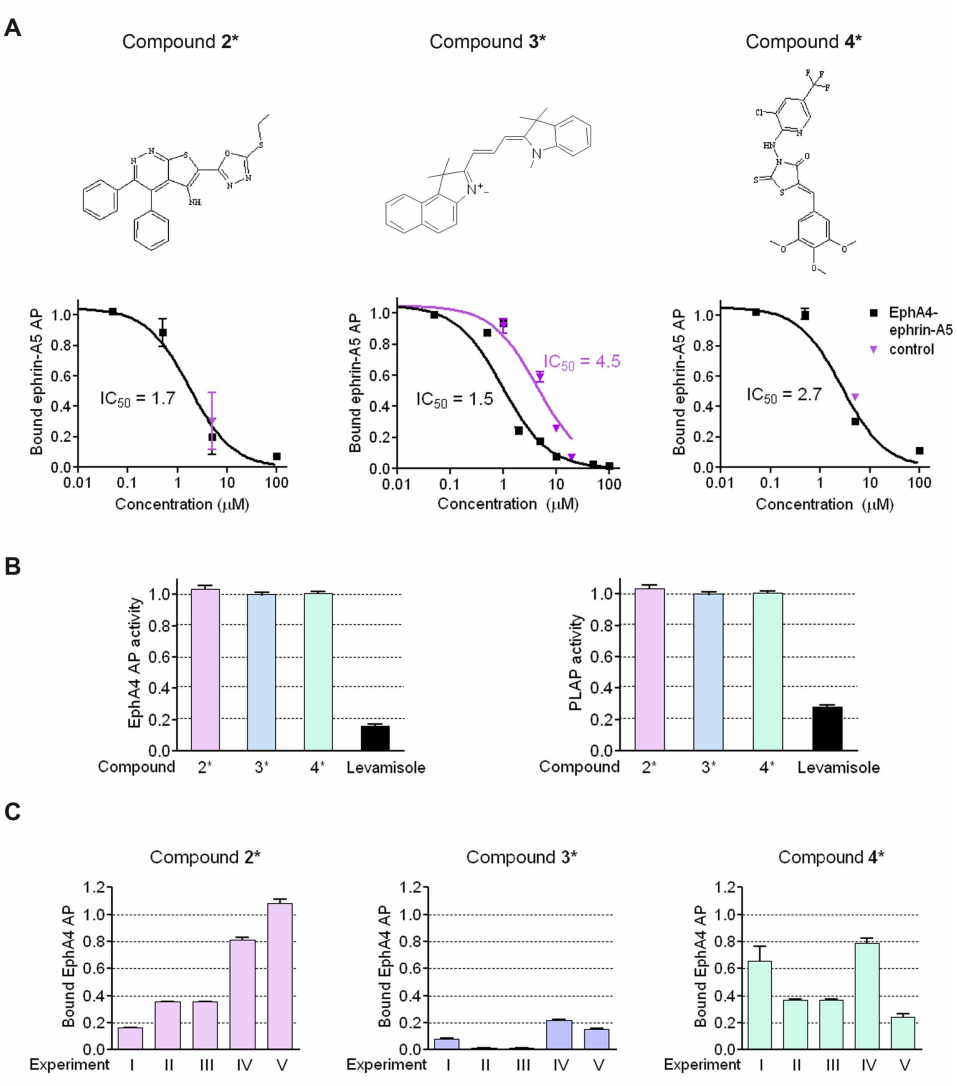

A
Compound 2*
Compound 3*
Compound 4*
Bound ephrin-A5 AP
IC50 = 1.7
IC50 = 1.5
IC50 = 4.5
IC50 = 2.7
Concentration (μM)
EphA4-ephrin-A5
control
B
EphA4 AP activity
PLAP activity
Compound 2* 3* 4* Levamisole
C
Compound 2*
Compound 3*
Compound 4*
Bound EphA4 AP
Experiment I II III IV V

Fig. 2.4. **Many compounds identified by HTS for EphA4 antagonists are non-specific inhibitors**. **(A)** Structures of compounds **2***, **3*** and **4***. EphA4 AP binding to ephrin-A5 Fc immobilized to protein A-coated plates was measured in the presence or in the absence of compounds and was detected by alkaline phosphatase activity (black). EphA4 AP was also immobilized to protein G coated plates through an anti-EphA4 antibody and alkaline phosphatase activity was measured in the presence or in the absence of compounds (purple), in order to measure inhibition of alkaline phosphatase activity or other non-specific inhibition. **(B)** Placental alkaline phosphatase or EphA4 AP in solution were incubated with 5 μM of compound **2*** and **4*** or 100 μM of compound **3***, followed by measurement of alkaline phosphatase activity. Levamisole a known inhibitor of alkaline phosphatase, was used as a control at 5 mM **(C)** Compounds **2***, **3*** and **4*** were tested in ELISA assays using EphA4 AP immobilized to protein G-coated plates through an anti-EphA4 antibody as described in (A). Experiment I – Compounds were added after EphA4 AP had been immobilized on the plate and the wells were washed before measuring alkaline phosphatase activity. Experiment II – The same experiment as in I was performed, but the wells were washed for 3 hours before measuring alkaline phosphatase activity. Experiment III – Compounds were added to the plate after immobilizing the anti-EphA4 antibody and the wells were washed before adding EphA4 AP. Experiment IV – Compounds were added to protein-G plates and the wells were washed before adding the anti-EphA4 antibody. Experiment V – The same experiment as in I was performed, but without washing the wells before measuring alkaline phosphatase activity. In all the experiments measurements were normalized to alkaline phosphatase activity in the absence of compounds. Bars indicate standard errors from triplicate measurements in all the panels.

Two 2,5-dimethylpyrrolyl benzoic acid derivatives selectively target the EphA4 and EphA2 receptors

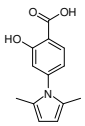
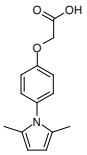
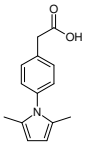
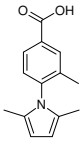
Among the hits identified in the high throughput screens for EphA4 inhibitors, 4 had a 2,5-dimethylpyrrolyl benzene scaffold (**Table 2.1**) and inhibited EphA4 AP binding to the KYL peptide with IC_{50} values ranging from 2 to 56 μM.

Importantly, compound **1**, 2-hydroxy-4-(2,5-dimethyl-1-pyrrolyl)benzoic acid also inhibited binding of ephrin-A5 AP to the EphA4 extracellular domain with an IC_{50} value of 13 μM (**Fig. 2.5**). The concentrations needed to inhibit ephrin binding were higher than those required to inhibit KYL binding, which was expected given that the ephrin binds EphA4 with higher affinity compared to the peptide.

Unlike compound **1***, compound **1** appeared to be a reversible inhibitor, because its activity was reverted by several hours of incubation in binding buffer before the addition of ephrin-A5 AP or EphA4 AP to EphA4 Fc or ephrin-A5 coated plates, respectively (data not shown). Furthermore, unlike compounds **2***-**4***, compound **1** did not show any activity in the control

assay involving EphA4 AP immobilized through an anti EphA4 antibody to protein G-coated plates (data not shown). These results suggest that compound **1** inhibits the binding of the EphA4 receptor to both a synthetic peptide ligand and a natural ephrin ligand by a specific and non-covalent reversible mechanism.

TABLE 2.I. Hits identified in the high throughput screen for EphA4 inhibitors

Structure	M.W.	% Inhib	IC_{50} (μM)	
		EphA4 KYL	EphA4 KYL	EphA4 ephrin-A5
 **1**	231	99 99	2.1	13
 **2**	245	81 90	25	>1000
 **3**	229	65 70	14	>1000
 **4**	229	58 66	56	>1000

Fifty-six additional compounds belonging to the same class as compound **1** were obtained from ChemBridge and other sources and were tested for inhibition of EphA4-KYL and EphA4-ephrin binding. Compound **5** – a 1,2-isomer of compound **1** – also inhibited binding of ephrin-A5 AP to immobilized EphA4. The IC_{50} value for inhibition of EphA4-KYL peptide binding by compound **5** was 3 μM and for inhibition of EphA4-ephrin-A5 binding was 9 μM (**Fig. 2.5**).

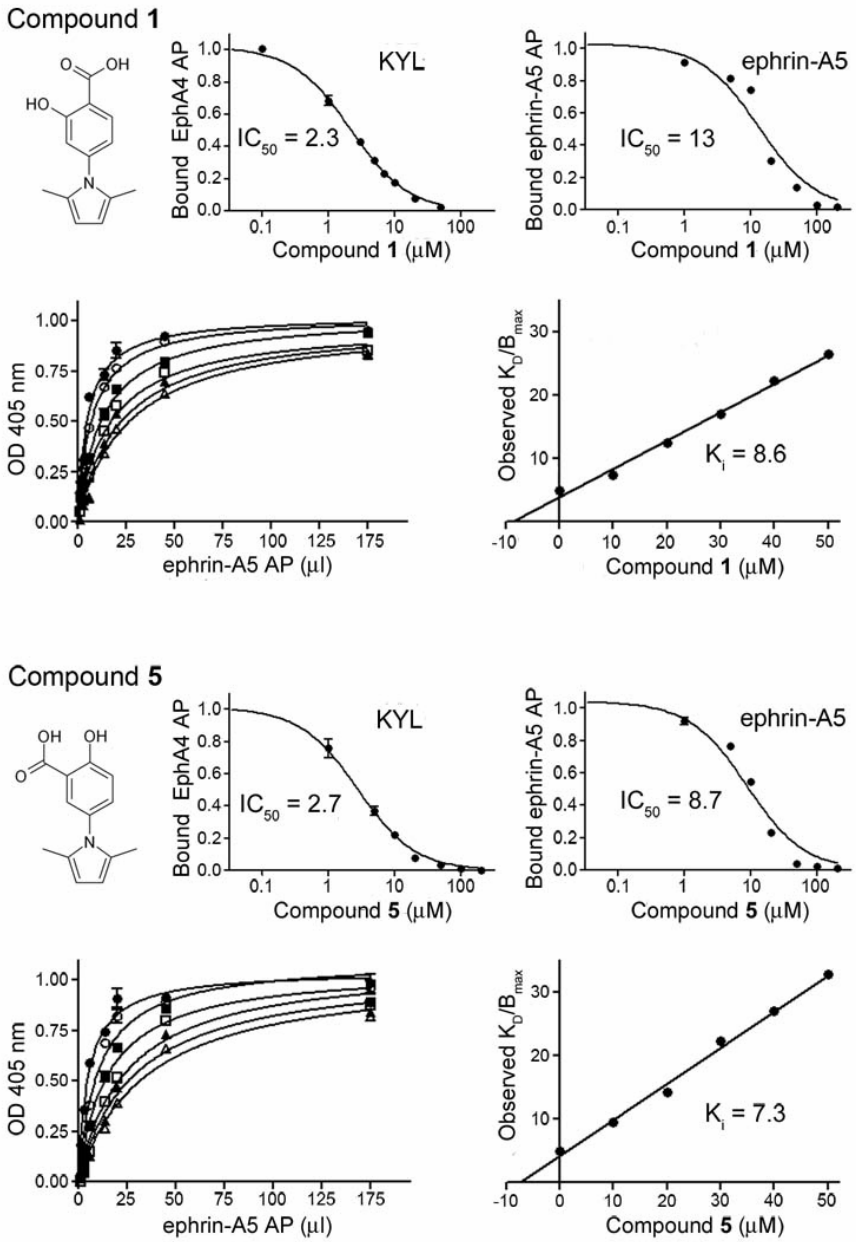


Fig. 2.5.**Small molecules identified by high throughput screening inhibit ephrin-A5 binding to EphA4 in a competitive manner**. Compound **1** and compound **5** inhibit EphA4 AP binding to immobilized biotinylated KYL peptide and ephrin-A5 AP binding to immobilized EphA4 ectodomain Fc in a concentration-dependent 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 (○) 10 μM (■) 20 μM (□) 30 μM (▲) 40 μM (Δ) 50 μM. In the 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.

Ephrin-A5 AP binding curves were measured at different compound concentrations, showing that compounds **1** and **5** competitively inhibit EphA4-ephrin-A5 binding with K_i values of 9 μM and 7 μM, respectively (**Fig. 2.5**). These data suggest that compounds **1** and **5** target the high affinity ephrin-binding site of the Eph receptors. The K_i value can also be obtained from the IC_{50} value and the dissociation constant (K_D) for receptor-ligand binding, using the Cheng-Prusoff equation (see Material and Methods). The K_i values for compound **1** and compound **5** calculated from the inhibition curves shown in Fig. 2.5 were 8 and 6 μM, respectively. Inhibition constants were also calculated from other IC_{50} values obtained using different ephrin concentrations, finding values ranging from 8 to 10 μM for compound **1** and from 6 to 8 μM for compound **5** (data not shown). These values are consistent with each other and with the K_i values calculated from the binding curves. The Cheng-Prusoff equation was also applied to EphA2-ephrin-A5 binding, obtaining K_i values ranging from 11 to 14 μM for compound **1** and from 10 to 13 μM for compound **5** (data non shown).

Interestingly, despite the small size of the compounds, which is 298 Da, and the ability of ephrin ligands to bind promiscuously to different Eph receptors, compounds **1** and **5** efficiently inhibited ephrin binding only to EphA4 and EphA2 but not any of the other EphA or EphB receptors examined (**Fig. 2.6A**). Both compounds inhibited binding to EphA4 of all ephrin ligands except ephrin-A4, ephrin-B1 and ephrin-B2, suggesting that the binding interactions of EphA4 with these ephrins are distinct from those with other ephrins. Similar inhibitory effects were obtained for EphA2-ephrin-A binding, suggesting that ephrin-A4 also interacts with EphA2 differently than other ephrins (**Fig. 2.6B**). The binding of ephrin-A2, ephrin-A3 and ephrin-A5 to EphA2 and EphA4 is similarly inhibited by the compounds, while ephrin-A1 binding to EphA2 is less easily inhibited compared the binding of this ligand to EphA4.

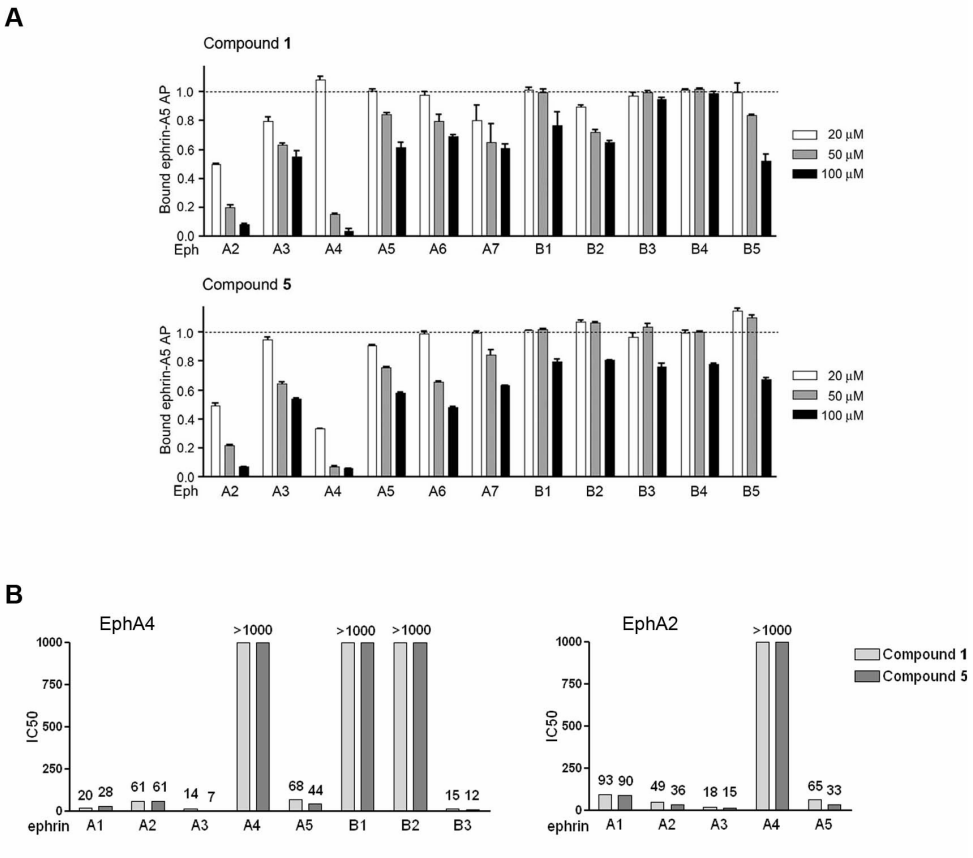


Fig. 2.6. **Compounds 1 and 5 show selectivity in their inhibition of different Eph receptors or ephrins. (A)** Ephrin-A5 AP or ephrin-B2 AP binding to immobilized EphA or EphB receptor Fc fusion proteins was measured in the presence of the indicated concentrations of compounds **1** and **5**. The ratio 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 compounds **1** and **5** inhibition of EphA4 AP or EphA2 AP binding to the indicated ephrins immobilized on protein A-coated plates.

Compound 1 and compound 5 inhibit EphA4 and EphA2 activation by ephrin in cells without showing toxicity

Given the promising results obtained in the ELISA assays, the two 2,5-dimethylpyrrolyl benzoic acid derivatives were tested for their ability to inhibit EphA4 and EphA2 activation in cells. HT22 neuronal cells stimulated with ephrin-A5 Fc and incubated with compound **1** or **5** showed a significantly lower level of EphA4 phosphorylation on tyrosine residues compared to cells treated with DMSO as a control (**Fig. 2.7A-B**).

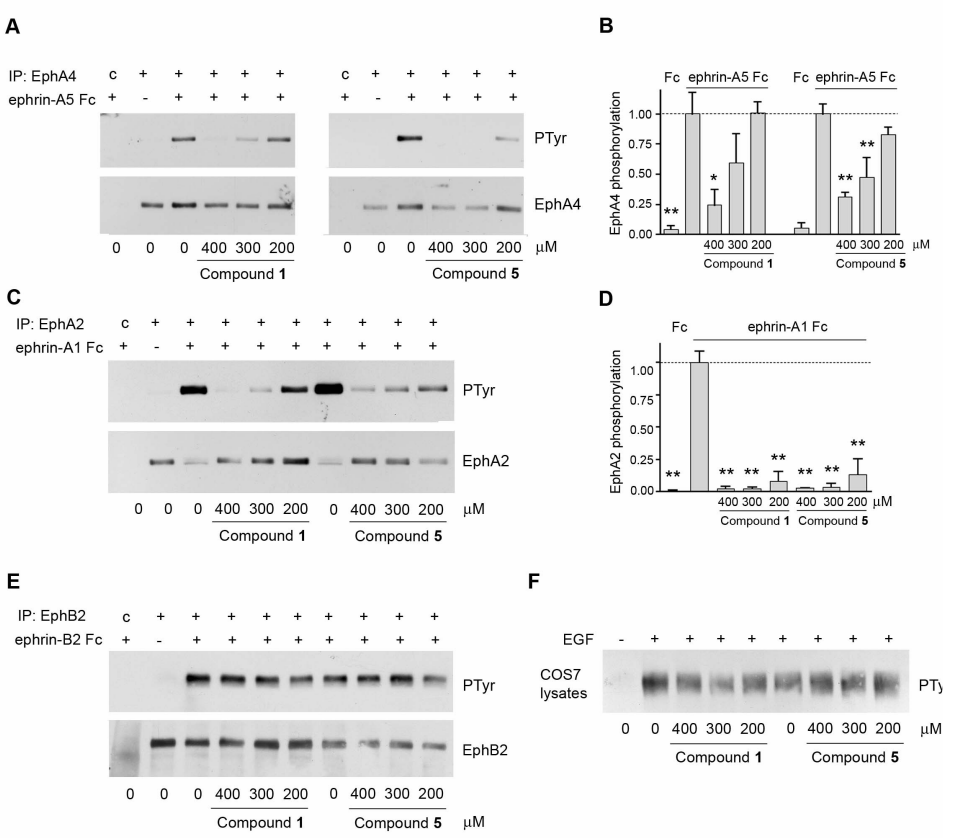


Fig. 2.7. **Compounds 1 and 5 inhibit the ligand-induced phosphorylation of EphA4 and EphA2 but not that of EphB2 or the EGF receptor in cells**. **(A)** HT22 neuronal cells pretreated with the indicated concentrations of compounds **1** or **5** for 15 min were stimulated with 0.5 μg/ml ephrin-A5 Fc (+) 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 concentrations of compounds **1** and **5**. 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 concentrations of compounds **1** or **5** 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 ** $P<0.01$.

Similar results were obtained for EphA2 in COS7 cells stimulated with ephrin-A1 Fc, where treatment with 200 μM of either compound almost completely abolished EphA2 phosphorylation (**Fig. 2.7C-D**). The presence of the compounds also counteracted ephrin-mediated internalization and degradation of EphA2. It is interesting to note that previously identified EphA2-targeting peptides were able to inhibit EphA2-ephrin binding in ELISA assays but, unlike the newly identified small molecules, stimulated EphA2 phosphorylation in cells and acted as agonists rather than antagonists (Koolpe et al., 2002). In a control experiment, COS7 cells were also stimulated with ephrin-B2 Fc in order to activate EphB2. Consistent with the results obtained in the ELISA assays, compounds **1** and **5** did not inhibit EphB2 phosphorylation (**Fig. 2.7E**). As an additional control, the effects of the compounds on EGF receptor phosphorylation were measured. Concentrations of up to 400 μM of compounds **1** and **5** did not alter EGF receptor activation in COS7 cells stimulated with EGF (**Fig. 2.7F**). The toxicity of the compounds was also assessed by using the MTT assay. These experiments showed that compounds **1** and **5** at concentrations up to 400 μM do not affect HT22 cell viability when incubated with the cells for up to 3 days (**Fig. 2.8**).

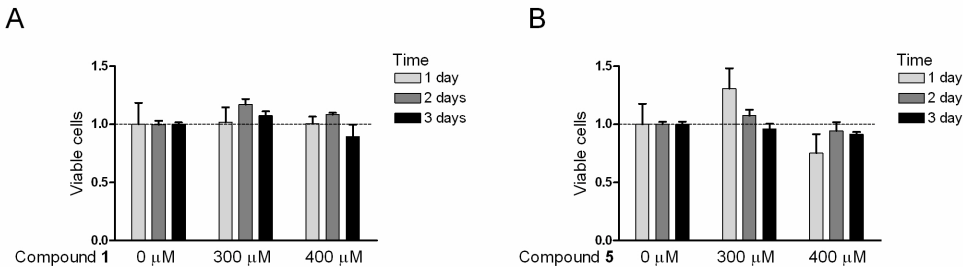
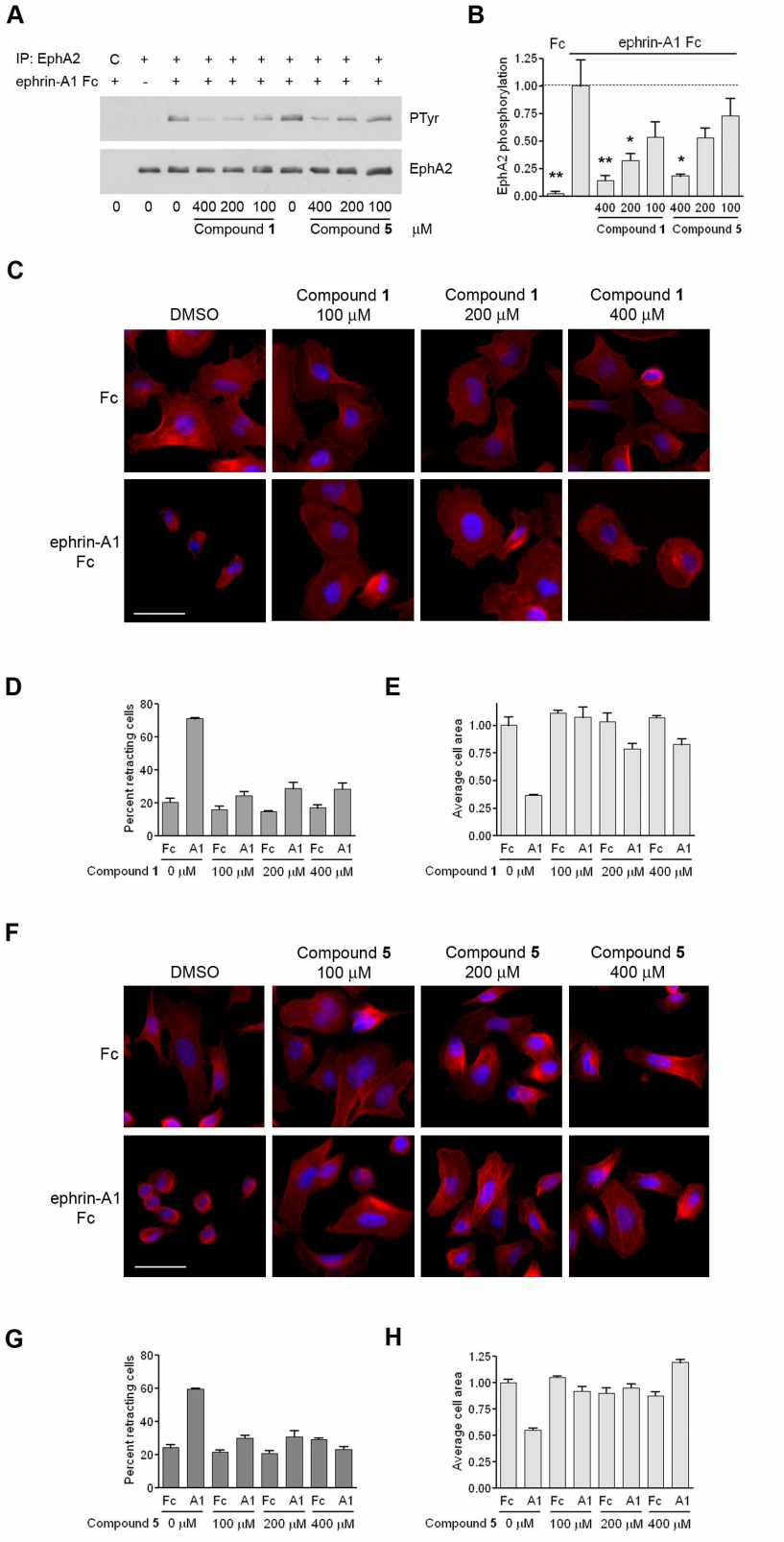


Fig. 2.8. **Compound 1 and compound 5 do not have toxic effects in cell culture**. HT22 neuronal cells were grown in the presence of compound **1 (A)** or compound **5 (B)** at the indicated concentration for 1, 2 or 3 days. DMSO in place of the compounds was used in the "0 μM" sample, as a control. 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.

Compound 1 and compound 5 inhibit EphA2-mediated retraction of the cell periphery

EphA2 is known to induce changes in cell morphology when activated by ephrin-A1 (Dail et al., 2006; Miao et al., 2000). PC3 prostate cancer cells express EphA2 (Miao et al., 2001) and have been shown to undergo retraction of the cell periphery and rounding after stimulation with ephrin A1 Fc (Parri et al., 2005). These cells were therefore used to examine whether compound **1** and compound **5** are able to interfere with EphA2-mediated cell retraction. Treatment with the compounds inhibited EphA2 activation (**Fig. 2.9A-B**) and ephrin-A1 induced PC3 cell retraction (**Fig. 2.9C-H**) without affecting cell morphology in the absence of ephrin treatment. Ephrin-A1 Fc treatment caused an increase in the percentage of retracting cells (**Fig. 2.9D, G**) and a decrease in the average cell area (**Fig. 2.9E, H**). Treatment with 100 μM of the dimethylpyrrolyl benzoic acid derivatives inhibited these effects. Interestingly, the compounds at 100 μM only partially inhibited EphA2 phosphorylation, suggesting that substantial levels of EphA2 activation may be required to promote the changes in cell morphology.

Fig. 2.9. **Compounds 1 and 5 inhibit ephrin-induced retraction of PC3 prostate cancer cells**. **(A-B)** PC3 cells pretreated for 15 min with the indicated concentrations of compound **1** or compound **5** 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 phosphorylation, which were normalized to the amount of EphA2 in the immunoprecipitates. Error bars indicate standard error from 3 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 with compound **1** as in (A) were stained with DAPI and rhodamine-phalloidin to label filamentous actin. DMSO was used as a control (0 μM). Bar, 50 μm. 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 compound **5**.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Compound 1 and compound 5 inhibit EphA4-mediated growth cone collapse in retinal neurons

Growth cones extending from retinal explants are well known to collapse in response to ephrin stimulation (Drescher et al., 1995). EphA4 is homogenously expressed in different parts of the retina, while other EphA receptors are present in the temporal but not the nasal region (Connor et al., 1998). Therefore, EphA4 is the predominant EphA receptor expressed in the nasal retina. For this reason, nasal retinal explants were chosen to test whether the 2,5-dimethylpyrrolyl benzoic acid derivatives are able to counteract EphA4-mediated growth cone collapse after ephrin-A stimulation. In explants treated with the KYL peptide (**Fig. 2.10A**), compound **1** (**Fig. 2.10B**) or compound **5** (**Fig. 2.10C**), the growth collapsing effects of ephrin-A5 Fc were completely blocked. Neither the peptide nor the compounds had any effect on growth cone shape on their own. This is consistent with the recent report that the KYL peptide protects rat neocortical growth cones from collapsing after ephrin-A5 Fc treatment (Fabes et al., 2007). The KYL peptide has also been shown to promote axonal growth and functional recovery after corticospinal tract injury of the rat spinal cord (Fabes et al., 2007). The effect of the KYL peptide and the 2 2,5-dimethylpyrrolyl benzoic acid derivatives on growth cones is an encouraging result, which suggests that they could be used to enhance axon regrowth after injury.

Structure-activity relationship analysis of analogs of compounds 1 and 5

In order to find compounds related to compounds **1** and **5** but with higher potency, 56 compounds having the 2,5-dimethylpyrrolyl benzene scaffold or the ortho-hydroxybenzoic acid moiety were tested for inhibition of ligand binding to EphA4. The compounds were obtained from commercial sources or from the Medicinal Chemistry Facility of the Burnham Institute for Medical Research. The structures and IC_{50} values for inhibition of EphA4-KYL binding of some of the compounds tested are shown in **Fig. 2.11**.

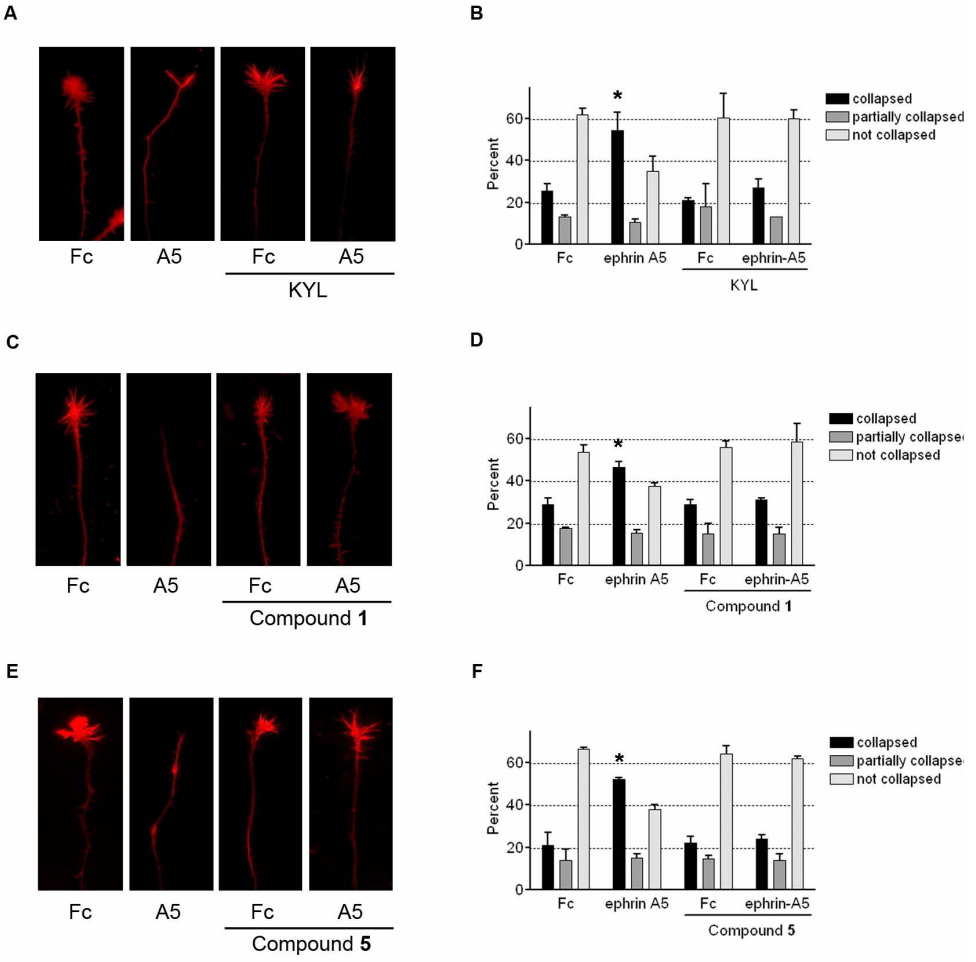

Fig. 2.10. KYL peptide, compound 1 and compound 5 prevent ephrin-induced growth cone collapse in retinal neurons. **(A,B)** Retina explants from E6 chicken embryos were preincubated with 5 µM of KYL peptide for 15 min, stimulated for 30 min with 1 µg/ml ephrin-A5 Fc, or Fc as a control, and stained with 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 Compound **1** (C-D) or **5** (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 experiment. Percent of collapsed growth cones in the different conditions were compared to the Fc control by one-way ANOVA and Newman-Keul's post test. * $P<0.05$

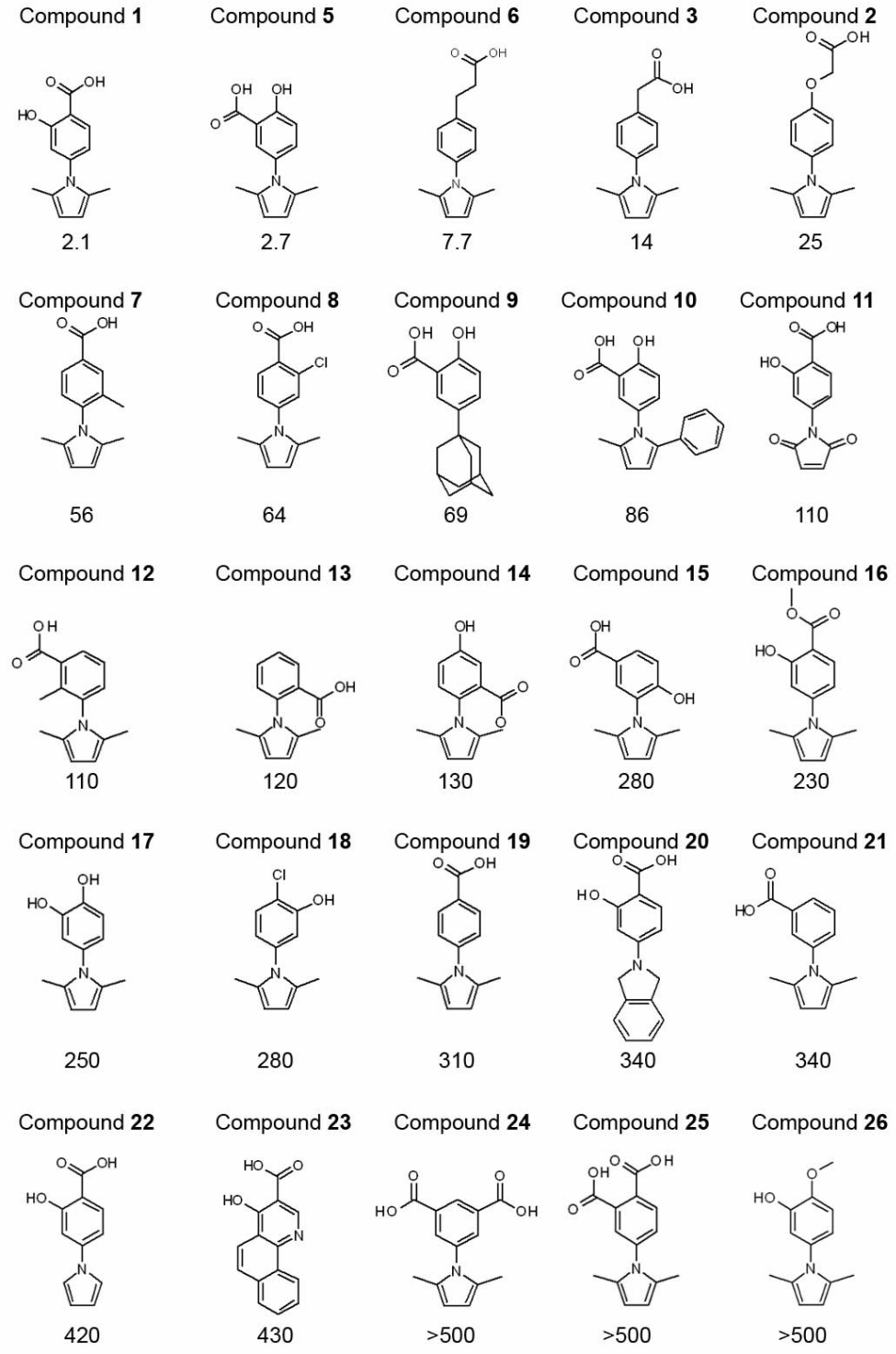

Fig. 2.11. Structures of 2,5-dimethylpyrrolyl benzoic acid analogs and IC_{50} values in μM for inhibition of EphA4 AP binding to immobilized biotinylated KYL peptide.

Although many of the compounds showed inhibitory activity with the peptide, none of them inhibited EphA4-ephrin binding. Even introducing small changes to the structure abolished the ability to inhibit ephrin binding. Indeed, compounds lacking the hydroxyl group at position 2 on the benzene ring (compounds **19**, **21**); compounds having the carboxylic group in place of the hydroxyl group (compounds **24**, **25**) or the hydroxyl group in place of the carboxylic group on the benzene ring (compound **17**); or compounds with the carboxylic group and the hydroxyl group placed at different positions in the benzene ring (compounds **14**, **15**) did not show any inhibition of EphA4-ephrin binding. These results suggest that the presence of the hydroxyl and carboxylic moieties and their position on the benzene ring are crucial for the activity of the compounds. This was also confirmed by the loss of inhibitory activity when the hydroxyl or carboxylic group in compound **1** was substituted with a chlorine atom (compounds **8**, **18**). In addition, no inhibition was observed with the methyl-ester derivative of compound **1** (compound **16**) or when a methoxy group (compound **26**) was put in place of the carboxylic group in para position with respect to the pyrrole ring, suggesting that some hydrogen bonding at this position may be involved in the binding of the compounds to EphA4.

Some alternatives to the 2,5-dimethyl pyrrolyl group were also tested. Eliminating or modifying the 2 methyl groups (compounds **11**, **22**) abolished the inhibitory activity with the ephrin and almost abolished the inhibitory activity with the KYL peptide in the case of compound **22**, presumably because the methyl groups cause a rotation of the plane containing the pyrrole with respect to the plane containing the benzene ring, which may be important for the binding of the compound. Another possibility to explain the behavior of compound **22**, which is inactive despite being very similar to the 2 active compounds, is that the absence of the methyl group makes the pyrrole ring more unstable. However, this hypothesis was found to be incorrect based on the results obtained using compounds where the pyrrole ring was substituted with an adamantyl or isoindolyl group (compounds **9**, **20**) or by 2 phenyl groups fused with the hydroxyl benzoic acid (compound

23). These compounds would be expected to be more stable than compounds with the pyrrole ring, but still do not inhibit EphA4 binding. Furthermore, inserting a bigger moiety, like a benzene ring (compound **10**) in place of one of the methyl groups also abolished activity, probably by creating steric hindrance.

Although the compounds that we screened did not detectably inhibit ephrin binding to EphA4 or EphA2, their IC_{50} values for inhibition of EphA4-KYL peptide binding could be used as a guide to design modified versions of compounds **1** and **5** that might have increased potency. By comparing the IC_{50} values of compounds **7** and **19** and compounds **12** and **21**, which only differ in a methyl group attached to the benzene ring, a 2-3 fold difference in the inhibitory activity can be observed. This suggests that adding a methyl group to the structure of the original compounds may improve EphA4-ephrin inhibition. In addition, compounds **2**, **3**, and **6**, which have a phenoxy acetic acid, a phenyl acetic acid and a phenyl propanoic acid, in place of the benzoic acid, show a 10 to 40 fold decrease in the IC_{50} values compared to compound **19**, suggesting that exchanging the carboxylic group of compounds **1** and **5** with these other groups may significantly improve inhibitory activity. Although the concentrations of compounds **1** and **5** required to observe an effect are rather high, the properties of these compounds are very promising: they are specific for only 2 out of the 16 Eph receptors, they behave like reversible competitive inhibitors and they show activity in cell-based and functional assays without showing any toxicity. Therefore, the improvement of the potency that the described structural modifications could achieve would make this class of compounds a promising lead to develop pharmaceuticals for the treatment of EphA4- and EphA2- mediated pathologies.

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Chapter 3

Platinum(II) tetraamines as potential inhibitors of the EphA2 receptor

ABSTRACT

Angiogenesis is a crucial process involved in normal development as well as in many diseases, such as cancer. Being overexpressed in many types of cancer and in tumor vasculature, the EphA2 receptor tyrosine kinase is a promising new target for inhibition of tumor angiogenesis and other forms of pathological angiogenesis. Some peptide inhibitors and monoclonal antibodies that inhibit EphA2 activity have shown to inhibit receptor-ligand interaction and decrease tumor angiogenesis and progression *in vivo*, but no chemical compound inhibitors, which would have more desirable pharmaceutical properties, have been identified so far. Here we report the screen of combinatorial libraries for inhibition of EphA2-ephrin binding and the identification of platinum(II) tetraamine compounds as potential therapeutics.

INTRODUCTION

Angiogenesis is a highly complex process that plays critical roles in normal development as well as in multiple pathological processes, including tumor neovascularization, ischaemic recovery after infarction (Schumacher et al., 1998), development of the advanced arterosclerotic plaque (Hughes, 1996), retinopathy (Steinle et al., 2003) and wound healing (Li et al., 2003). Several growth factors and their receptors are involved in the angiogenic process, including the Eph receptor tyrosine kinases. This family of receptors and their ligands, the ephrins, are well known regulators of vascular remodeling in the embryo, and more recent evidence also associates them with post-natal angiogenesis and pathological forms of angiogenesis.

EphA2 is a member of the A class of Eph receptors and it is activated by binding to the 6 GPI-linked ephrin-A ligands. This receptor, previously known as Eck, for epithelial cell kinase, was originally identified in a screen for tyrosine kinases expressed in epithelial cells (Lindberg and Hunter, 1990) and was subsequently characterized as an important player in the angiogenic response in the adult using the corneal angiogenesis assay (Pandey et al., 1995). Inflammatory cytokines such as tumor necrosis factor alpha (TNFα) stimulate EphA2 activity through the upregulation of ephrin-A1 expression in endothelial cells. Furthermore, EphA2 is required for endothelial capillary tube formation *in vitro* (Ogawa et al., 2000), and promotes the formation of blood vessel-like structures by melanoma cells (Hess et al., 2001). The angiogenic effect of EphA2 involves the activation of the Rho-family GTPase Rac by phosphatidylinositol 3-kinase (Brantley-Sieders et al., 2004). EphA2 expression seems to be restricted to adult angiogenic blood vessels, since this receptor is not expressed in quiescent blood vessels or in embryonic vasculature (McBride and Ruiz, 1998; Ogawa et al., 2000). Consistent with this observation, EphA2 knock-out mice show defective angiogenic responses in the adult, without showing any obvious defect during development (Brantley-Sieders et al., 2004; Brantley-Sieders et al., 2006). Ephrin-A1 is also not present in adult quiescent blood vessels,

although it has been detected in the embryonic vasculature (McBride and Ruiz, 1998). The differential expression of EphA2 suggests that targeting the pathological effects of EphA2 would not affect the normal vasculature.

EphA2 and ephrin-A1 appear to also play an important role in tumor development, since they are overexpressed in numerous types of cancer, including breast, cervix, colon, esophagus, lung, ovary, prostate, kidney carcinomas and melanoma (Ireton and Chen, 2005). The level of EphA2 expression often correlates with the malignancy of the tumor and with poor patient outcome. EphA2 and ephrin-A1 are expressed both in the tumor cells, where the receptor localization also seems to be altered (Ireton and Chen, 2005) and in the tumor vasculature (Brantley et al., 2002; Ogawa et al., 2000). The mechanism of action of EphA2 and ephrin A1 in tumors is not well understood, but presumably ephrin-A1 expressed in tumor endothelial cells and in tumor cells acts as a pro-angiogenic factor by interacting with EphA2 expressed in tumor endothelial cells.

Supporting this hypothesis, blocking the activation of EphA2 with soluble recombinant EphA2 (EphA2 Fc) inhibits angiogenic response and counteracts the effect of proangiogenic factors as TNF-α and VEGF (Brantley et al., 2002; Cheng et al., 2003; Cheng et al., 2002; Dobrzanski et al., 2004; Pandey et al., 1995). The decreased angiogenesis influences tumor growth, which is also reduced due to the lack of oxygen and nutrients. Consistent with the idea that the observed diminished tumor growth is a secondary effect of decreased angiogenesis, soluble EphA Fc receptors show no effect on growth and survival of tumor cells in culture (Cheng et al., 2003). Similar reduction of tumor size and vascularization is also observed when the EphA2 gene is inactivated (Brantley-Sieders et al., 2006) or when monoclonal antibodies are used to stimulate EphA2 endocytosis and degradation (Carles-Kinch et al., 2002).

Two peptides have also been identified, which selectively bind to the EphA2 receptor. Despite being apparently monomeric, these peptides stimulate EphA2 phosphorylation in cells through unknown mechanisms (Koolpe et al., 2002). Recent work has shown that the knockdown of EphA2

in the retina by antisense nucleic acids reduces retinal neovascularization (Shen et al., 2007). This effect could be exploited for the treatment of diseases characterized by abnormal vascularization, such as retinopathy.

The results described above show how targeting EphA2 could be useful for the treatment of cancer and other forms of pathological angiogenesis. However, inhibitors with better pharmacological properties compared to the large molecules that have been used so far would be desirable.

Mixture-based synthetic combinatorial libraries have emerged in the last few years as a valuable tool for the identification of compounds that show activities in a variety of systems. Combinatorial libraries allow the testing of millions of compounds without requiring classical high throughput means and have been shown to work in many different assays (Houghten et al., 1999). We tested mixture-based combinatorial libraries in a screening for inhibitors of EphA2 and we identified platinum(II) tetraamine mixtures as promising molecules for EphA2 specific targeting.

MATERIALS AND METHODS

Mixture-based combinatorial library screen for EphA2 inhibitors

A 96-well format ELISA assay was used for the screening of a sampler of 30 libraries of compounds obtained from the Torrey Pines Institute for Molecular Studies (La Jolla, CA). Protein A-coated plates (Pierce Biotecnology, Rockford, IL) were incubated with 1 μg/ml of ephrin-A1 Fc or ephrin-A5 Fc diluted in binding buffer (Tris-buffered saline (TBS) (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) containing 1 mM $CaCl_2$ and 0.01% Tween 20). The mixtures, which were dissolved in dimethylformamide (DMF), were then added at a concentration of 100 μg/ml together with cell culture medium containing the ephrin-binding domain of EphA2 fused with alkaline phosphatase (EphA2 AP) diluted 1:10 in binding buffer. The mixtures were incubated for 3 hours at room temperature before adding *p*-

nitrophenylphosphate (pNPP) (Pierce Biotecnology, Rockford, IL) as a substrate and measuring the absorbance at 405 nm. Alkaline phosphatase activity from wells where Fc was added instead of ephrin Fc-fusion proteins was subtracted as the background. The inhibitory activity of the mixtures was calculated by dividing the absorbance observed in the presence of each mixture and the absorbance from wells where only DMF was added.

Synthesis of tetraamine and platinum(II) tetraamine libraries

Tetraamine libraries were prepared using the "tea bag" method for simultaneous multiple synthesis (Houghten et al., 1999). Resin-bound mixtures were synthesized using ratios of amino acids previously determined in order to have equimolar ratios (Ostresh et al., 1994). A tripeptide bound to a 4-methylbenzhydrylamine hydrochloride salt (MBHA) resin was used as starting material. The tripeptide combinatorial sub-libraries were prepared in the positional scanning format using the "reagent mixture method" (Ostresh et al., 1994) and 58 different amino acids. The obtained tripeptides were exhaustively reduced by a 72 hour treatment with BH_3-THF at 65°C, followed by overnight treatment with neat piperidine at 65°C (Ostresh et al., 1998). Resin-bound mixtures were cleaved using hydrogen fluoride procedures (Houghten et al., 1996) and were extracted using 95% acetic acid. Synthetic combinatorial libraries were lyophilized and reconstituted in DMF at 10 mg/ml. Platinum(II) tetraamine mixtures were synthesized from tetraamine combinatorial libraries by solution-phase interaction with K_2PtCl_4, as previously described. (Nefzi et al., 2006). Individual compounds were prepared using the methods described above and their purity and identity were characterized by reverse phase HPLC and laser desorption mass spectrometry. Some libraries were filtered using 0.75 μm MWCO pore size filters in order to remove any traces of free platinum present in the platinum(II) tetraamine mixtures.

Platinum(II) tetraamine inhibition of EphA2-ephrin-A binding

The ability of the tetraamine and platinum(II) tetraamine mixtures and single compounds to inhibit EphA2 interaction with ephrin-A1 or ephrin-A5 was assessed as described above. Different concentrations of the mixtures of compounds were added to plates coated with protein A and ephrin-A ligands, together with EphA2 AP. As a control for non-specific inhibition of protein-protein interactions or alkaline phosphatase activity, a modified ELISA assay was performed. High binding capacity plates were coated overnight at 4°C with an anti-human IgG conjugated with AP (Promega, Madison, WI) diluted to 0.1 μg/ml in borate buffer 0.1 M pH 8.7. Compounds were then added for 3 hours, before adding pNPP as the substrate and measuring the absorbance at 405 nm. Absorbance from wells where no anti-human IgG-AP was added was subtracted as background.

RESULTS AND DISCUSSION

Platinum(II) tetraamine libraries inhibit ephrin binding to EphA2

Mixture-based combinatorial libraries are mixtures of compounds having the same structural scaffold but different functional groups at some variable positions (indicated by R). The higher the number of variable positions in the molecule and the number of functionalities used for the synthesis of the mixture, the higher the complexity of the mixture. Each library can contain thousands or even millions of compounds, which can be screened using a "low throughput" approach.

A sampler of 30 mixture-based combinatorial libraries of compounds having different structural scaffolds was tested for inhibition of EphA2 binding to ephrin-A1 or ephrin-A5 (**Fig. 3.1**). The libraries contained 2 to 4 variable positions, each corresponding to a mixture having all but one defined functional groups. In total, 91 mixtures were screened. The most effective at inhibiting EphA2-ephrin interaction, at a concentration of 100 μg/ml, were the three mixtures corresponding to the platinum(II) tetraamine

scaffold (mixtures 89-91), which showed an average inhibition of 18% and 50% with ephrin-A1 and ephrin-A5, respectively (**Fig. 3.1**).

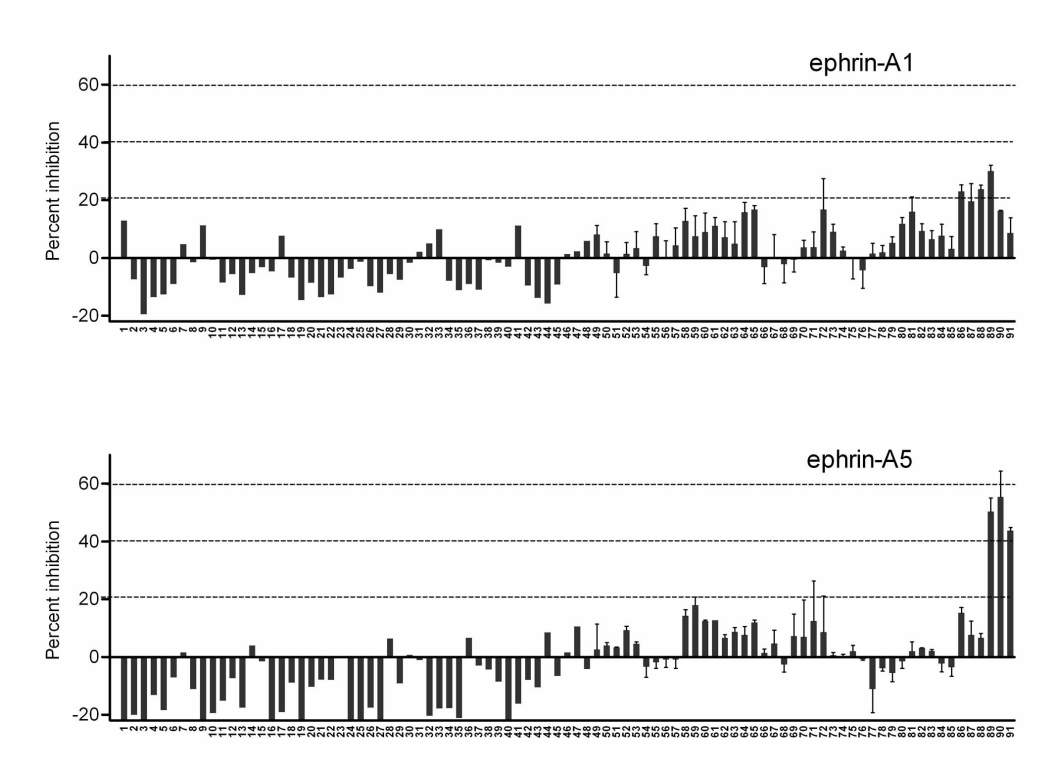

Fig. 3.1. **Screening of positional scanning combinatorial libraries for inhibition of EphA2-ephrin-A binding**. Ninety-one mixtures at 100 µg/ml were added together with EphA2 AP to ELISA plates precoated with protein A and ephrin-A1 or ephrin-A5. Percentage inhibition of EphA2 binding to the ephrins is shown. Averages from 2 independent experiments ± SE are shown.

These compounds consist in a tetraamine scaffold, which is derived from exhaustive reduction of a tripeptide, with a platinum atom coordinated at the center of the molecule. Three variable positions (R1, R2 and R3) are present (**Fig 3.2A**). Controls verified that the platinum(II) tetraamine libraries do not inhibit alkaline phosphatase activity or the pre-formed protein A-Fc complexes (data not shown). Interestingly libraries 86-88, which contain tetraamines having the exact same structure as the platinum(II) tetraamines but without the metal, exhibited substantial lower activity when tested with ephrin-A5, suggesting that coordination of the central platinum is important for the inhibitory activity of these complexes. The results obtained with the

two ephrin ligands were qualitatively similar. However, EphA2 binding to ephrin-A5 was more easily inhibited by the platinum(II) tetraamine complexes and less easily by the metal-free tetraamine compared to EphA2 binding to ephrin-A1. Furthermore, the platinum(II) tetraamine libraries appear to be selective for EphA2. Indeed when they were tested for inhibition of ephrin binding to two related Eph receptors, EphA4 and EphB4, no activity was observed (**Fig. 3.2B**).

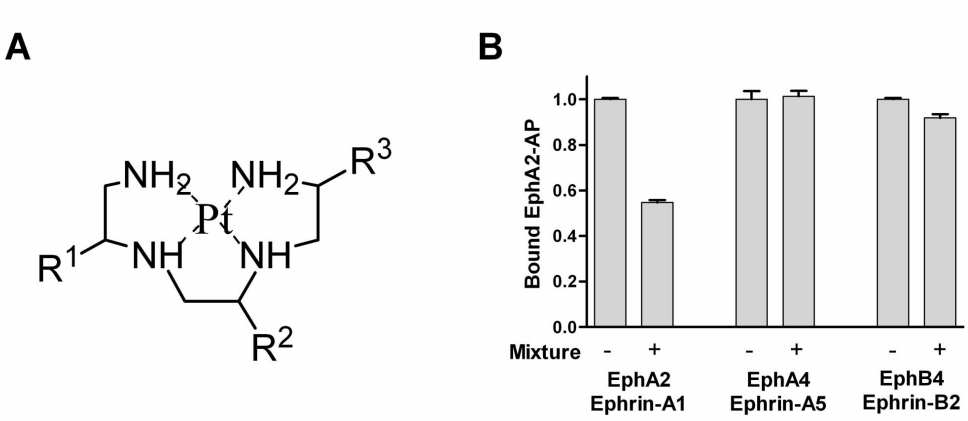

Fig. 3.2. **The platinum(II) tetraamine library selectively inhibits binding of EphA2 to ephrin-A ligands. (A)** Platinum(II) tetraamine scaffold. **(B)** EphA2 AP, EphA4 AP and EphB4 AP were incubated on ELISA plates coated with ephrin-A1 Fc, ephrin-A5 Fc or ephrin-B2 Fc, respectively. (-) Indicates that DMF was added in place of the libraries as a control; (+) indicates that the R1 platinum(II) tetraamine sub-library mixture was added at 100 μM/ml together with the Eph receptor AP fusion proteins. Averages from 3 measurements ± SE are shown.

Positional scanning screen of the platinum (II) tetraamine libraries

The platinum(II) tetraamine library was synthesized in the positional scanning format (Houghten et al., 1999). Positional scanning synthetic combinatorial libraries are composed of one sublibrary for each variable position, each further divided into mixtures having a different functional group at that variable position.

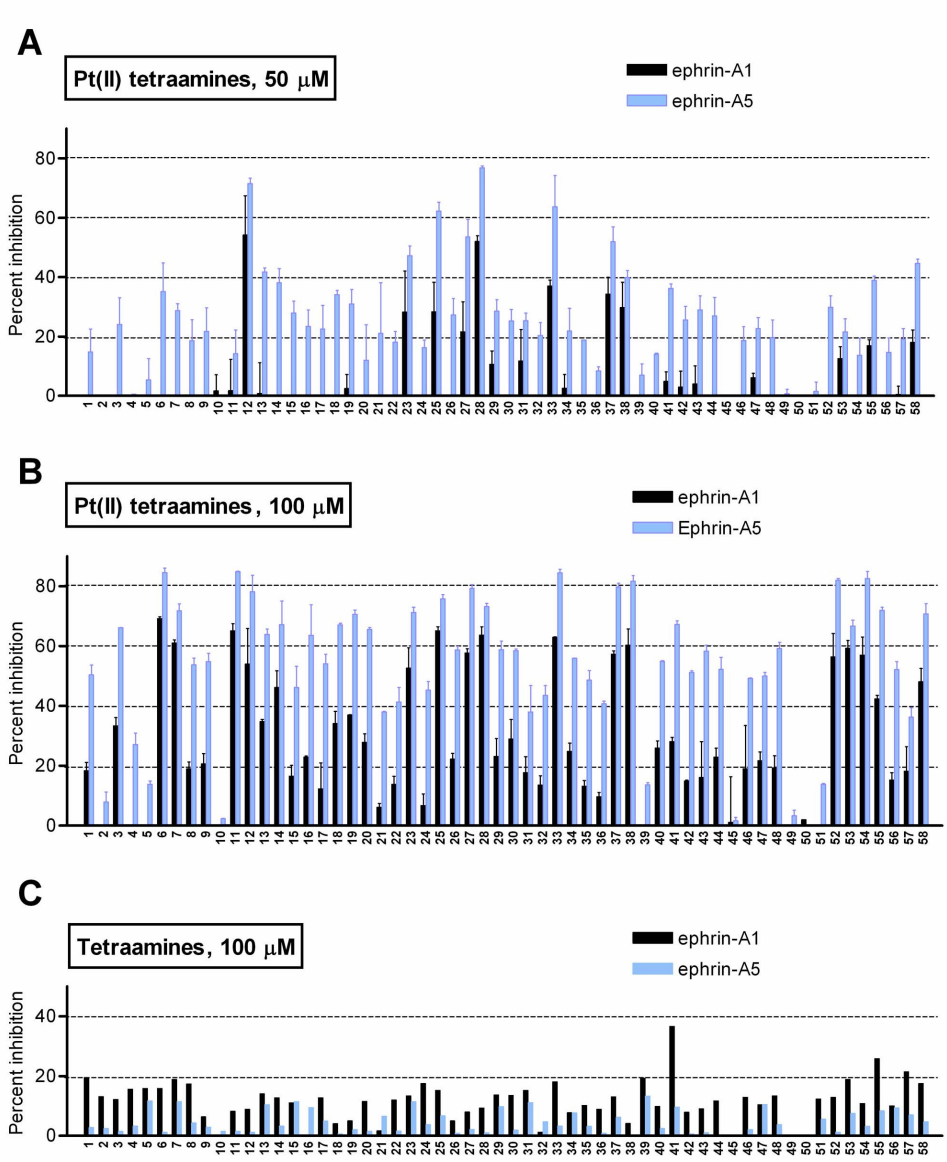

Fig. 3.3. **Positional scanning mixtures of the platinum(II) tetraamine library inhibit EphA2-ephrin-A interaction**. **(A-B)** The platinum(II) tetraamine mixtures were screened at 50 μg/ml (A) or 100 μg/ml (B) for inhibition of EphA2 AP binding to ephrin-A1 (black) or ephrin-A5 (blue) immobilized on ELISA plates. The histograms show averages from 2 independent experiments ± SE. **(C)** The tetraamine mixtures were screened at 100 μg/ml for inhibition of EphA2 binding to ephrin-A1 (black) or ephrin-A5 (blue). Percent inhibition of EphA2 binding to ephrins is shown in all the panels. Pt(II), platinum(II).

Each compound present in a given mixture has an individual building block at a given variable position and mixtures of all the building blocks used for the synthesis of the library at the other variable positions. The sublibraries for each position contain the same individual compounds, but they differ by the location of the variable position. For the synthesis of the platinum(II) tetraamine libraries 58 functional groups were used, therefore 58 mixtures were present for each variable position, each one containing 3,364 individual compounds. The mixtures corresponding to the R1 position, having defined functionalities at the R1 position and mixtures of functional groups at the R2 and R3 positions, were screened at a concentration of 50 μg/ml (**Fig. 3.3A**) and 100 μg/ml (**Fig. 3.3B**) using either ephrin-A1 or ephrin-A5. This second screen confirmed that the platinum(II) tetraamine libraries contain compounds able to inhibit EphA2-ephrin interaction and that the extent of the inhibition depends on the concentration of the compounds and on the functional group present at the variable position. As observed in the initial screen, the results obtained with ephrin-A1 and ephrin-A5 were qualitatively similar, with a higher inhibition level observed using ephrin-A5. The 58 R1 positional scanning mixtures of the tetraamine library were also screened (**Fig 3.3C**). The extent of inhibition was significantly lower, particularly in the case of ephrin-A5, confirming that the presence of platinum is crucial for the activity of these compounds, presumably by conferring a specific three-dimensional conformation.

The R2 and R3 positional scanning mixtures containing platinum(II) tetraamines were then screened at 100 μg/ml in order to complete the positional scanning analysis of the library (**Fig 3.4**). Only few of the mixtures showed substantial inhibition, suggesting that only few of the compounds present in each mixture account for the observed activities.

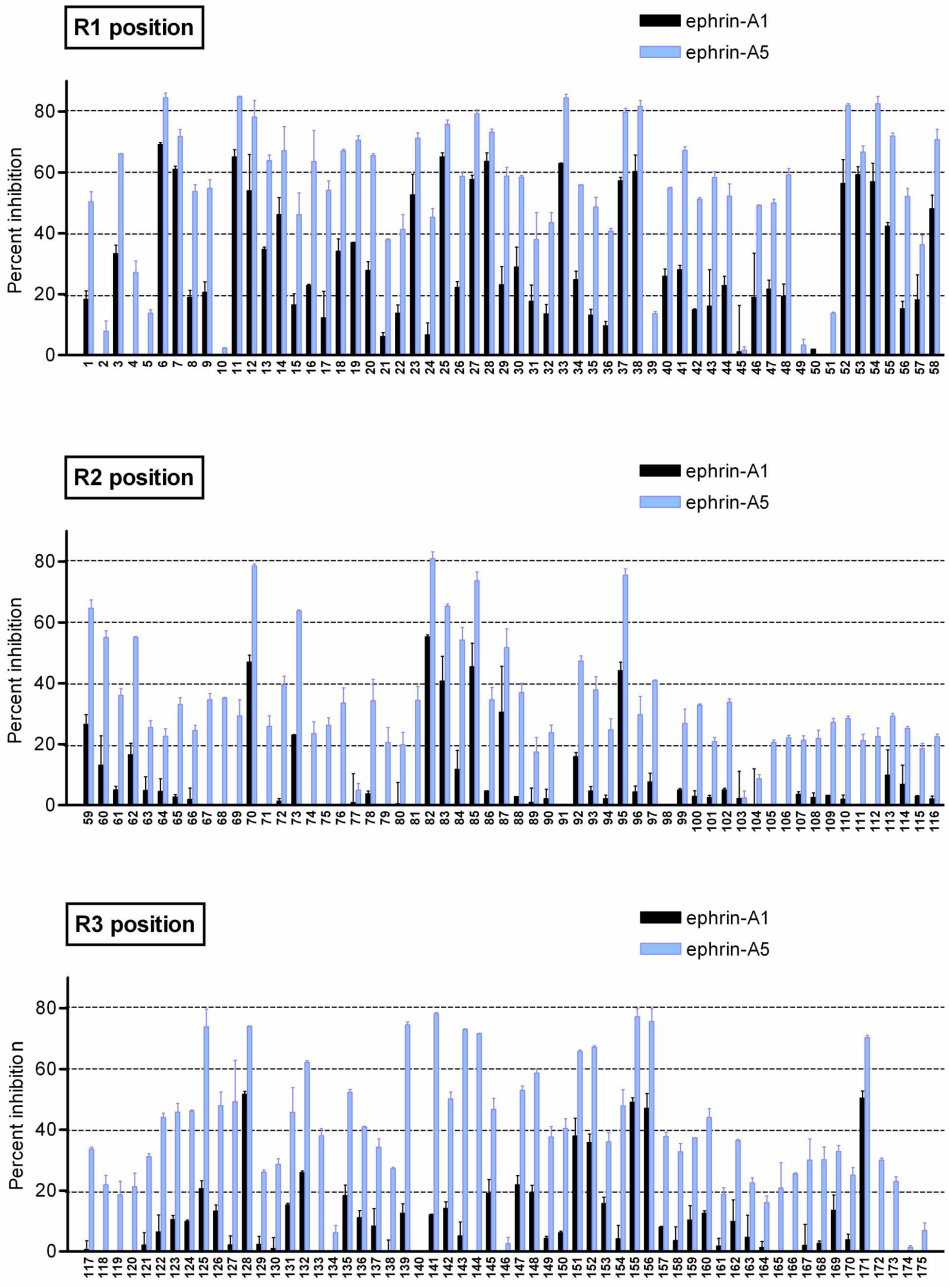

Fig. 3.4. **Screen of 3 positional scanning platinum(II) tetraamine mixtures**. EphA2 binding to ephrin-A1 (black) or ephrin-A5 (blue) was measured in the absence or in the presence of platinum(II) tetraamine mixtures added at 100 µg/ml. Mixtures corresponding to R1, R2 and R3 positions were tested. R1 is the same as B in Fig. 3. Averages from 2 experiments ± SE are shown

Table 3.I. IC_{50} values for inhibition of EphA2-ephrin-A1 interaction by platinum(II) tetraamine mixtures and mixtures chosen for the synthesis of individual compounds (blue = R1 position, yellow = R2 position; green = R3 position)

Mixture	Variable position	Functional group	IC_{50} (μM)
6	R1	Boc-L-Ile	>300
7	R1	Boc-L-Lys(CBZ)	110
11	R1	Boc-L-Gln	>300
12	R1	Boc-L-Arg(Tos)	47
14	R1	Boc-L-Thr(Bzl)	150
23	R1	Boc-D-Lys(CBZ)	>300
25	R1	Boc-D-Asn	67
27	R1	Boc-D-Gln	65
28	R1	Boc-D-Arg(Tos)	31
33	R1	Boc-D-Tyr(BrZ)	48
37	R1	Boc-D-Norleucine	79
38	R1	aN-CBZ-L-Lysine(Boc)	67
52	R1	Boc-D-4-Chlorophenylalanine	58
53	R1	Boc-L-4-Fluorophenylalanine	48
54	R1	Boc-D-4-Fluorophenylalanine	52
58	R1	Boc-O-ethyl-D-Tyrosine	130
59	R2	Boc-L-Ala	210
70	R2	Boc-L-Arg(Tos)	90
82	R2	Boc-D-Leu	68
83	R2	Boc-D-Asn	38
85	R2	Boc-D-Gln	60
87	R2	Boc-D-Ser(Bzl)	50
95	R2	Boc-D-Norleucine	86
128	R3	Boc-L-Arg(Tos)	86
132	R3	Boc-L-Trp(CHO)	260
151	R3	Boc-D-Norvaline	160
152	R3	Boc-L-Norleucine	120
155	R3	Boc-L-Ornithine(CBZ)	160
156	R3	aN-CBZ-L-Ornithine(Boc)	160
171	R3	Boc-L-a-tButylglycine	170

Synthesis of individual platinum(II) tetraamines

The positional scanning screen revealed an activity profile, which was used to guide the deconvolution approach. The concentration dependence of the inhibition of EphA2-ephrin-A1 interaction for the most active compounds was determined (**Table 3.I**). Taking into account the obtained IC_{50} values and the results from the positional scanning screening, 11 functional groups were chosen for the synthesis of individual compounds: 5 for the R1

position and 3 for each of the R2 and R3 positions (**Table 3.I**). Compounds with all the possible combinations of the chosen functional groups were synthesized and tested for inhibition of EphA2-ephrin-A1. Among the 45 individual compounds tested only 4 showed some inhibition, with IC_{50} value comprised between 140 and 260 μg/ml, values much higher than what expected from the results obtained in the positional scanning screening. It is possible that functional groups singularly active at one of the variable positions are not as active when combined together. Indeed, the activity of a mixture is due to the presence of specific active compounds and not to the functional groups as separate independent entities.

Iteration of the platinum (II) tetraamine libraries

Another deconvolution method that can be used is the iterative method, which consists in fixing one of the variable positions with the functional group corresponding to the most active mixture, while testing all the functional groups at the other positions. The process is repeated sequentially until all the positions are defined. A significant improvement in the activity should be observed upon defining each additional position. This method requires more steps compared to the positional scanning method, but allows the screening of all the sublibraries associated with one particular group.

The iterative deconvolution method was applied to the platinum(II) tetraamine library. Since the higher activity had been observed with the mixture corresponding to the R1 position, the first fixed position was R1. The synthesis of mixtures containing L-Arg at the R1 position, one of the 58 building blocks at the R2 position and mixture of all building blocks at the R3 position was performed in parallel with the synthesis of the original sub-library containing L-Arg at the R1 position and mixtures of functional groups at the R2 and R3 positions, as a control. The results of the screening of the iteration mixtures are shown in **Fig. 3.5**. Although the mixtures tested at 100 μg/ml showed inhibition of EphA2-ephrin-A1 binding, the activity was not greatly improved compared to the previously tested mixtures having the R2

position defined (compare to Fig. 3.4, second panel). In addition, there was no clear differentiation among the mixtures, since 38 mixtures showed inhibition comprised between 20 and 50%. It is possible that the lack of differentiation is due to the fact that the presence of the L-Arg at the R1 position confers a certain degree of activity to the compounds regardless of the chemical group present at the other positions. However, some differentiation could be observed in the activities of the mixtures tested at 50 μg/ml, differentiation that will guide the synthesis of the individual compounds for the next step of the iterative deconvolution.

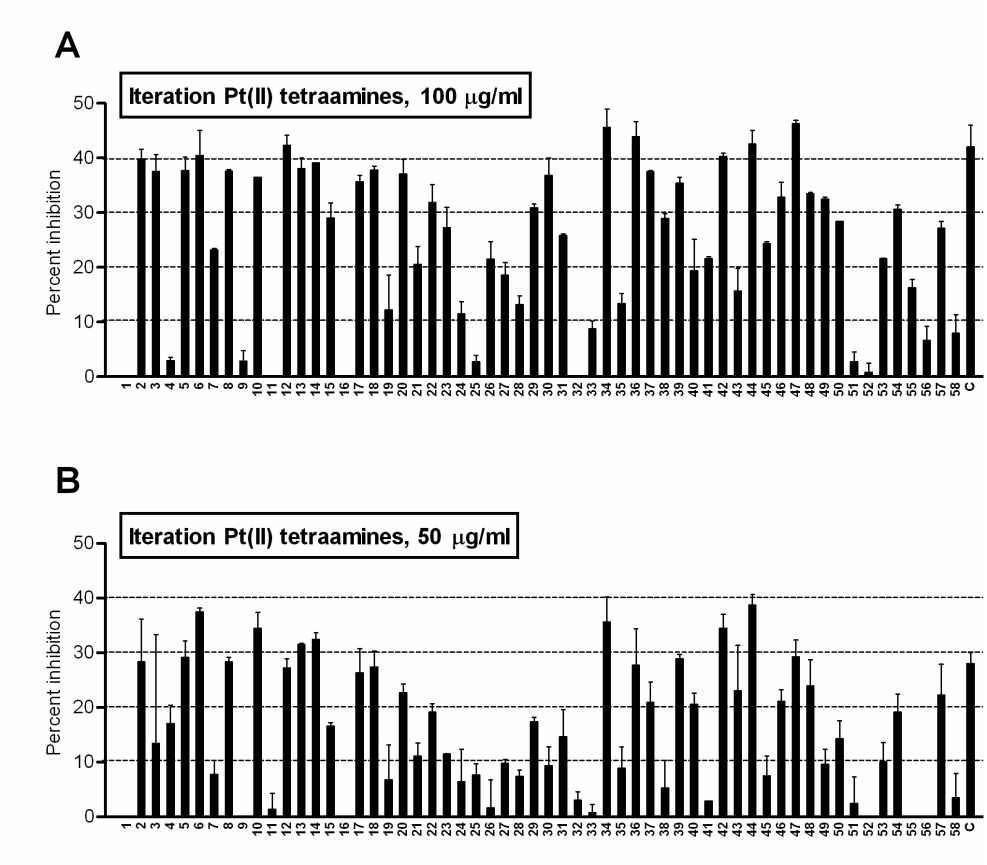

Fig. 3.5. **Screen of iteration platinum(II) tetraamine mixtures**. EphA2 binding to ephrin-A1 was measured in the absence or in the presence of L-Arg iteration platinum(II) tetraamine mixtures added at 100 or 50 μg/ml. C represents the re-synthesis of the mixture having L-Arg at the R1 position and mixture of functional groups at the R2 and R3 positions. Eleven mixtures were filtered in order to remove any traces of free platinum and retested. The same results as before the filtration were obtained for all the mixtures except the C sample. For this sample the inhibition after filtration is shown. Averages from 2 measurements ± SE are shown. Pt(II), platinum(II).

Is has to be noted that 2 of the 3 functionalities previously chosen for the synthesis of single compounds, D-Leu and D-Asn, which correspond to mixtures 24 and 25, showed no activity in the iteration mixtures screen, explaining why the single compounds having these chemical moieties at the R2 position and L-Arg at the R1 position did not inhibit EphA2-ephrin binding. However, the presence at the R2 position of L-Arg (mixture 12), which was also used for the synthesis of the individual compounds, confers activity, suggesting that the group present at the third position is crucial for the activity of these compounds. In addition to the synthesis of individual compounds, the synthesis of other 2 iteration libraries will be carried out, using 2 functional groups in order to compare their activities in the next iteration step. D-Arg and D-Tyr will be used in this synthesis, in order to compare the effect of different amino acid chirality and different substituents and therefore identify the functional groups responsible for the activity of the platinum(II) tetraamine library.

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Chapter 4

Targeting the EphB4 receptor in cancer with TNYL-RAW, an antagonistic peptide

ABSTRACT

The Eph receptors are a family of tyrosine kinases involved in many pathological processes and have emerged as promising drug target candidates. EphB4 in particular is overexpressed in many types of cancer and inhibition of the interaction with its ligand, ephrin-B2, could be useful for the treatment of cancer and for inhibiting tumor angiogenesis and other forms of pathological angiogenesis. The TNYN-RAW 15-mer peptide is a potent and specific antagonist of EphB4 and could, therefore, be exploited in the treatment of pathologies involving the receptor. However, unmodified peptides are rapidly lost *in vivo* due to a variety of processes, including proteolytic degradation, instability and clearance through the kidney and the reticuloendothelial system. Coupling to polyethylene glycol (PEG) molecules or to the Fc portion of human IgG_1 has been shown to reduce these processes, thus increasing the stability and the circulating half-lives of peptides. Here we describe the synthesis of PEGylated and Fc fused forms of the TNYL-RAW peptide and analyze the effects of such modifications on the binding properties of TNYL-RAW, its stability in cell conditioned medium and its retention in the mouse blood. Our results show that PEGylation and fusion to Fc improve the pharmacological properties of the peptide.

INTRODUCTION

The Eph receptors comprise a large family of tyrosine kinase receptors involved in a multitude of biological processes, both in developing and adult tissues. Besides their role in a variety of physiological functions, the Eph receptors have been implicated in different pathological processes, including cancer progression and metastasis (Dodelet and Pasquale, 2000; Nakamoto and Bergemann, 2002), pathological forms of angiogenesis (Brantley-Sieders and Chen, 2004), inhibition of spinal cord regeneration after injury (Goldshmit et al., 2004) and human congenital malformations (Twigg et al., 2004; Wieland et al., 2004). Therefore, inhibition of Eph-ephrin interaction could be useful for diverse medical application. Given the variety of processes mediated by this class of receptors, however, high specificity is desirable in order to inhibit the pathological effects of a specific Eph receptor without affecting the physiological functions of the other closely related receptors.

Despite the promiscuity observed for Eph receptors-ephrin interactions (see Chapter 1), several peptides that selectively target only one of the Eph receptors and inhibit ephrin binding have been identified (Koolpe et al., 2005; Koolpe et al., 2002; Murai et al., 2003). The most potent peptide identified so far is TNYL-RAW, which targets EphB4 (Koolpe et al., 2005). The TNYL-RAW peptide, a 15 amino acid long peptide identified by phage display, shows a remarkably high binding affinity for EphB4 (with a K_D of 1-2 nM). This affinity is comparable to that of ephrin-B2, the preferred ligand for EphB4. TNYL-RAW binds in the hydrophobic binding cleft that constitutes the high affinity binding site for ephrin-B2. Surprisingly, given that the peptide and ephrin-B2 contain four identical consecutive amino acids, the conformation and orientation of TNYL-RAW in the EphB4 binding pocket is distinct from that of the ephrin-B2 G-H loop, which binds in the same position (Chrencik et al., 2006)

The EphB4-ephrin-B2 interaction is known to play a critical role in blood vessel remodeling during embryonic development (Adams et al., 1999; Foo et al., 2006) and more recent evidence also shows its

involvement in cancer angiogenesis and other forms of pathological angiogenensis (Dodelet and Pasquale, 2000; Masood et al., 2005; Nakamoto and Bergemann, 2002; Noren et al., 2004; Walker-Daniels et al., 2003). EphB4 is upregulated in prostate, colorectal, lung, ovarian and breast cancer and in mesothelioma (Berclaz et al., 2003; Liu et al., 2002; Xia et al., 2005; Xia et al., 2006), consistent with a role in tumor development and progression. However, little is known about the mechanism of action of this receptor in the oncogenic process. EphB4 expressed in the tumor cells has been shown to stimulate tumor angiogenesis by activating ephrin-B2 reverse signal in the surrounding blood vessels (Noren et al., 2004). EphB4 is also present in endothelial cells, where it promotes tumor angiogenesis by stimulating ephrin-B2 reverse signaling (Erber et al., 2006). As suggested by this evidence, interfering with EphB4-ephrin-B2 interaction decreases tumor angiogenesis and tumor growth. Indeed, a soluble monomeric form of EphB4, which blocks EphB4-ephrin-B2 bidirectional signaling, inhibits capillary sprouting in cultured endothelial cells and decreases the growth of breast tumors and other types of tumor xenografts in mice (Kertesz et al., 2006; Martiny-Baron et al., 2004). A soluble form of EphB4, as well as a soluble form of ephrin-B2, also inhibits neovascularization in a model of retinophaty (Zamora et al., 2005). Recent evidence also shows that the inhibition of EphB4-ephrin-B2 signals in cultured melanoma cells decreases the invasiveness of these cells (Meyer et al., 2005; Yang et al., 2006).

TNYL-RAW, which inhibits EphB4 activation in breast cancer cells (Koolpe et al., 2005) and blocks capillary-like tube formation in HUVE endothelial cells in culture (Salvucci et al., 2006), is a good drug candidate for the treatment of EphB4-mediated diseases. However, unmodified peptides are known to have a short life in cell-culture or when systemically administered *in vivo* (Ladner et al., 2004; Torchilin and Lukyanov, 2003). Peptides are rapidly degraded by proteases in biological fluids, cleared through the reticuloendothelial system or filtered by the kidneys. Polyethylene glycol (PEG) covalent coupling has been shown to improve

the pharmacodynamic and phamacokinetic properties of drugs. Indeed, PEG-conjugated biomolecules show increased stability, higher solubility, lower clearance and better surface shielding from antigen-recognizing cells, antibodies and proteolitic enzymes, which result in longer *in vivo* circulating half-lives (Hamidi et al., 2006; Harris et al., 2001). Another approach to improve the peptide stability and retention in the blood circulation is the fusion with the Fc part of human IgG_1 (Kim et al., 1998) . Numerous molecules have been coupled to the immunoglobulin Fc domain, showing retention of their biological activity and increased circulating half-lives. This is at least in part due to the ability of Fc to bind to FcRn, the neonatal Fc receptor, which protects the molecules bound to it from catabolism (Junghans and Anderson, 1996).

In this study we describe the conjugation of the TNYL-RAW peptide with 10 or 40 kDa PEG molecules or with the Fc portion of human IgG_1. We then analyze the effect of these modifications on the binding capacity, stability in cell culture medium and retention in the mouse blood circulation of the peptide.

MATERIALS AND METHODS

Reagents

The TNYL-RAW peptide was synthesized with a carboxy-terminal aminoexanoic acid linker with biotin attached to a lysine side chain and was obtained from the Protein Facility of the Burnham Institute for Medical Research. Activated monofunctional polyethylene glycol molecules, PEG-succinimydil-α-methybutanoate ((SMB)-PEG, MW~10 kDa) and PEG-succinimidyl-glutarate (NHS-glutaryl PEG, branched molecule, MW~40 kDa)) were obtained from Nektar Therapeutics (San Carlos, CA) and NOF Corporation (Tokyo, Japan), respectively. The anti-PEG antibody AGP3 was purchased from Academia Sinica, Taiwan. The secondary horseradish peroxidase (HRP)- conjugated anti-mouse IgG was from Morphosys

(Munich, Germany). The Fc fusion-proteins were obtained from R&D Systems, (Minneapolis, MN).

PEGylation of the TNYL-RAW peptide

TNYL-RAW peptide in phosphate buffer saline (PBS) at a concentration of 200 to 500 μM was mixed with activated PEG molecules, which were also dissolved in PBS at a concentration between 2.7 and 11 μM. Either the 10 kDa (SMB)-PEG or the 40 kDa branched NHS-glutaryl PEG were used, at different molar ratios to the peptide. The mixture was incubated for 30 min at room temperature, followed by overnight incubation at 4°C. The reactions were then dialyzed for 2 days at 4°C against PBS using 5,000 MWCO microdialysis units (Pierce Biotecnology, Rockford, IL), in order to remove the uncoupled peptide. PEGylation efficiency was analyzed by measuring absorbance at 280 nm.

TNYL-RAW fused with human Fc

cDNA encoding the TNYL-RAW peptide was cloned into a pcDNA3-based vector preceded by a signal sequence for secretion into the medium and followed by a GSGSK linker and human Fc. The DNA was used to transfect embryonic kidney HEK293 cells, which were then grown for 4 days in DMEM with 10% FBS before collecting the cell supernatant. TNYL-RAW Fc was purified using protein A coupled to sepharose beads (GE Healthcare, UK).

Peptides binding to EphB4

After immobilizing the EphB4-Fc fusion protein to protein A-coated wells (Pierce Biotecnology, Rockford, IL), TNYL-RAW or TNYL-RAW-PEG were added at different concentrations in binding buffer (Tris-buffered saline (TBS) (150 mM NaCl; 50 mM Tris-HCl, pH 7.5) with 1mM $CaCl_2$ and 0.01% Tween 20). Horseradish peroxidase (HRP)-conjugated streptavidin diluted 1:1,000 in binding buffer was then added, and the absorbance at 405 nm

was measured after 1 hour using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich, Steinheim, Germany) in citric acid as a substrate. In the case of TNWL-RAW coupled with the 40 kDa NHS-glutaryl PEG, which could not be detected using streptavidin-HRP, Ni-NTA coated plates (Qiagen, Valencia, CA) were used to immobilize EphB4 Fc (which also contains an hexa-histidine tag) and the bound PEGylated peptide was detected using 1.5 μg/ml AGP3 anti-PEG antibody in binding buffer, followed by 0.5 μg/ml secondary anti-mouse IgM-HRP. The absorbance from wells where no peptide was added was subtracted as the background and the data were analyzed using non linear regression.

As an alternative, protein L-coated plates (Pierce Biotecnology, Rockford, IL) were incubated with AGP3 antibody followed by the PEGylated peptide. Five μl of cell culture medium containing EphB4 alkaline phosphatase fusion protein (EphB4 AP) diluted 1:10 in binding buffer were then added. Bound EphB4 AP was detected using *p*-nitrophenylphosphate (pNPP) (Pierce Biotecnology, Rockford, IL) as a substrate by measuring the absorbance at 405 nm.

For the detection of TNYL-RAW Fc binding to EphB4, protein A-coated plates were incubated with 0.03 μg/well TNYL-RAW Fc or ephrin-B2 Fc, and different amounts of EphB4 AP were then added. Absorbance was measured at 405 nm. Human Fc was used as a negative control.

Inhibition of EphB4-ephrin-B2 binding

Protein A-coated plates were used to immobilize ephrin-B2 Fc fusion protein. TNYL-RAW, TNYL-RAW-PEG and TNYL-RAW Fc at different concentrations were incubated for 3 hours with EphB4 AP culture medium diluted 1:10 in binding buffer and the amount of bound AP fusion protein was quantified by adding pNPP as the substrate and measuring the absorbance at 405 nm. Alkaline phosphatase activity from wells where human Fc was added instead of EphB4 Fc was subtracted as the background.

Measurement of alkaline phosphatase activity

Cell culture medium containing placental alkaline phosphatase (PLAP) fusion proteins was incubated for 10 min at 60°C in order to inactivate endogenous alkaline phosphatases. Different amounts of conditioned medium were incubated at 37°C with the pNPP substrate. Alkaline phosphatase fusion proteins concentration was calculated from the linear regression of the absorbance measured at 405 nm plotted versus the amount of conditioned medium, assuming that the activity of PLAP is 1,500 U/mg and that 1 mU causes a 0.04 increase in the absorbance at 405 nm at 37°C.

Detection of TNYL-RAW in cell culture medium

PC3 prostate cancer cells were grown overnight in RPMI 1640 medium (Mediatech, Inc, Herndon, VA) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and Pen/Strep. Biotinylated TNYL-RAW or TNYL-RAW-PEG peptides were added to the cells at a concentration of 1 μM for different times. The medium was collected and the peptide present in it was captured using neutravidin-coated plates (Pierce Biotecnology, Rockford, IL) and detected using EphB4 AP and pNPP substrate. The same experiment was also performed after replacing the cell medium with fresh medium just before the addition of the peptides or by adding to the cell medium protease inhibitors (10 μg/ml aprotinin, 5 μg/ml pepstatin and 10 μg/ml leupeptin) mixed together or added individually. In another format of the experiment, TNYL-RAW peptide or TNYL-RAW-PEG were added to the PC3 cell conditioned medium removed from the cells and the peptide was incubated for different times at 37°C. The same experiments were also carried out using C6 glioma cells grown overnight in Dulbecco's Modified Eagle's Medium (DMEM) (Irvine Scientific, Santa Ana, CA) with 10% FBS, Pen/Strep and Sodium Pyruvate and BPH1 prostate epithelial cells grown in DMEM/F-12 with 5% FBS.

Retention of TNYL-RAW peptide in mouse blood

TNYL-RAW and TNYL-RAW-PEG were administered to mice by intravenous injection diluted in sterile PBS at a concentration of 40 μM in a 150 μl volume. Blood was collected from the mouse orbital sinus 30 min or 2 hours after injection and by cardiac puncture 6 hours after injection. Blood was kept at 4°C for few hours and then centrifuged for 30 min at 5,000 rpm at 4°C in order to isolate the serum, which was then stored at -20°C. TNYL-RAW and TNYL-RAW-PEG binding to EphB4 was measured by ELISA assay as described above, using wells without peptide but with the same amount of mouse serum as the background.

RESULTS AND DISCUSSION

TNYL-RAW stability in cell culture medium

Peptides have short half-lives in biological fluids, due to diverse processes, including proteolitic degradation. The stability of the TNYL-RAW peptide in PC3 prostate cancer cell conditioned medium was tested, confirming that the peptide is rapidly lost (**Fig. 4.1A**). The kinetics of peptide loss depend on the cell density and on the amount of time the culture medium remained in contact with the cells. Indeed, replacing the culture medium with fresh medium just before addition of the peptide slows down the degradation of the peptide, which remains intact for several hours (**Fig 4.1B**). Presumably after this time proteases have been secreted into the medium by the cells and begin degrading the peptide. The peptide was also lost when it was incubated with conditioned medium in the absence of the cells (**Fig. 4.1C**), confirming that peptide loss is due to some components present in the medium and not to uptake by cells. Supporting the idea that proteases secreted by cells in the medium are responsible for peptide loss, the addition of proteases inhibitors to the cell culture medium reduced peptide loss (**Fig 4.1D**). Furthermore, when the protease inhibitors were administered individually, peptide loss was reverted by aprotinin and leupeptin but not pepstatin (**Fig. 4.1E**).

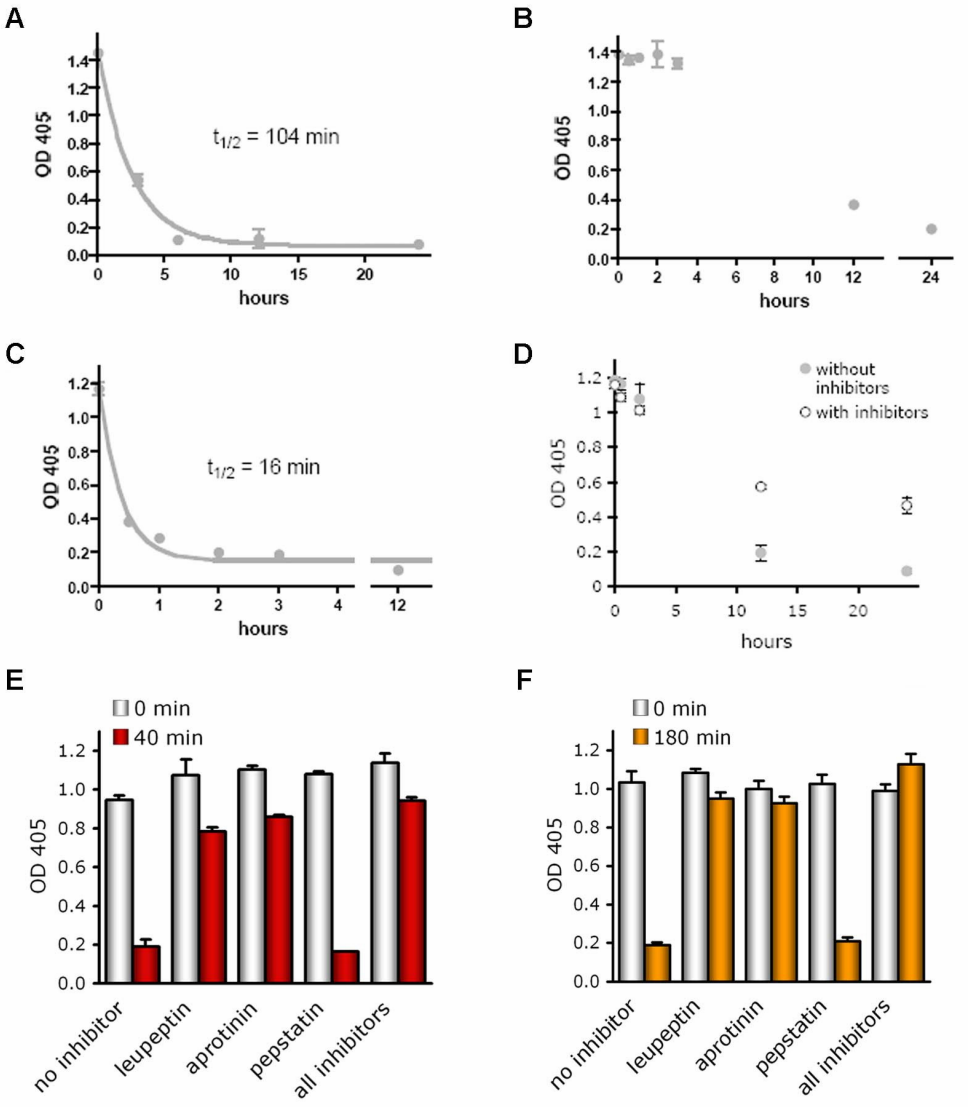

Fig. 4.1. Uncoupled TNYL-RAW peptide is rapidly lost in cell culture medium, unless protease inhibitors are added. (A) Biotinylated TNYL-RAW peptide was added at a concentration of 1 μM to PC3 prostate cancer cells cultured overnight. Functional (EphB4- and streptavidin-binding) peptide remaining in the cell culture medium at the indicated times was captured in ELISA plates coated with neutravidin. Bound peptide was detected with EphB4 AP. The peptide is rapidly lost. **(B)** The same experiment as in (A) was carried out, except that the cell culture medium was replaced with fresh medium just before adding the peptide. The peptide remains intact for several hours before being degraded. **(C)** Conditioned medium was collected from cells and analyzed as in (A). The peptide was also lost in the absence of the cells. **(D)** The peptide was incubated with cell conditioned medium with and without a mixture of protease inhibitors including aprotinin, PMSF, leupeptin and pepstatin. The protease inhibitors reduce peptide loss. **(E)** PC3 cells conditioned medium containing proteases inhibitors all together or individually added was collected 40 min after the addition of TNYL-RAW and the peptide present was measured as described in (A). **(F)** The same experiment described in (E) was performed using medium from G6 glioma cells collected 180 min after the addition of TNYL-RAW. Averages from 3 measurements ± SD are shown in all the panels

Similar results were also obtained with C6 glioma cells (**Fig. 4.1F**) and BPH1 prostate epithelial cells (data not shown). Aprotinin is a well known inhibitor of serine protease, such as trypsin and chymotrypsin, which preferentially cleave peptidic bonds at the C terminus of arginine and lysine or aromatic residues, respectively. Leupeptin is an inhibitor of various proteases, including trypsin and cathepsin B, the latter preferentially cleaving bonds two-residues away from aromatic or positively charged residues. Being inhibited by both aprotinin and leupeptin, which reduced the inactivation of the peptide, trypsin-like proteases are possible candidates for the degradation of TNYL-RAW. The sequence of TNYL-RAW peptide is TNYLFSPNGPIARAW. If trypsin-like proteases are involved in TNYL-RAW degradation, the molecular bond at the C terminus of Arg 13 may be particularly susceptible to proteolysis, and could be therefore modified in order to improve the stability of the peptide. Examples of such modifications would be selective substitution of an amino acid with the corresponding D-amino acid or chemical modifications such as methylation of a side chain.

TNYL-RAW-PEG stability in cell culture medium

Another known method to reduce peptide degradation and delay clearance through the reticuloendothelial system and the kidneys when administered *in vivo* is the coupling with PEG chains. TNYL-RAW was coupled with a 10 kDa PEG molecule containing a succinimidyl α-methylbutanoate reactive group, which allows the coupling to the amino terminus of peptides and proteins. This approach was chosen because the crystal structure of TNYL-RAW in complex with EphB4 showed that the amino-terminal T and N amino acids are not involved in binding to EphB4 (Chrencik et al., 2006). After the coupling reaction, the uncoupled peptide was removed by dialysis and the amount of PEGylated peptide was measured by optical density at 280 nm. When an excess of PEG molecules was used, essentially all the peptide was coupled and the ratio between the PEGylated and the uncoupled peptide initially present in the reaction was about 1 (**Fig. 4.2A**).

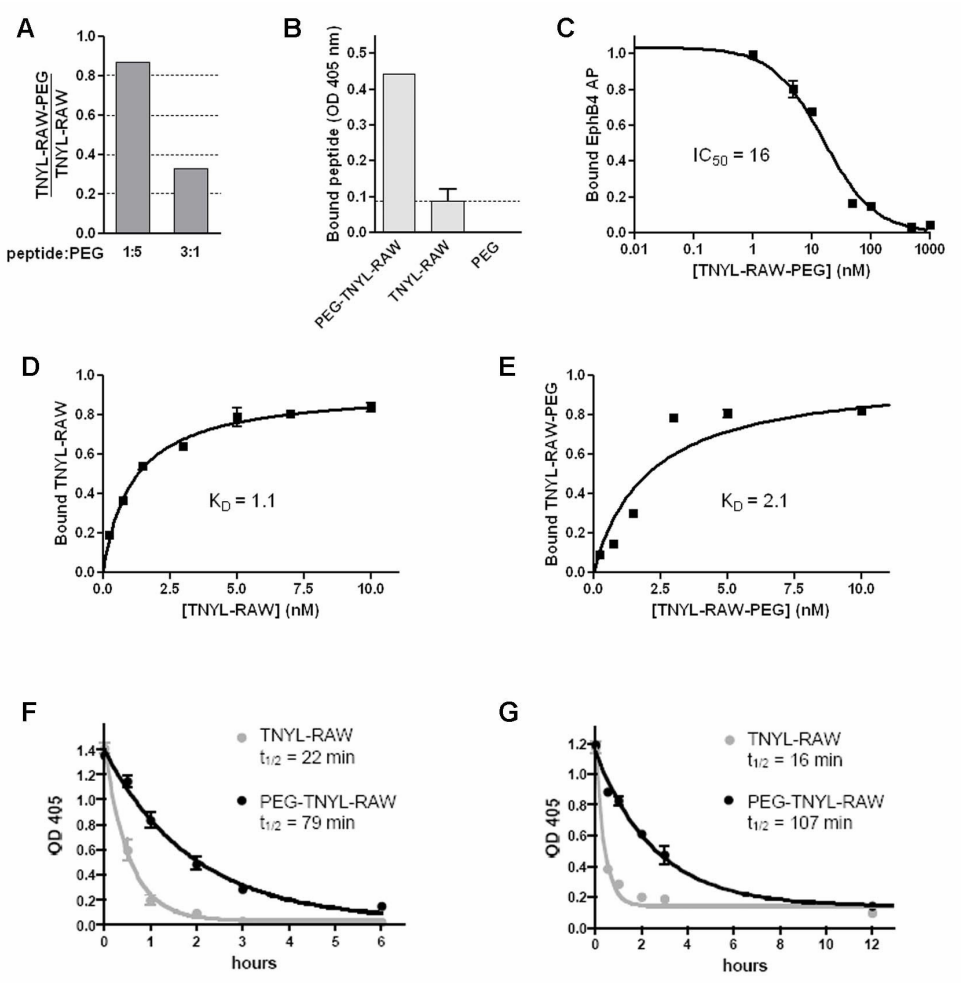

Fig. 4.2. PEGylated TNYL-RAW peptide binds with high affinity to EphB4 and shows higher stability in cell culture medium compared to the uncoupled peptide. (A) TNYL-RAW peptide was covalently coupled to monofunctional 10 kDa PEG using 1:5 and 3:1 ratios of peptide to PEG. After dialysis to remove unbound peptide, almost all the peptide was coupled to PEG (ratio between PEGylated TNYL-RAW and TNYL-RAW initially added to the reaction ~1) when an excess peptide was present during the coupling reaction (1:5 peptide to PEG ratio). In contrast, when an excess peptide was used (3:1 peptide to PEG ratio) approximately one third of the peptide initially added was coupled. **(B)** PEGylated TNYL-RAW peptide was captured in ELISA wells coated with anti-PEG antibodies and detected with EphB4 AP. The signal with PEGylated peptide was higher than the background signal with peptide alone (dashed line). Averages from 2 measurements ±SE are shown. **(C)** The PEGylated TNYL-RAW peptide was incubated at the indicated concentrations together with a constant concentration of EphB4 AP in ELISA wells pre-coated with protein A and ephrin-B2 Fc. The ratio of EphB4 AP bound in the presence and in the absence of peptide is shown. **(D,E)** Uncoupled (D) and PEGylated (E) TNYL-RAW peptide were incubated at the indicated concentrations in ELISA wells pre-coated with protein A and EphB4 Fc. Bound peptide was detected with streptavidin-HRP. Averages from 3 measurements ±SE are shown in (B), (C), (D) and (E). **(F)** Uncoupled or PEGylated TNYL-RAW peptide was added to PC3 prostate cancer cells in culture. Functional (EphB4- and streptavidin-binding) peptide remaining in the cell culture medium at the indicated times was captured on neutravidin coated-plates and detected using EphB4 AP. **(G)** Conditioned medium was collected from cells and analyzed as in (F). Averages from 3 measurements ±SD are shown in (F) and (G).

On the contrary, when a 1:3 ratio of PEG molecules to peptide molecules was used, only one third of the peptide was PEGylated, as expected (**Fig. 4.2A**). Coupling was verified by capturing the PEGylated peptide on ELISA protein L plates coated with anti-PEG antibody (AGP3) followed by detection with streptavidin-HRP (**Fig. 4.2B**). TNYL-RAW-PEG showed a significantly higher signal compared to the unmodified TNYL-RAW, demonstrating that the coupling reaction was successful. The PEGylated peptide retained the ability to inhibit EphB4-ephrin-B2 binding (**Fig. 4.2C**) and a high binding affinity for EphB4. The calculated dissociation constant (K_D) for TNYL-RAW-PEG is comparable to the K_D for the uncoupled peptide (**Fig. 4.2D-E**). Furthermore, the stability of the PEGylated peptide was tested in PC3 cell culture medium, showing an increased resistance to degradation compared to the non-PEGylated peptide (**Fig. 4.2F**), with an approximate 4 fold increase in the peptide half-life ($t_{1/2}$). When the PEGylated peptide was tested in cell culture supernatants in the absence of cells (**Fig. 4.2G**), an improvement in the half life was also observed, which was approximately 7 fold higher compared to TNYL-RAW.

TNYL-RAW and TNYL-RAW-PEG retention in mouse blood

The stability of the unmodified and PEGylated TNYL-RAW was also assessed *in vivo*. In this case a 40 KDa branched PEG was used, containing a succinimidyl-glutarate reactive group, which also allowed the coupling with the amino terminus of the peptide. A higher molecular weight was chosen for the *in vivo* experiments because it may help decrease filtration through the kidneys. *In vitro*, TNYL-RAW coupled with the 40 kDa PEG efficiently inhibited EphB4-ephrin-B2 interaction (**Fig. 4.3A**). However, peptide binding to EphB4 could not be detected by ELISA using streptavidin-HRP, presumably because the higher molecular weight PEG interferes with streptavidin binding to the biotin tag on the peptide. A slightly different assay was then used to detect the peptide, which involves the use of the AGP3 anti-PEG antibody followed by a secondary anti mouse IgM. The K_D value obtained is comparable to that of the uncoupled peptide (**Fig.**

4.3B), showing that the binding to EphB4 is not altered by the presence of the larger PEG molecule.

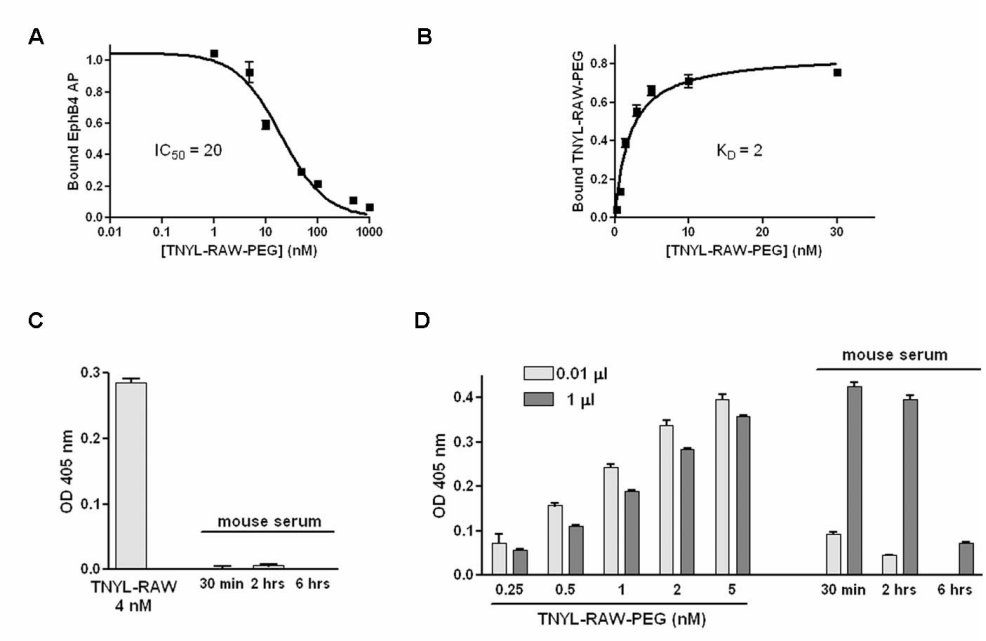

Fig. 4.3. Coupling of TNYL-RAW to a 40 KDa branched PEG increases the peptide half-life in mouse serum. (A) TNYL-RAW peptide coupled to a 40 kDa PEG was incubated at the indicated concentrations together with a constant concentration of EphB4 AP in ELISA wells pre-coated with protein A and ephrin-B2 Fc. Ratio of EphB4 AP bound in the presence and in the absence of peptide is shown. **(B)** TNYL-RAW-PEG diluted in 100 µl of binding buffer to the indicated concentrations was incubated in ELISA Ni-coated wells pre-coated with EphB4 Fc. Bound peptide was detected with anti-PEG antibody and a secondary anti-mouse IgM. **(C)** Serum collected at the indicated times from mice injected with TNYL-RAW was incubated at a dilution of 1:10 in ELISA wells pre-coated with protein A and EphB4 Fc. Bound peptide was detected with streptavidin-HRP. TNYL-RAW peptide at a concentration of 4 nM was used as a control. **(D)** 0.01 or 1 µl of serum collected at the indicated times from mice injected with TNYL-RAW-PEG was incubated in ELISA wells as described in (B) (right portion of the histogram). TNYL-RAW-PEG peptide at different concentrations in the presence of 0.01 or 1 µl of non-treated mouse serum was used as a control (left portion of the histogram). Averages from 3 measurements ±SE are shown in all the panels.

Six µmol of the peptides were injected intravenously in mice and the blood was collected after 30 min, 2 hours and 6 hours. Serum was separated and the amount of peptide present in the serum was measured by ELISA after capturing it with EphB4 Fc. TNYL-RAW could not be detected in the serum at any of the time points (**Fig. 4.3C**), suggesting that

the unmodified peptide is rapidly lost. On the contrary, TNYL-RAW-PEG could be detected in mouse serum diluted up to 10,000 fold (**Fig. 4.3D**). By comparing the absorbance of peptide contained in the mouse serum with the absorbance of TNYL-RAW-PEG in the presence of equal amount of serum, the approximate concentration of peptide retained in mouse blood can be deduced. After 30 min, approximately all the peptide injected was still in the mouse serum (calculated concentration of 2 μM), after 2 hours the absorbance was reduced to about a half and after 6 hours the calculated concentration was about 0.025 μM. More time-points must be tested in order to better quantify the half-life of the PEGylated peptide, nevertheless the results obtained so far show that the presence of the PEG molecule highly enhances the *in vivo* stability of the peptide.

Characterization of TNYL-RAW Fc

A synthetic DNA sequence encoding the TNYL-RAW peptide was cloned in a vector encoding a signal peptide allowing secretion of the peptide in the culture medium and the Fc portion of human IgG_1. The TNYL-RAW Fc fusion protein purified from HEK293 cell culture supernatants bound to EphB4 with substantial affinity (**Fig. 4.4A**). However, the K_D of EphB4 AP binding to TNYL-RAW Fc and the IC_{50} value for inhibition of EphB4-ephrin-B2 binding were approximately 17 and 14 times lower, respectively, compared to ephrin-B2 Fc (**Fig. 4.4B-C**). One possible explanation for the decreased affinity and inhibitory activity is that a portion of the TNYL-RAW Fc protein may become inactivated in the HEK293 cell culture medium before purification. Regardless, since the Fc fusion protein was purified from medium in which the producing HEK293 cells were grown for 4 days, the half-life of TNYL-RAW Fc appears to be much longer compared to the synthetic peptide, which is lost within several hours in cell culture medium. Therefore, fusion to Fc appears to effectively stabilize the peptide.

In order to verify that the fusion with the Fc portion of human IgG_1 improves the stability of TNYL-RAW peptide also *in vivo*, TNYL-RAW Fc retention in mouse blood will be examined. The binding properties and the

stability of TNYL-RAW-PEG and TNYL-RAW Fc will be compared and the best form of the peptide will be selected to be tested in tumor xenograft studies.

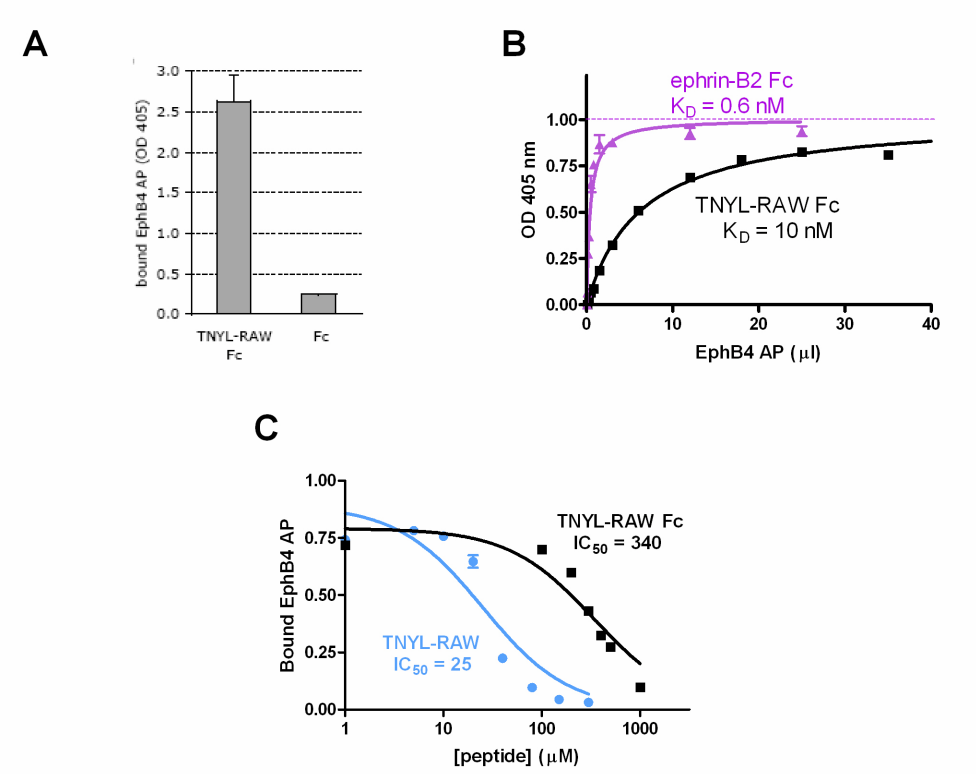

Fig. 4.4. Characterization of TNYL-RAW peptide fused to human Fc. (A) TNYL-RAW Fc immobilized on protein A-coated ELISA plates binds EphB4 AP; human Fc was used as a control. **(B)** The indicated amounts of EphB4 AP were added to ELISA wells precoated with 0.03 µg/well of ephrin-B2 Fc or TNYL-RAW Fc. Measured AP activity was normalized to the maximum binding for each curve calculated using non linear regression. K_D values were based on EphB4 AP concentration as calculated from AP activity. **(C)** TNYL-RAW or TNYL-RAW Fc were incubated at the indicated concentrations together with a constant amount of EphB4 AP in ELISA wells pre-coated with protein A and ephrin-B2 Fc. Ratio of EphB4 AP bound in the presence and in the absence of peptide is shown. Averages from 3 measurements ±SE are shown in all the panels.

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Chapter 5

Conclusions

The Eph receptors and their ligands, the ephrins, are involved in many physiological and pathological processes. Inhibitors of their interaction could therefore be useful to help elucidate the mechanism of receptor-ligand interaction, for characterizing the functions of Eph receptors and ephrins in disease, and as leads for drug development. Agents targeting the extracellular domain of the Eph receptors are advantageous with respect to tyrosine kinase inhibitors in that they can act without penetrating inside the cells and they can be highly selective. Although ephrin binding to Eph receptors is very promiscuous, the characterization of peptides identified by phage display demonstrated that it is possible to selectively target only one of the Eph receptors. This is a highly desirable feature in order to inhibit the pathological function of a specific receptor, without affecting the numerous physiological functions of the other receptors.

In this study we applied 2 different screening approaches, high throughput screening and combinatorial library screening, to the identification of chemical compounds that target EphA4 and EphA2. Two 2,5-dimethylpyrrolyl benzoic acid derivatives were identified in the high throughput screen for EphA4 antagonists. These compounds inhibit ephrin binding to EphA4 and EphA2 but no other Eph receptors. These results are completely novel because no small molecule inhibitors of Eph receptor-ephrin interaction have been identified so far. Importantly, they demonstrate that the Eph receptors can be successfully targeted by small molecules. The activity and selectivity of the two compounds are particularly impressive, given the large size of the ephrin-binding pocket in the Eph receptors and the small size of the compounds. Intriguingly, the 2 2,5-dimethylpyrrolyl benzoic acid derivatives also show selectivity in their inhibition of the binding of different ephrins to EphA4 and EphA2. This

suggests that ephrin-A4, ephrin-B1 and ephrin-B2 bind differently to EphA4 compared to other ephrins of the same class. Virtual docking or NMR analysis of the compounds in complex with EphA4 and EphA2 will help clarify the molecular basis for these differences.

The 2 2,5-dimethylpyrrolyl benzoic acid derivatives also inhibit EphA4 and EphA2 phosphorylation in cells as well as the characteristic biological activities of the 2 receptors, such as retraction of the cell periphery and growth cone collapse. On the other hand, the compounds do not affect cell and growth cone morphology in the absence of ephrin treatment or cell viability. Thus, these compounds could be useful as inhibitors of EphA4 or EphA2-dependent pathological processes, such as cancer progression, pathological blood clotting and inhibition of spinal cord regeneration after injury provided that a higher potency is achieved. The design other 2,5-dimethylpyrrolyl benzene derivatives with structural modifications based on the structure-activity relationships observed for inhibition of EphA4-KYL binding is anticipated to result in the identification of compounds retaining the original functional activity and selectivity but having increased potency.

By combinatorial library screening, we also identified platinum(II) tetraamines as potential inhibitors of EphA2 activity. EphA2 inhibitors can have application in the treatment of tumor angiogenesis and other forms of pathological angiogenesis. The platinum(II) tetraamine library seems to inhibit ephrin-A1 and ephrin-A5 binding to EphA2 in a specific manner because no effect was observed when the platinum(II) tetraamine library was tested for inhibition of 2 other Eph-ephrin interactions. All the other receptors will be tested once the compounds responsible for the activity observed in the mixtures will be identified. Nevertheless, this preliminary result suggests that the compounds are likely to be selective for one or few Eph receptors. In particular, the platinum(II) tetraamines did not inhibit EphA4, a receptor that is highly related to EphA2 and that is not discriminated from EphA2 by the 2,5-dimethylpyrrolyl benzoic acid derivatives. In addition, the same tetraamine libraries without the coordinated platinum have poor activity, indicating that the structural

conformation conferred by the metal is necessary for the activity of these compounds. A drawback of platinum-containing compounds, however, is that the release of platinum could create some toxicity problems in cell culture and *in vivo*. In any case, these compounds could represent useful leads for the development of other, platinum-free compounds with similar structures.

TNYL-RAW, a peptide antagonist that targets the EphB4 receptor also has potential applications for the treatment of different forms of cancer and pathological forms of angiogenesis. The TNYL-RAW peptide shows promising features, since it is highly selective, has nanomolar affinity for EphB4 and has been shown to inhibit capillary-like structures *in vitro*. However, peptides are known to be highly unstable in physiological fluids. This was confirmed by our results, which show that the unmodified TNYL-RAW peptide is rapidly lost both in cell culture medium and in the mouse blood. This effect is due at least in part to proteolitic degradation, because the peptide persists longer in cell culture medium when protease inhibitors are present. Further studies using different protease inhibitors will help identify the peptide bonds that are particularly susceptible of proteolitic degradation, enabling synthesis of peptides carrying modifications at those bonds. Coupling to PEG or Fc were also found to increase the stability of the peptide in cell culture medium and, in the case of PEGylation, also in mouse blood. The *in vivo* stability of TNYL-RAW Fc remains to be examined, although Fc fusion proteins including antibodies are typically extremely stable *in vivo*. These results are encouraging for the possible use of TNYL-RAW *in vivo* to inhibit cancer progression and angiogenesis.

Pharmacological tools to manipulate Eph receptor function will open new avenues of research and therapy. By identifying new chemical compounds with expected better pharmacological properties compared to the previously identified agents and by improving the properties of a known peptide inhibitor we obtained some promising results for Eph receptor targeting.

Furthermore, our studies are the first to show the feasibility of approaches to identify agents targeting the extracellular domain of this large and important class of receptors, approaches that can be used with other receptors and have therefore wide application.

LIST OF PUBLICATIONS

Fantuzzi A. et al., Retinol Release in the Presence of Liposomes and Conformational State of its Carrier, Human Plasma Retinol Binding Protein. Manuscript in preparation.

Noberini R. et al., Small Molecules That Selectively Inhibit Ephrin Binding to the EphA4 and EphA2 Receptors. Manuscript in preparation.

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